with cells incubated with 10% FCS at 4 °C and with chloramphenicol, valinomycin, nonactin, and gramicidin at 37 °C show that intact *M. capricolum* cells incorporate much less cholesterol under conditions where growth is inhibited. The decrease in cholesterol incorporation under these conditions suggests that growing mycoplasmas may possess a mechanism that catalyzes cholesterol incorporation into the inner half of the bilayer. That this mechanism is not operative in nongrowing cells is supported by the high $k_{cell}/k_{membrane}$ ratios obtained in the chloramphenicol- and ionophore-treated cultures (0.7–0.9), indicative of a preferential localization of the newly acquired cholesterol into the outer half of the lipid bilayer. The adapted strain offers advantages in the study of cholesterol movement in mycoplasmas, and further experiments may lead to a better understanding of the factors that influence the lipid distribution in this model biological membrane.

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Proposed Structure for Coenzyme F_{420} from *Methanobacterium*†

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ABSTRACT: The low-potential electron carrier, coenzyme F_{420}, was purified from *Methanobacterium* strain M.o.H. A yield of 160 mg/kg of wet-packed cells was obtained. Results of analysis of hydrolytic fragments and periodate oxidation products of the coenzyme, by infrared, UV–visible, ^1H and ^{13}C NMR spectrometry, mass spectrometry, and quantitative elemental analyses indicate that coenzyme F_{420} is: *N*-[*N*-[*O*-[5-(8-hydroxy-5-deazaisoalloxazin-10-yl)-2,3,4-trihydroxy-4-pentoxyhydroxyphosphinyl]-L-lactyl]-γ-L-glutamyl]-L-glutamic acid. A convenient trivial name would be the *N*-(*N*-L-lactyl-γ-L-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin 5′-phosphate. Proof of structure by organic synthesis was not performed; the stereochemical configuration of the hydroxyl groups on the side chain as well as the position of the hydroxyl group on the aromatic ring require confirmation by organic synthesis of the molecule.

The purification and properties of a fluorescent compound, Factor$_{420}$ (F_{420}) from *Methanobacterium* strain M.o.H., were reported by Cheeseman et al. (1972). The yellow compound had a strong absorption maximum at 420 nm; upon reduction, the 420-nm absorption maximum and fluorescence were lost. A molecular weight of about 630 was estimated by Sephadex G-15 column chromatography. Acid hydrolysis released glutamic acid, phosphate, an acid-stable chromophore, and an ether-soluble phenolic compound. F_{420} was easily photolyzed aerobically, but not anaerobically, under neutral to basic conditions. The coenzyme role of F_{420} was defined by Tzeng et al. (1975a) who reported the presence of an F_{420}-dependent NADP[1]-linked hydrogenase system in cell extracts of *Methanobacterium ruminantium* strain PS and *Methanobacterium* strain M.o.H. An F_{420}-dependent formate hydrogenlyase system also was described in *M. ruminantium* (Tzeng et al., 1975b); electrons were transferred by formate dehydrogenase to F_{420} which served as a substrate for hydrogenase. However, electrons from reduced F_{420} were preferentially

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[1] Abbreviations used are: F_{420}, coenzyme F_{420}; FA, FO, F+, N-1, N-2, and SAC, hydrolytic fragments of F_{420}; PA and ALD, periodate oxidation fragments of F_{420}; γLGLG, γ-L-glutamyl-L-glutamic acid; DEAE, diethylaminoethyl; QAE, quaternary aminoethyl; TSP, sodium 3-trimethylsilylpropionate; EI, electron impact; FD, field desorption; IR, infrared; NMR, nuclear magnetic resonance; UV, ultraviolet; ESR, electron-spin resonance; NAD, nicotinamide adenine dinucleotide; NADP, NAD phosphate; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.

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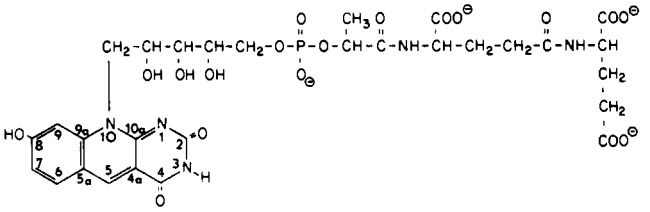


FIGURE 1: Proposed structure for coenzyme F_{420}.

transferred to NADP by an F_{420}:NADP oxidoreductase. The presence of high amounts of F_{420} in methanogens contributes to the fluorescence of these cells under UV light (Edwards and McBride, 1975).

Possession of F_{420} is a characteristic of all methanogens now in culture; the coenzyme has not been reported in other organisms. F_{420} is the second unique coenzyme from the methanogens to be described, the first being coenzyme M, 2-mercaptoethanesulfonic acid (McBride and Wolfe, 1971; Taylor and Wolfe, 1974).

Here we propose a structure for coenzyme F_{420}. Analysis of the hydrolysis and periodate oxidation products of F_{420}, as well as data from infrared, UV-visible, ^1H and ^{13}C NMR spectrometry, mass spectroscopy, and quantitative elemental analyses enable us to propose the structure shown in Figure 1. Evidence for this structure is presented in two parts: (1) the side chain; (2) the ring system.

Experimental Procedure

Methods

Organism. Methanobacterium strain M.o.H. was grown, harvested, and stored as described by Taylor and Wolfe (1974). The cell extract for use in the hydrogenase-catalyzed reduction of F_{420} was prepared as described by Gunsalus and Wolfe (1977).

Extraction of F_{420}. Whole cells (320 g) were suspended in water (1:1, w/v) and broken in a French pressure cell at 15 000 psi. The lysate was rendered less viscous by a brief sonication and was then heated with stirring to 95 °C. Denatured polymers and cell debris were removed by centrifugation, the pellet being extracted again by the same procedure. After lyophilization of the supernatant solutions, the dry powder was dissolved in 500 mL of water and applied to a 21 × 4 cm column of QAE-Sephadex A25 (bicarbonate form), and by application of a linear gradient of ammonium bicarbonate (3 L, 0–1.5 M) fraction II was eluted between 1.25 and 1.45 M. Ammonium bicarbonate was removed by lyophilization, yielding fraction II which was dissolved in a minimal amount of water and applied to a second column of QAE-Sephadex A25 (16 × 1.25 cm). By application of 1 L of a 0–1 M ammonium bicarbonate linear gradient, fraction III was obtained, which after lyophilization was dissolved in water and applied to a 19 × 1.5 cm column of DEAE-Sephadex A25 (bicarbonate form), and fraction IV was eluted with a linear gradient of 0–0.8 M ammonium bicarbonate, yielding the ammonium salt of F_{420}. Material for analysis was prepared by the addition of a solution of 54 mg of fraction IV in 20 mL of 0.1 M ammonium acetate to a Sephadex G-15 column (112 × 5 cm). F_{420} eluted at 1200 mL of 0.1 M ammonium acetate and was lyophilized.

Acid Hydrolysis and Electrophoresis of F_{420}. A 0.5-mL amount of a solution of F_{420} (480 μg/mL in 1 M HCl) was added to each of ten vials which were sealed and subjected to 110 °C, a separate vial being removed to an ice bath after each of the following periods: 0.0, 0.12, 0.25, 0.5, 1.0, 2.5, 4.0, 8.0, 12.0, and 24 h. After the final time period, each vial was opened, the contents were dried under a stream of argon, and 0.11 mL of 0.1 M ammonium hydroxide was added. A 5-μL amount of each hydrolyzed sample and 10-μL amounts of the unhydrolyzed solution were subjected to thin-layer electrophoresis.

A Desaga–Brinkman electrophoresis apparatus was operated at 400 V to separate F_{420} and its hydrolytic derivatives on Eastman cellulose (6064) or Merck cellulose (5577) plates; buffer B (acetic acid–pyridine–water, 16:8:976) at pH 4.4 was used. After electrophoresis, fluorescent spots were outlined, and the plate was sprayed with ninhydrin (0.2% in methanol) followed by a molybdate–stannous chloride spray (Krebs et al., 1969); fluorescent spots were labeled FA, FO, and F+; ninhydrin-positive spots were labeled N-1 and N-2; inorganic phosphate positive spots were labeled P.

Purification of Hydrolytic Fractions F+, N-1, and N-2. F_{420} (115 mg in 125 mL of 1 M HCl) was hydrolyzed for 45 min at 100 °C. The hydrolyzed products were rotary evaporated to remove acid, and the residue was dissolved in a small amount of water. This solution was applied to a 28 × 5.0 cm QAE-Sephadex A25 column (acetate form) which had been equilibrated overnight with 1500 mL of water. A linear gradient of ammonium acetate (5 L, 0–2.5 M) at a flow rate of 54 mL/h was employed to elute the main hydrolytic products of F_{420}; F+, FO, N-1, and N-2 eluted at 1.35, 1.1, 0.5, and 0.76 M ammonium acetate, respectively. Appropriate fractions were pooled and desalted by rotary evaporation at 40 °C. The crude FO fraction obtained by this procedure was discarded. F+ was further purified by application to a second QAE-Sephadex A25 column (bicarbonate form, 22 × 1.5 cm) followed by elution with a 0–0.5 M ammonium bicarbonate gradient (1000 mL) at a flow rate of 57 mL/h; F+ eluted at 0.47 M ammonium bicarbonate. The appropriate F+ fractions were pooled, desalted by rotary evaporation, and lyophilized to yield a bright-yellow powder.

N-2 was purified by application to a 15 × 1.0 cm DEAE-Sephadex A25 column (acetate form) which had been equilibrated with 250 mL of water. Elution was accomplished by use of a 0–1.0 M ammonium acetate gradient (500 mL) at a flow rate of 28.5 mL/h, N-2 eluting at an ammonium acetate concentration of 0.13 M. The ammonium salt of N-2 failed to form a powder after lyophilization; preparation of the calcium salt yielded a colorless solid. N-1 was purified further by application to a 20 × 1.0 cm DEAE-Sephadex A25 column (acetate form) which had been equilibrated with water. A 0–0.5 M ammonium acetate gradient flowing at a rate of 32.4 mL/h eluted N-1 at 0.11 M ammonium acetate. The pooled fractions were rotary evaporated and lyophilized to dryness. N-1 failed to form a solid after lyophilization; preparation of the sodium salt yielded a white solid after lyophilization.

Purification of Hydrolytic Fraction SAC. Another hydrolytic fraction SAC was obtained by short-term hydrolysis (10 min) of F_{420} in 1 M HCl at 100 °C. SAC, which was ninhydrin negative, was located when a sample of the hydrolysate was subjected to thin-layer electrophoresis and the plate was sprayed with a chlorine–starch–potassium iodide spray (Rydon and Smith, 1952). To purify SAC the hydrolysate was cooled and rotary evaporated to dryness at 25 °C. The dry residue was suspended in 50 mL of water, and ammonium hydroxide was added (1 drop) to facilitate solubility. This solution was applied to a 20 × 1.5 cm DEAE-Sephadex A25 column (bicarbonate form). Elution was accomplished by means of a 0–0.5 M ammonium bicarbonate gradient (1000 mL) flowing at a rate of 60 mL/h. SAC eluted at 0.26 M ammonium bicarbonate. The partially purified SAC was applied to a 20 × 1.5 cm DEAE-Sephadex A25 column (bicarbonate form) which had been

equilibrated with water. Elution was accomplished by applying a 0–0.5 M ammonium bicarbonate gradient (1000 mL) at a flow rate of 65.5 mL/h. SAC eluted at 0.24 M ammonium bicarbonate. The ammonium salt of SAC failed to yield a powder upon lyophilization; preparation of the calcium salt yielded a white solid.

Purification of FO. Since FO was difficult to obtain in pure form by column fractionation of hydrolyzed F_{420}, it was preferentially obtained by treatment of F+ with alkaline phosphatase. The ammonium salt of F+ (13 mg) was dissolved in 0.1 M ammonium acetate (5 mL, with 0.5 mM $MgCl_2$ added) at pH 10.5. Alkaline phosphatase (10 μL) was added, and the progress of the reaction was followed by subjecting samples to thin-layer electrophoresis. When the reaction was complete (1 h), the reaction mixture was diluted fivefold and applied to a 15 × 1 cm QAE-Sephadex A25 column (bicarbonate form) which had been equilibrated with 200 mL of water. A 0–0.1 M ammonium bicarbonate gradient (1000 mL), flowing at a rate of 66 mL/h, eluted FO. The yellow material was dried. This partially purified preparation was dissolved in 50 mL of water, acidified with concentrated HCl to approximately pH 1.0, and applied to a column of Florisil (32 × 4 cm). Prior to preparation of the column, the Florisil had been washed with 1000 mL of 6 M HCl, followed by water and then by acetone; the dried Florisil was suspended in water, and the column was poured as an aqueous slurry which was washed extensively with water before applying the sample. FO bound to the first few centimeters of column material. The column was washed a second time with water (1500 mL). FO was eluted with a 50% aqueous acetone solution and was dried as a yellow powder. A second QAE-Sephadex A25 column prepared and employed in an identical manner to the first column was used to complete the purification of FO. The material which eluted from this column was dried, dissolved in water, and lyophilized; the yield of FO was 8 mg.

Periodate-Treated F_{420}: Fractions PA and ALD. F_{420} (151 mg) was dissolved in 30 mL of water and sodium periodate (173 mg) was added. Shortly after mixing, an orange precipitate began to form; the reaction was allowed to proceed for 22 h at room temperature in the dark. A sample of the reaction mixture (4 μL) was subjected to thin-layer electrophoresis in buffer A (pyridine–acetic acid–water, 20:5:975, plus 0.025 M NaCl at pH 5.7) for 45 min at 400 V. PA was observed as a yellow-colored, blue-fluorescent spot which moved approximately 1 cm to the cathode. ALD was detected after spraying the thin-layer plate with a silver nitrate–sodium hydroxide spray reagent (Krebs et al., 1969) as an aldehyde-positive spot which was located 7 cm toward the anode. The precipitate of the reaction mixture contained PA; ALD remained in solution. PA was dissolved in 50 mL of water to which a few drops of concentrated HCl were added. Under these conditions PA dissolved readily, and the low pH allowed it to bind to the 21 × 1.5 cm Sephadex SP-C25 column (ammonium form) to which it was applied. Elution was accomplished by means of a 0–0.1 M ammonium bicarbonate gradient (1000 mL). PA eluted in a very broad band centered at a salt concentration of 0.05 M. Due to the insolubility and lability of this compound, it was not purified further, but the pooled PA-containing fractions were desalted by lyophilization and were stored as a yellow solid at −15 °C.

The ALD-containing filtrate was applied to a 25 × 1.5 cm QAE-Sephadex A25 column (bicarbonate form) which had been equilibrated with 150 mL of water. Elution of ALD was accomplished by means of a 0–0.5 M ammonium bicarbonate gradient (1000 mL) flowing at a rate of 63 mL/h. ALD was found to elute at 0.46 M ammonium bicarbonate, and the compound was desalted by lyophilization. For further purification, ALD was dissolved in about 30 mL of water and applied to a 20 × 1.5 cm DEAE-Sephadex A25 column (bicarbonate form) which had previously been equilibrated with water. An ammonium bicarbonate gradient (0–0.5 M, 1000 mL) was applied, and ALD eluted at 0.36 M ammonium bicarbonate, yielding a white powder after lyophilization.

Enzymatic and Chemical Assays. Alkaline phosphatase (EC 3.1.3.1) was used to distinguish between mono- and diester phosphate linkages in F_{420} and its derivatives. The sample to be tested was dissolved in 0.5 mL of 0.5 mM $MgCl_2$ in 0.1 M ammonium acetate (pH 10.5), followed by the addition of alkaline phosphatase (10 μg); the reaction was allowed to proceed for 6 h at 25 °C. Phosphate was determined by the method of Fiske and SubbaRow (1925). L-Glutamic acid was determined by the use of glutamate dehydrogenase (EC 1.4.1.3). The assay mixture (3 mL) contained: potassium phosphate buffer (pH 8.0), 500 μmol; nicotinamide adenine dinucleotide (NAD), 5.7 μmol; glutamate dehydrogenase (Boehringer), 20 μg. Hydrolyzed compounds were added in amounts equivalent to 350–400 nmol of L-glutamic acid. The reaction was followed at room temperature with a Cary 118 recording spectrophotometer. Lactic acid was determined with lactic dehydrogenase (EC 1.1.1.27) (Marbach and Weil, 1967). Standard manometric techniques were used to measure hydrogen uptake and the reduction of F_{420} by crude extract (16 mg of protein) in phosphate buffer at pH 7.0. Formic acid was determined by the method of Barker and Somers (1966) with the modification that absorbance of the reaction mixture was measured with a Gilford 300 N spectrophotometer immediately after development of the chromophore. The amount of periodate reduced by F_{420} was determined by the method of Aspinal and Ferrier, as described by Dryhurst (1970). (Interference with the assay was negligible, since the chromophore of F_{420}, which was produced by periodate oxidation, precipitated from solution.) To detect peptides on thin-layer electrophoresis plates the chlorine–starch–potassium iodide spray reagent of Rydon and Smith (1952) was used.

Spectrometric Assays. Low-resolution electron-impact (EI) mass spectra were obtained with a Varian MAT spectrometer, Model CH-5DF, employing the direct-probe technique; conditions: accelerator voltage, 3 kV; emission current, 100 A; resolution, 800; source temperature, 300 °C; solids probe temperature, 20–250 °C; electron energy, 70 eV. High-resolution EI and FD mass spectra were obtained on a Varian MAT mass spectrometer, Model 731, equipped with a combination EI–FD field emission ion source, and a Varian mass spectrometry data system, Model SS100. The FD mass spectrum was obtained at a resolution of 800 and with a source temperature of 90 °C. The high-resolution EI spectrum was measured by peak matching; conditions: accelerator voltage, 8 kV; emission current, 1600 μA; resolution, 20 000; source temperature, 240 °C; solids probe temperature, 20–280 °C; electron energy, 90 eV.

Proton NMR spectra were obtained with a Varian HR-220 spectrometer equipped with a Nicolet Instrument Co. TT-220 Fourier transform accessory; each dried sample was dissolved in deuterium oxide, and chemical shifts are reported in parts per million from 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$ (TSP) as an internal standard. To obtain a spectrum of reduced F_{420}, 15 mg of the ammonium salt of F_{420} was dissolved in 5 mL of water which contained 20 mg of ammonium bicarbonate under a hydrogen atmosphere. The cell extract (500 μg of protein) was added, and after reduction was complete 100 μL of oxygen-free 2 M HCl was added. Reduced F_{420} was frozen under anaerobic conditions and lyophilized in the reduced state. The

TABLE I: Purification of F_{420} from *Methanobacterium* Strain M.o.H.

fraction	mass of fraction (mg)	F_{420} content (mg)[a]	purity (%)	purification	% recovery
(I) crude F_{420} prep	20 000	(61.6)[b]	0.31	1	100
(II) 1st QAE-Sephadex A25 eluate	190	59.5	31.3	102	97
(III) 2nd QAE-Sephadex A25 eluate	66	55.8	84.5	275	91
(IV) DEAE-Sephadex A25 eluate	54.1	51.8	95.6	310	84

[a] Calculated from the extinction coefficient of F_{420} (sodium salt) at 420 nm of 51.5 for a 1 mg/mL solution at pH 8.0. [b] Estimated.

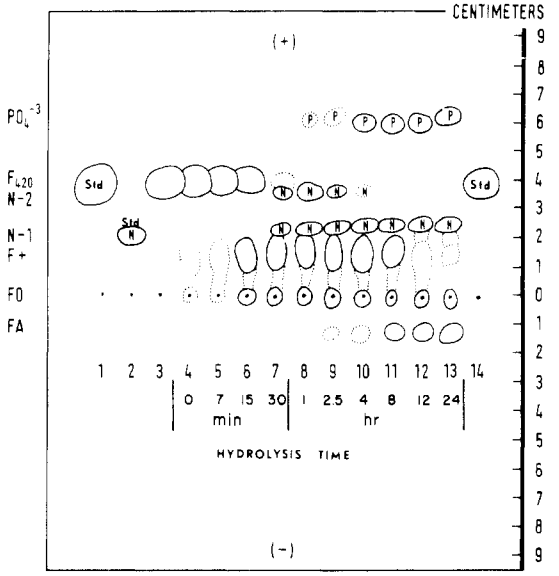


FIGURE 2: Thin-layer electrophoresis of acid-hydrolyzed coenzyme F_{420}; time-dependent formation of hydrolysis products and their intermediates. The hydrolysis procedure was performed as described in the text. Electrophoresis was accomplished on Eastman cellulose plates in buffer B for 45 min at 400 V. Numbers 1, 3, and 14 are F_{420} standards; 2 is an L-glutamic acid standard; 4 to 13 represent samples of F_{420} hydrolyzed in 1.0 N HCl in 110 °C for the indicated lengths of time. N indicates ninhydrin-positive spots. P represents inorganic phosphate spots. Dashed lines indicate spots of low intensity. Symbols for the hydrolysis products of F_{420} are presented in the left margin; each symbol corresponds to the position of the compound it represents.

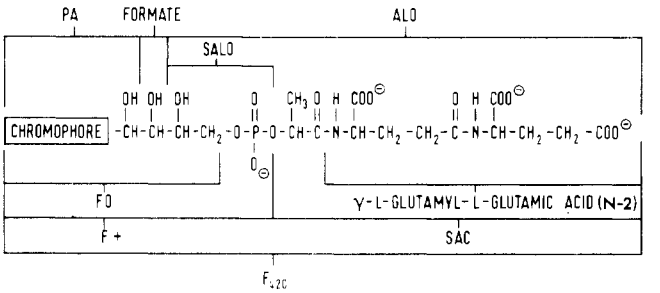


FIGURE 3: Fragments of the side chain of coenzyme F_{420}; symbols for the products of acid hydrolysis of F_{420} are indicated below the structure; symbols of the periodate oxidation fragments are shown above the structure. FA, the product of prolonged hydrolysis, is not shown.

sample was prepared for spectral analysis as described above. ^{13}C nuclear magnetic resonance spectra were obtained with a Varian Model XL-100-15 which was interfaced with Digilab NMR-3 data; a 256K disk was operated at 25.2 MHz. The proton-decoupled ^{13}C NMR spectrum was determined with an internal D_2O lock; a coaxial capillary of tetramethylsilane in CCl_4 was employed as a reference. Electron-spin resonance (ESR) spectra were recorded with a Varian E-9 instrument. The ammonium salt of F_{420} (20 mg) was reduced for analysis in 1 mL of 0.2 M potassium phosphate buffer at pH 8 under a hydrogen atmosphere with the crude cell extract (1.5 mg of protein). The reduced sample was transferred in an anaerobic chamber to a 4-mm quartz spectrometer tube which was then sealed. Ultraviolet-visible spectra were obtained with a Cary Model 14 or 118 recording spectrophotometer. For determination of pK_a values, the pH of each solution of F_{420} or its derivative was adjusted to a known pH value between 0.1 and 13.5 by an appropriate addition of HCl, acetic acid, potassium phosphate, or potassium hydroxide. Infrared (IR) spectra were obtained with a Beckman Model 12 or a Perkin-Elmer Model 472 infrared spectrometer. Each KBr pellet contained 1 mg of sample.

Materials

FMN, FAD, L-lactic acid, L-glutamic acid, and γ-L-glutamyl-L-glutamic acid were purchased from Sigma Chemical Co. Alkaline phosphatase, glutamate dehydrogenase, lactic dehydrogenase, and NAD were purchased from Boehringer Chemical Co. α-L-Glutamyl-L-glutamic acid was purchased from Schwarz/Mann Chemical Co. QAE-Sephadex A25 and DEAE-Sephadex A25 and G-15 were purchased from Pharmacia Fine Chemicals. Florisil was obtained from Fischer Scientific Co. Deuterated water was purchased either from Merck Sharp and Dohme, Canada Limited, or from Diaprep, Inc. All gasses were acquired from Linde Co. and were made oxygen free by passage through a glass column filled with copper filings at 350 °C.

Results

Purification of F_{420}. Typical results for the purification of F_{420} are presented in Table I. The yield of F_{420} was about 160 mg/kg of wet-packed cells.

The Side Chain of F_{420}. Evidence is presented below to support the structure of the side chain as diagrammed in Figure 1. A time-course study of the acid hydrolysis of F_{420} was performed as described under Methods. After hydrolysis, a sample from each vial was subjected to thin-layer electrophoresis (Figure 2). (To aid the reader in evaluating the evidence, the position of each fragment of F_{420} is diagrammed in Figure 3.) Between 15 and 30 min of hydrolysis, two main hydrolytic products were observed. The first (F+) was yellow, fluorescent, and anionic. Another fragment (SAC) was determined from a separate hydrolysis experiment and is not shown on the thin-layer plate shown in Figure 2. It was a strongly anionic compound which moved approximately 1.3 times faster than F_{420} at pH 4.4. It was ninhydrin negative and was detected with a chlorine-starch-potassium iodide spray reagent for peptides. After 30 min, SAC was almost completely hydrolyzed, and two ninhydrin-positive compounds (N-1 and N-2) began to appear. N-2 was acid labile and disappeared after 2 to 4 h of hydrolysis.

TABLE II: Quantitative Determinations of Phosphate, L-Lactate, and L-Glutamate in Hydrolyzed Preparations of F_{420} and Its Derivatives.

compd	expd mol wt	amt released/mol of acid-hydrolyzed compd (mol)		
		phosphate	L-lactate[a]	L-glutamate[b]
F_{420} (NH_4 salt)	860	1.04	0.99	2.15
F+ (NH_4 salt)	496	1.07	0.00	0.00
FO	363	0.00	0.00	0.00
SAC (Ca salt)	386	0.00	0.93	2.20
ALD (Ca salt)	546	0.75	0.88	2.05

[a] The compounds were hydrolyzed in 1.0 M HCl for 24 h at 110 °C and then neutralized with 10 N NaOH. [b] The compounds were hydrolyzed in 1.0 M HCl for 24 h at 110 °C, lyophilized to dryness, and dissolved in 0.2 M potassium phosphate (pH 8.0).

N-1 reached its maximal intensity at 2 to 4 h of hydrolysis and remained at a constant level throughout the remainder of the experiment. F+ was hydrolyzable and was found to decrease in intensity after 4 h of hydrolysis. This was the same interval during which inorganic phosphate began to appear in high amounts. FO, which also was yellow and fluorescent, was only slightly charged at pH 4.4 and was difficult to quantitate on the thin-layer plate. FA, the only compound found to be cationic, was yellow and fluorescent and began to appear in large amounts after 8 h of hydrolysis; evidence for the structure of this derivative is not presented, since it is not essential to our proposal.

N-1 had the same electrophoretic mobility as glutamic acid and was further identified and quantitated with L-glutamate dehydrogenase. F_{420} released 2.15 mol of L-glutamic acid per mol of F_{420} hydrolyzed (Table II); no free glutamic acid was found in unhydrolyzed F_{420} samples. The electrophoretic mobility (Eastman cellulose, buffer B, 90 min) of purified N-2 was compared with that of standard α- and γ-L-glutamyl-L-glutamic acid. At pH 4.4, α- and γ-L-glutamyl-L-glutamic acid have different electrophoretic mobilities (5.2 and 7.7 cm to the anode, respectively). N-2 was found to have an electrophoretic mobility identical to that of γ-L-glutamyl-L-glutamic acid. The NMR spectrum of purified N-2 was found to be identical to that of authentic γ-L-glutamyl-L-glutamic acid.

The phosphate contents of F_{420}, F+, and FO are presented in Table II. Each mol of F_{420} and F+ both released approximately 1 mol of phosphate. No phosphate was released from either compound after 7 min of hydrolysis in 0.5 M H_2SO_4, indicating the presence of a stable phosphate ester. FO released no phosphate. Alkaline phosphatase released no phosphate from F_{420} and did not change the electrophoretic mobility of it but did release phosphate from F+ (about 86% of the amount released after total hydrolysis of F+). Since the alkaline phosphatase preparation was free of phosphodiesterase activity, it was evident that F_{420} is a phosphate diester. F+, however, when treated with the enzyme was hydrolyzed, yielding a product with an electrophoretic mobility (Merck cellulose, buffer B, 2.5 h) identical to that of FO (1.0 cm to the cathode). Thus, F+ lost phosphate by hydrolysis or by phosphatase treatment to yield FO.

By comparing the elemental analysis data (Table III) of F_{420} and F+, it was evident that 13 carbons were lost when F_{420} was hydrolyzed to F+. Loss of the γLGLG moiety alone accounted for only ten carbons. A comparison of the NMR spectra of oxidized F_{420} (Figure 4) with that of F+ and N-2 revealed that the doublet in 1.57 ppm (3 protons), which was present in the spectrum of F_{420}, was absent in the spectra of both F+ and γLGLG. This doublet represented a three-carbon moiety, lactic acid. As shown in Table II, 1 mol of L-lactic acid was released during the hydrolysis of 1 mol of F_{420}. These data indicated that SAC was *N*-(*N*-L-lactyl-γ-L-glutamyl)-L-glutamic acid. The lactyl group was bound to the primary amine of γ-L-glutamyl-L-glutamic acid (γLGLG) to form SAC, which was ninhydrin negative. The results presented in Table II confirmed that 1 mol of L-lactic acid and 2 mol of L-glutamic acid were released per mole of SAC hydrolyzed. Since F_{420} was shown to be a phosphodiester which released F+ and SAC after a short period of hydrolysis, it was evident that the lactyl moiety was attached to the free primary amine group of γLGLG by an amide linkage, thus allowing the free alcohol group of the lactyl moiety to form an ester linkage with the phosphate moiety of F+.

The peaks located between 3.6 and 4.2 ppm in the NMR spectrum of F_{420} (Figure 4) indicated the presence of a polyalcohol moiety. When F_{420} and periodate-treated F_{420} were subjected to thin-layer electrophoresis, intact F_{420} had an electrophoretic mobility (4.5 cm to the anode) very much different from that to the chromophore (0.7 cm to the cathode) that was found after treatment of F_{420} with sodium periodate. In addition, a strongly anionic aldehydic compound (6.9 cm to the anode) was detected with 2,4-dinitrophenylhydrazine. Thus, F_{420} was oxidized to yield a somewhat cationic chromophoric (PA) and a strongly anionic aldehyde (ALD) (Figure 3). ALD, when acid hydrolyzed, was found to release approximately 1 mol of phosphate and L-lactic acid and 2 mol of L-glutamic acid (Table II). Results indicated that 2.15 mol of periodate was reduced per mole of F_{420} oxidized, and 5.4 mg

TABLE III: Quantitative Elemental Analyses of F_{420} and F+.

compd	mol formula	mol wt	comp (%)		
			C	H	N
F_{420} (NH_4^+ salt)					
found			40.34	5.60	12.28
expect $(NH_4^+)_3$	$C_{29}H_{45}N_8O_{18}P{\cdot}2H_2O$	860.7	40.47	5.74	13.02
F_{420} (Na^+ salt)					
found			38.96	4.35	7.58
expect $(Na^+)_4$	$C_{29}H_{32}N_5O_{18}PNa_4{\cdot}2H_2O$	897.6	38.81	4.04	7.80
F+ (NH_4^+ salt)					
found			38.67	4.92	12.93
expect NH_4^+	$C_{16}H_{21}N_4O_{10}P{\cdot}2H_2O$	496.4	38.71	5.07	11.28
F+ (Na^+ salt)					
found			35.86	3.93	7.36
expect $(Na^+)_2$	$C_{16}H_{16}N_3O_{10}PNa_2{\cdot}3H_2O$	541.3	35.50	4.10	7.76

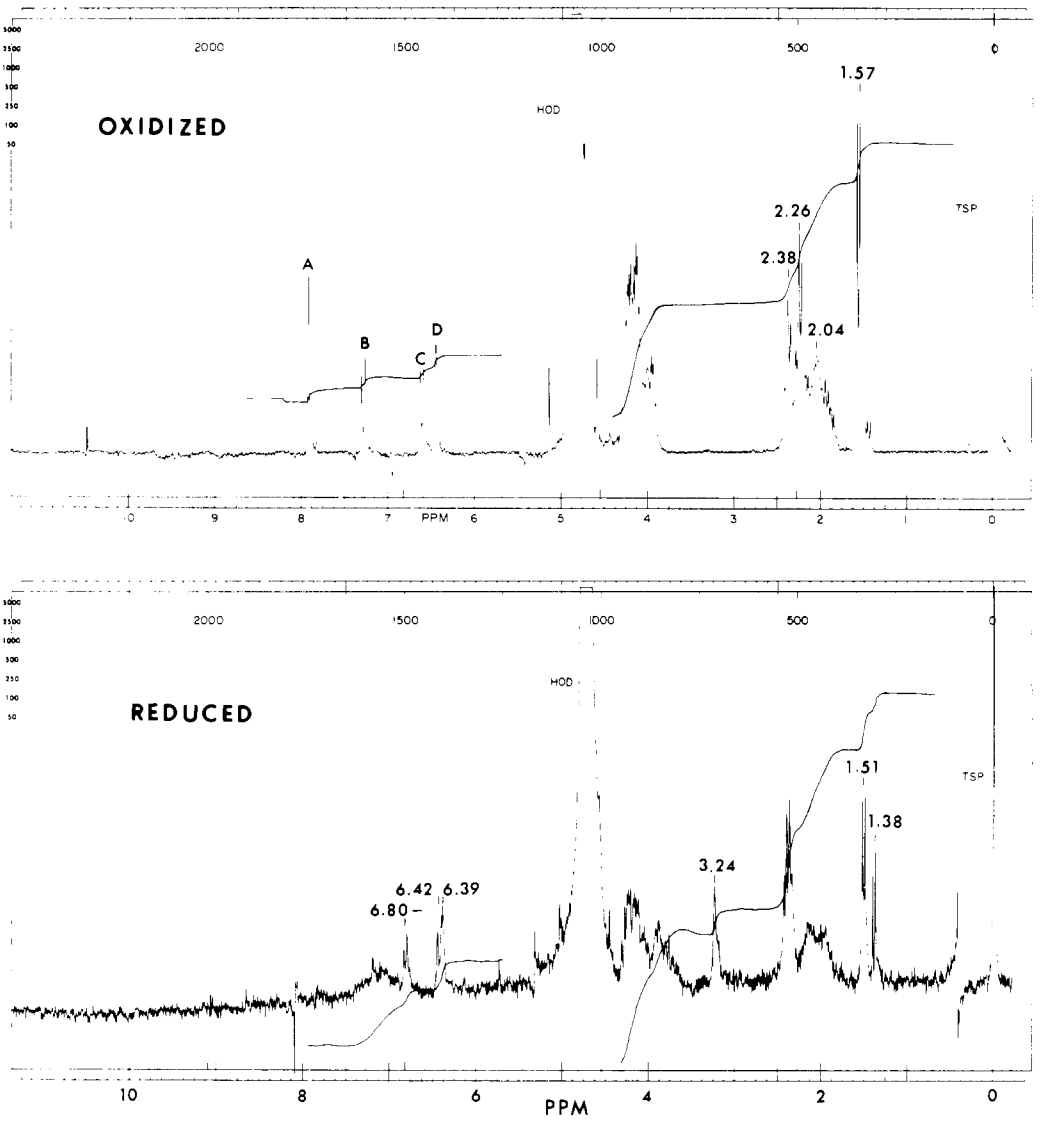
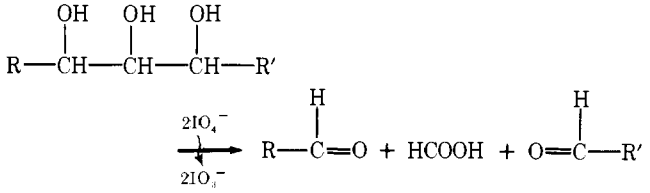


FIGURE 4: The proton NMR spectra of oxidized and reduced coenzyme F_{420}. The 220-MHz NMR spectra of oxidized (upper trace) and reduced (lower trace) F_{420} were obtained under the following conditions: (oxidized and reduced) amount F_{420}, 10 and 10 mg; sodium carbonate, 10 and 0 mg; deuterium oxide, 0.2 and 0.5 mL; spectral width, 3012 and 3012 Hz; pulse sequences, 200 and 1200; pulse width, 20 and 5 μs; pulse interval, 3 and 0.5 s; acquisition delay, 20 and 20 μs; points, 8000 and 8000; line broadening, 0.4 and 0.5 Hz; temperature, 27 and 28 °C.

of periodate-treated F_{420} was found to release formic acid, 6.1 μmol being detected. No formic acid was found in untreated F_{420} samples. These results suggested that periodate reacted as shown below.



PA was RCHO and ALD was R′CHO. F_{420}, when treated with periodate, released PA, formic acid, and ALD. The fact that 2 mol of periodate was reduced and 1 mol of formic acid was released during the oxidation of F_{420} indicated the presence of an α,γ-disubstituted, α,β,γ-triol group in F_{420} and F+. A fourth alcohol group was postulated to be involved in an ester linkage to bind phosphate; thus, FO would contain an α-substituted tetritol moiety and ALD would contain glycol aldehyde as the oxidized residue of the erythritol group linked to phosphate.

Ring Structure of F_{420}. Evidence is presented below to support the structure of the chromophore of F_{420} as presented in Figure 1. The data from elemental analyses of F_{420} and F+ are shown in Table III. The calculated values for the assigned structures of these compounds have been included for comparative purposes. The experimentally determined values are in good agreement with the calculated values. Since no attempt was made to isolate F_{420} and F+ in one particular ionized form, some values may deviate from the calculated values due to a mixture of two salt forms. These data indicate that the dihydrates of the salts of F_{420} and F+ were formed (trihydrate for the sodium salt of F+). The molecular weight values presented in Table III were used in calculating the molar concentrations of these compounds. The elemental analysis data of F+ agreed well with the previous conclusion that F+ was formed from F_{420} by the loss of a lactyl-γLGLG moiety (13 carbons, 2 nitrogens, and 8 oxygens).

The structure proposed for PA has a molecular weight of 271. An electron-impact mass (EI) spectrum of PA lends strong support for this structure. Although a molecular ion was not observable, peaks at *m/e* 253 ($M - H_2O$) and 229 ($M - C_2H_2O$) were observed. The high-resolution EI spectrum of

TABLE IV: UV-Visible Spectral Properties and pK_a Values of F_{420} and 8-Hydroxy-FMN.[a]

compd	pH	λ_{max} (ϵ), nm (mM^{-1} cm^{-1})			pK_a values
F_{420}	0.1	375 (32.8)	267 (21.2)	250 (22.6)	1.7 ± 0.2
		230 (39.7)			
	4.5	395 (27.4)	385 sh (25.7)	267 (25.4)	6.3 ± 0.1
		250 (24.0)	235 (41.5)		
	8.85	420 (45.5)	295 (12.6)	267 (25.8)	12.2 ± 0.2
		247 (37.0)			
	13.5	420 (54.3)	290 (11.4)	245 (54.6)	
8-OH FMN	−1.0	422 (33.6)	258 (25.0)	223 (32.0)	0.7
	3.0	435 (26.0)	262 (31.0)	220 (37.0)	4.8
	7.0	472 (41.0)	300 (10.7)	267 (25.5)	11.5
		252 (51.0)			
	13.0	472 (53.2)	283 (11.1)	248 (58.0)	

[a] The UV-visible spectra and pK_a values were determined as outlined under Materials and Methods. The spectral properties of 8-hydroxy-FMN were obtained from Ghisla and Mayhew (1976).

these two peaks yielded *m/e* values of 253.0480 and 229.0485. These masses agree well with the formulas $C_{13}H_7N_3O_3$ and $C_{11}H_7N_3O_3$, respectively. Therefore, the peak at *m/e* 229 probably represents the ion of the aromatic chromophore plus a proton (C + H). Since FO yielded no interpretable results from EI mass spectrometry, field-desorption (FD) mass spectrometry was employed. The low-resolution FD spectrum (15 mA) showed a main peak at *m/e* 364 (M + H). A second FD spectrum (20 mA) showed a peak at *m/e* 345 (M − H_2O) as the main peak. These results agree with the molecular weight proposed for FO of 363.

In Table IV are presented the UV-visible spectral properties and the pK_a values for the different ionic forms of F_{420} and 8-OH-FMN (Ghisla and Mayhew, 1976). The long-wavelength absorption bands for the ionic forms of 8-hydroxy-FMN (472, 435, and 422 nm) are about 50 nm greater than the analogous bands for the ionic forms of F_{420} (420, 395, and 375 nm), although the extinction coefficients for these bands are similar. This 50-nm difference is due to the nitrogen at the 5 position in 8-hydroxy-FMN. A 50-nm difference has also been observed by Spencer et al. (1976) between 5-deazariboflavin (338 and 396 nm) and riboflavin (375 and 452 nm). A comparison of the absorption bands in the UV region of the spectra show 5- to 10-nm shifts, either to the blue or to the red, but the extinction coefficients for analogous bands are quite similar. Dissociation constants were found to be similar; F_{420} yielded pK_a values of 1.7, 6.3, and 12.2, whereas 8-hydroxy-FMN showed values of 0.7, 4.8, and 11.5. The chromophore of F_{420} appeared to be in its neutral form between pH 3 and 4. The band at 395 nm and shoulder at 340–350 nm in the spectrum of F_{420} at pH 4.5 (Figure 5) are reminiscent of the 396- and 338-nm peaks (ϵ 12 000 and 11 500, respectively) for 5-deaza-FMN. The extinction coefficients are not comparable, however.

Figure 4 shows a proton NMR spectrum; oxidized F_{420} was dissolved in a 5% solution of sodium carbonate in D_2O. In this spectrum, the resonances of the methyl group of the lactyl moiety (doublet, δ 1.57, J = 7 Hz) as well as the β- (multiplet centered at δ 2.04) and the γ-methylenes (2 triplets, δ 2.26 and 2.38, J = 7 Hz) of the γ-L-glutamyl-L-glutamic acid group were evident. The complex set of peaks centered at 4.14 ppm represent nine protons (probably seven from the ribityl moiety and two from the methine groups of the γLGLG group). The methine group of the lactyl moiety may be buried under the large HOD peak.

Four aromatic protons were observable in the spectrum of F_{420}. Protons B and C (doublets, δ 7.29 and 6.61, J = 8 Hz)

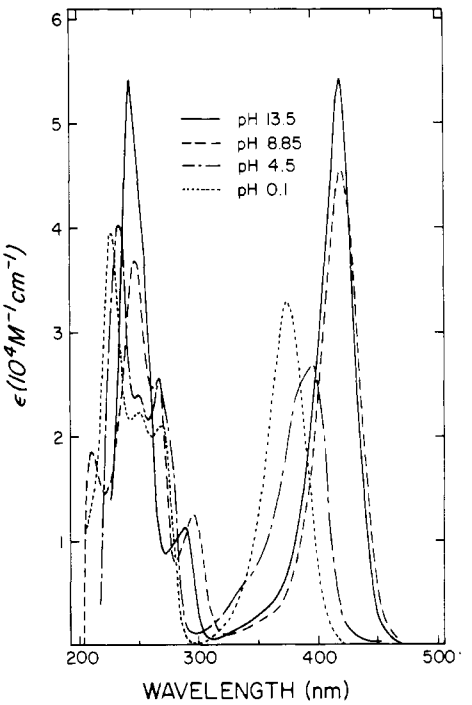


FIGURE 5: UV-visible absorption spectra of coenzyme F_{420} obtained at the indicated pH values.

appeared to be situated ortho to one another; this is indicated by their fairly large coupling constant. Protons C and D (singlet, δ 6.45) also appeared to be coupled to one another. This is evidenced by the fact that the peaks were broader and shorter than those of protons A and B. This coupling was quite small, however, and was found to be 1.4 Hz from an expanded spectrum of F_{420} dissolved in a 50% deuterated trifluoroacetic acid solution. The upfield positions of the peaks for protons C and D (relative to the peaks for protons A and B), the similarity of their chemical shifts, and the low coupling observed between these two protons indicated that protons C and D were located at positions meta to one another and that they were separated by a functional group, such as a phenolic or carbonyl group, which gave increased shielding. Protons B, C, and D were, therefore, assigned to carbons 6, 7, and 9, respectively, of the proposed structure of F_{420}. Proton A (singlet, δ 7.92) appeared not to be coupled and was, therefore, interpreted as being on a different ring from that of protons B, C, and D. It was assigned to carbon 5 of F_{420}. The observed chemical shift was in

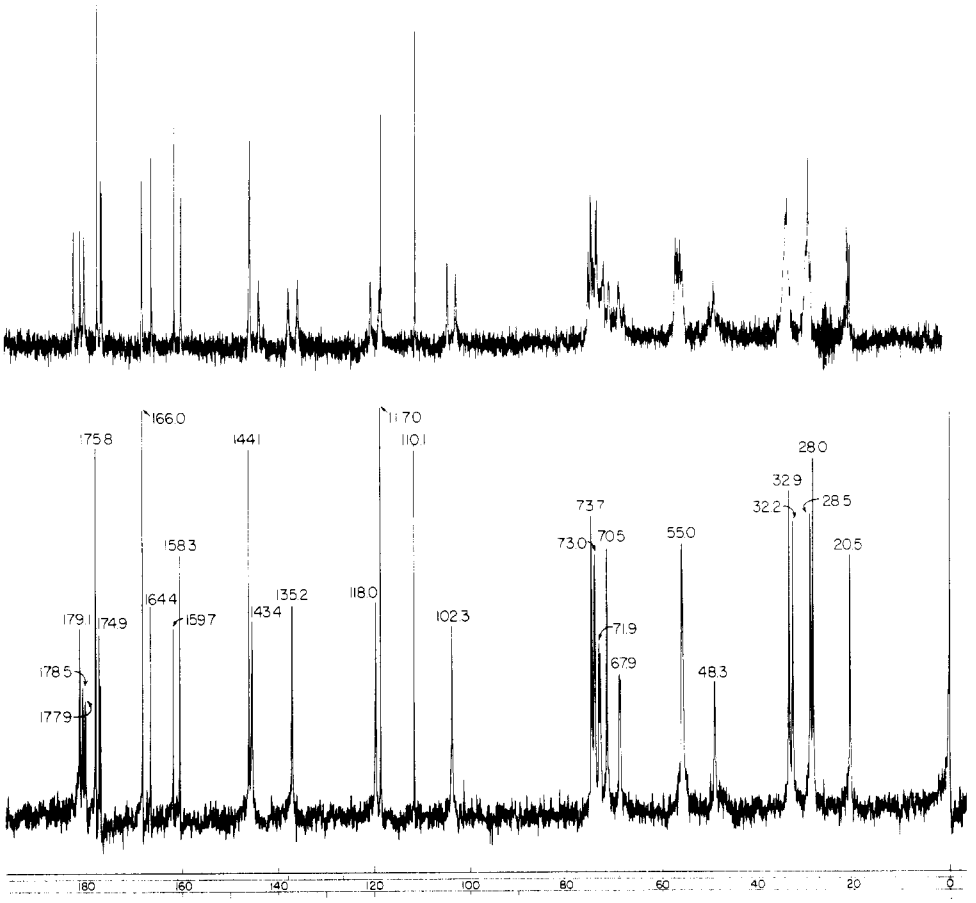


FIGURE 6: ^{13}C NMR spectra of coenzyme F_{420}. The proton-decoupled spectrum (lower trace) was determined on a 250-mg sample of the ammonium salt of F_{420} in 3.5 mL of D_2O in a 12-mm tube at a probe temperature of 28 °C with an internal deuterium lock (D_2O). A coaxial capillary of tetramethylsilane (Me_4Si) in CCl_4 was the reference. Conditions: sweep width, 6000 Hz; pulse width, 30 μs; acquisition time, 1.36 s; spin decoupler offset 45 460 Hz; noise bandwidth, 1.5 kHz; sampling 16 000 points. The off-resonance proton-decoupled spectrum (upper trace) was determined on a 250-mg sample of F_{420} (ammonium salt) in 3.5 mL of D_2O at a temperature of 30 °C in a 12-mm tube. Conditions were the same as for the proton-decoupled spectrum, except that the spin decoupler was offset 38 000 Hz.

TABLE V: Comparison of the Chemical-Shift Values of the Aromatic Protons in the NMR Spectra of F_{420} and Its Derivatives.

sample	solvent[a]	chem shifts (ppm) of aromatic protons A	B	C	D
F_{420}	50% CF_3COOD[b]	9.27	8.15	7.43	7.63
	D_2O	8.29	7.60	6.93	7.02
	5% Na_2CO_3	7.92	7.29	6.61	6.45
F+	5% Na_2CO_3	7.98	7.32	6.62	6.48
	7.5% Na_2CO_3	7.68	7.07	6.46	6.32
FO	7.5% Na_2CO_3	7.69	7.09	6.47	6.31

[a] All solutions were prepared with D_2O as solvent. [b] This spectrum was obtained on a Bruker, Model WH-90, NMR spectrometer. All other spectra were obtained as described under Materials and Methods.

agreement with its position in the proposed structure.

Table V shows a comparison of the chemical-shift values for the aromatic protons of F_{420}, F+, and FO. A comparison of the resonances of the aromatic protons of F_{420} in solvents with different pH values showed that the chemical shifts of the peaks in this region were pH dependent. The aromatic region of the NMR spectrum of FO was almost identical to that of F+. Also present in the spectra of F+ and FO were seven protons between 3.5 and 4.5 ppm, indicating the presence of the ribityl moiety.

Figure 6 presents the proton-decoupled and off-resonance proton-decoupled ^{13}C NMR spectra of F_{420}. Since the off-resonance proton-decoupled spectrum allows ^{13}C–^{1}H coupling, the methyl, methylene, methine, and quaternary carbon resonances could be established.

The chemical-shift assignments for F_{420} shown in Figure 7 (compound A) were deduced from the ^{13}C–^{1}H couplings observed in the off-resonance proton-decoupled spectrum, as well as by comparing the chemical shifts for F_{420} resonances with those of FMN and quinine (Johnson and Jankowski, 1972). The four doublets, located at 174.9, 73.0, 71.9, and 67.9 ppm in the proton-decoupled spectrum, indicated a ^{13}C–^{31}P coupling (J = 6.1, 5.2, 7.9, and 5.1 Hz, respectively) between the carbon atoms of the ribityl and lactyl moieties assigned to these resonances and the phosphorus of the phosphate group.

The proton-decoupled spectrum of F_{420} shows the expected 29 singlet absorptions. By comparison of the proton-decoupled and off-resonance proton-decoupled spectra, it is obvious that the two α-methine resonances of the glutamyl moieties are located under the broad peak located at 55.0 ppm.

The resonances for the two glutamic acid groups were assigned by comparison with the resonances of L-leucyl-L-seryl-L-glutamic acid (Gurd et al., 1971) compound C, at pH 4.5. This was also the pH of the F_{420} solution with which the ^{13}C NMR spectra were obtained. Due to their similarities, the

resonances for the α-carboxylic acid carbons (177.9 and 178.5 ppm) could not be assigned unequivocally to a specific glutamyl moiety. This was true also of the resonances for the α-methine and β- and γ-methylene carbons. The amide carboxyl carbons for the lactyl and glutamyl moieties showed resonances at 174.9 and 175.8 ppm, respectively. The lactyl carbonyl carbon resonance was assigned on the basis of its apparent coupling with phosphorus. The lactyl methine and methyl carbon resonances (73.0 and 20.5 ppm, respectively) were assigned on the basis of a comparison with the assigned resonances for acetoin (Johnson and Jankowski, 1972). A comparison with the resonances of the ribityl carbons of FMN (Breitmaier and Voelter, 1972) compound B allowed the assignment of the resonances for the ribityl carbons of F_{420}.

By comparing the observed resonances with those assigned (Johnson and Jankowski, 1972) for FMN, nicotinamide, 2 methyl-6-methoxyquinoline, and quinine, tentative assignments for the 11 ring carbons of F_{420} were made. The resonances assigned to carbon-2, -4, and -10a of F_{420} were compared with the resonances of analogous carbons for FMN. The chemical shifts observed for FMN, however, were at somewhat higher fields (5–7 ppm). The resonance at 158.3 ppm was assigned to carbon-10a, and the resonance at 159.7 was assigned to carbon-8. The resonances at 144.1, 117.0, and 110.1 ppm in the proton-decoupled spectrum remained singlets in the off-resonance proton-decoupled spectrum, indicating the quaternary nature of their respective carbons. The low-field resonance, 144.1 ppm, was best assigned to carbon-9a, since it is attached to a deshielding nitrogen. The resonances at 110.1 and 117.0 ppm were assigned to carbon-5a and -4a, respectively. Carbon-4a was assigned the low-field resonance due to its proximity to the deshielding nitrogens of the pyridopyrimidinoid part of the ring. The remaining four carbons showed resonances at 102.3, 118.0, 135.2, and 143.4 ppm, which split in the off-resonance decoupled spectrum (doublets, J = 40–50 Hz). They corresponded to the four aromatic resonances observed in the proton NMR spectrum of F_{420}. The resonances at 102.3, 118.0, and 135.2 ppm were assigned to carbon-9, -7, and -6, respectively. These assignments [made on the basis of a comparison with the assigned resonances for 2-methyl-6-methoxyquinoline, quinine, and nicotinamide (Johnson and Jankowski, 1972)] allowed the assignment of the remaining resonance at 143.4 to carbon-5 of F_{420}. Since resonances can be assigned to the carbon atoms of F_{420} with a fair degree of certainty, the structure proposed for F_{420} was supported by the ^{13}C NMR spectra.

The infrared (IR) spectra of F+ and FMN showed many similarities, giving evidence for the close relationship between their structures. Two carbonyl bands (positions 2 and 4 in the ring) were observed at 1730 and 1660 cm^{-1} for FMN and at 1700 and 1665 cm^{-1} for F+. An intense band at 1610 cm^{-1} in the spectrum of F+ was absent in the spectrum of FMN. This band may be due to a carbon–nitrogen double-bond absorption. A strong band at 1540 cm^{-1} (1550 cm^{-1} in the spectrum of FMN) probably represented an amide II absorption band.

Reduced F_{420}. The stoichiometric transfer of electrons from hydrogen to F_{420} was measured manometrically with the hydrogenase reaction; 13.6 μmol of hydrogen was utilized during the reduction of 13.6 μmol of F_{420}, resulting in the net transfer of two electrons per molecule of F_{420}.

Spencer et al. (1976) observed that 5-deazariboflavin, when reduced with sodium borohydride, lost a peak (singlet, 1 proton, δ 8.8) in the aromatic region of its NMR spectrum. This peak had been assigned to the proton attached to carbon-5 of the deazaisoalloxazine ring. An additional peak (singlet)

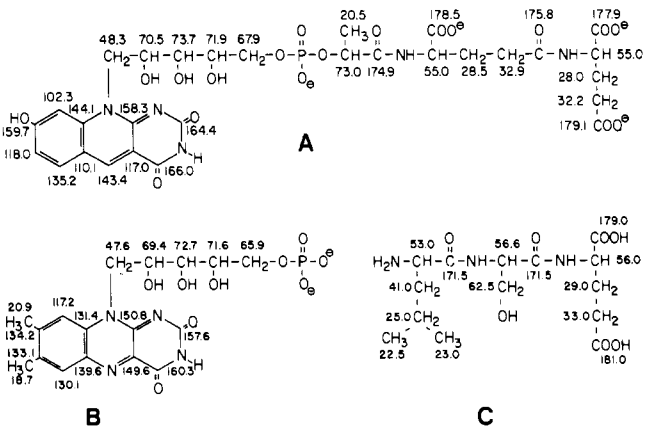


FIGURE 7: ^{13}C NMR resonances for F_{420} and model compounds.

representing two protons appeared at 3.57 ppm. They interpreted this to indicate that one of the sites of reduction for dihydrodeazariboflavin was at the C-5 position. A similar observation was made with the NMR spectrum of reduced F_{420} (Figure 4). Peak A of F_{420} was lost upon reduction with hydrogenase; however, peaks B, C, and D (6.80, 6.42, and 6.39 ppm, respectively) remained. An additional peak (singlet) representing two protons appeared at 3.24 ppm. Air oxidation of the same sample of reduced F_{420} resulted in peaks at 8.6, 7.78, 7.15, and 7.03 ppm. These resonances were typical of protons A, B, D, and C, respectively, for F_{420} at low pH. The peak at 3.24 ppm was lost upon oxidation. During the preparation of reduced F_{420}, some hydrolysis appears to have taken place, as evidenced by the two doublets at 1.51 and 1.38 ppm. These data support the proposal that F_{420} is an analogue of 5-deazariboflavin and that, like 5-deazariboflavin, one of the sites of reduction is the C-5 position.

The absorption spectra of reduced F_{420} at pH 5.9 and 8.8 are shown in Figure 8. The insert shows the absorption spectra of 1,5-dihydro-5-deazariboflavin at pH 5.7 and 8.6 (Spencer et al., 1976). Except for small differences in absorption maxima and extinction coefficients, the spectra appear to be almost identical. Spencer et al. (1976) determined a pK_a value of 7.2 for dehydrodeazariboflavin. This was based upon the loss of the 262-nm band when going from pH 8.6 to 5.7. They assigned the spectrum at pH 5.7 to the neutral form and the spectrum at 8.6 to the anion. A similar pK_a near neutrality in dihydroisoalloxazine structures is indicative of ionization, as well as reduction at the N-1 position (Ghisla et al., 1973). Thus, the pK_a value of 7.2 supported the 1,5-dihydro structure. A similar pK_a value of 6.9 was determined for reduced F_{420}. This determination was made by following the increase in fluorescence (λ_{max} 405 nm) when the pH was raised from 6.0 to 8.6. The 258-nm band in the spectrum of reduced F_{420} at pH 8.8 was lost when the pH was lowered to 5.9; this also indicated a pK_a near neutrality. The extreme similarity between the absorption spectra as well as the pK_a values of reduced F_{420} and 1,5-dihydro-5-deazariboflavin support the proposal that F_{420} is an analogue of 5-deazariboflavin and that the sites of reduction are at positions 1 and 5 of the proposed structure of F_{420}. No electron-spin resonance (ESR) signal was observed with a 12 mM solution of F_{420} (in 0.2 M phosphate buffer, pH 8.0) either in the fully oxidized state or as an approximately 95% reduced solution.

Discussion

To our knowledge this is the first report of a naturally occurring deazaflavin coenzyme. That it should be found in a methanogen may be significant in light of the recent work of

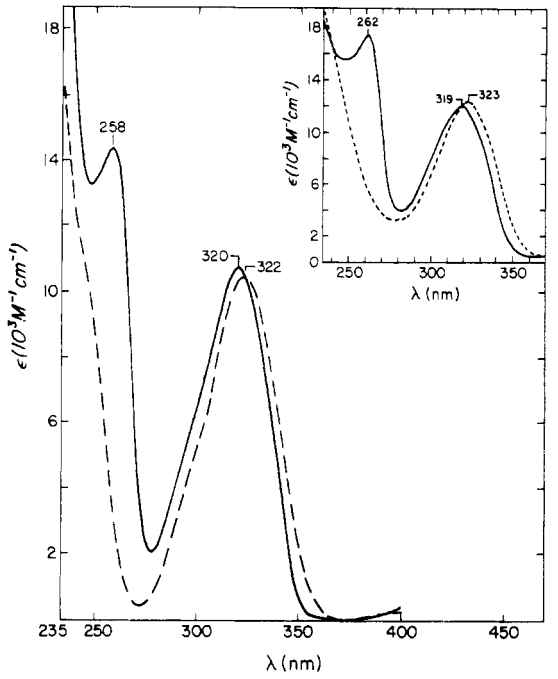


FIGURE 8: The UV-visible absorption spectra of reduced F_{420} and 1,5-dihydro-5-deazariboflavin. The spectra were obtained in 0.1 M phosphate buffer at pH 5.9 (broken line) and 8.8 (solid line). The spectra were measured in 1-cm anaerobic quartz cuvettes. F_{420} in a hydrogen atmosphere was reduced by the addition of 110 μg of *Methanobacterium* cell extract. A buffer solution, with enzyme but without F_{420} added, was used as a blank. The insert shows the UV-visible spectra of 1,5-dihydro-5-deazariboflavin at pH 5.7 (broken line) and 8.6 (solid line), which were obtained from Spencer et al. (1976).

Fox et al. (1977), which indicates that the methanogens are only distantly related to conventional prokaryotes.

F_{420} is a curious coenzyme with a long side chain ending in a diglutamyl moiety, reminiscent of the folates, and with a modified flavin chromophore which appears capable of participating only in two electron-transfer reactions. Proof of the proposed structure of F_{420} must await chemical synthesis of the coenzyme. We were unable to obtain FO in sufficient quantities to carry out the pertinent spectrometric assays to establish unequivocally that the isomeric structure of the poly(hydroxypentyl) moiety is identical to that of riboflavin. However, the evidence presented makes this structure certainly a likely one. The fluorescence properties of the chromophore in the oxidized and reduced forms will be presented elsewhere.

That F_{420} reduces at the 1 and 5 positions and has been shown to be involved in low-potential electron-transport reactions is significant, since 5-deazariboflavin has been found to have oxidation-reduction properties significantly different from those of riboflavin. Although anaerobic precautions were taken in studying the properties of the reduced chromophore, its reactivity with O_2 was very slow.

Fischer et al. (1976) measured the redox potential for 5-deazariboflavin, $E_0' = -0.310$ V at pH 8.6, and Stankovich and Massey (1976) determined the value at pH 7.0 to be −0.275 V. Blakenhorn (1976) has established a value of −0.32 V from electrochemical measurements of the 5-deazaflavin 3-sulfonate; the sulfonate moiety lowers the potential by about 30 mV (Müller and Massey, 1969). The redox potential of F_{420} ($E_0' = -0.373$ V) was determined from electrochemical measurements (Doddema and Vogels, personal communication). This potential is in agreement with the findings of Tzeng et al. (1975a,b) that F_{420} can rapidly reduce NADP with electrons generated from H_2. In addition, hydrogenase catalyzes a rapid reduction of protons with electrons generated from reduced F_{420}.

Flavins are known for their ability to form a free radical when partially reduced; a 10^{-3} M solution of partially reduced 5-deazariboflavin (approximately 50% reduced) was reported by Edmondson et al. (1972) to possess no stable free radical form, as determined by ESR spectroscopy, either in the free or bound state. They did report, however, that a neutral free radical could be formed during the photoreduction of 5-deazariboflavin. Massey and Hemmerich (1978) have reported on the use of photoreduced deazaflavins to reduce a variety of redox proteins. A 12 mM solution of F_{420}, when reduced approximately 95%, also shows no ESR signal. Gibson et al. (1962) easily observed an ESR signal in a 1.3 mM solution of FMN (dissolved in 0.12 M phosphate buffer, pH 6.2, at 25 °C) which was 90% reduced using sodium dithionite. Because partially reduced 5-deazariboflavin shows no ESR signal, it has been concluded by Spencer et al. (1977) that 5-deazaflavins have no thermodynamically accessible one-electron chemistry (except by photoreduction) and that they are restricted to net two-electron processes. The UV-visible absorption maxima at 570 and 900 nm of the flavin semiquinone can be readily observed in solutions (approximately 50% reduced) of 4 mM FMN dissolved in 0.12 M phosphate buffer (pH 6.3) (Gibson et al., 1962). No long-wavelength absorbance is observed in similar solutions of 5-deaza-FMN (Edmondson et al., 1972) or F_{420} when 50% reduced, providing additional evidence that neither compound forms a stable semiquinone upon reduction.

Recent, additional evidence supports our conclusion that F_{420} is a derivative of 8-hydroxy-7-demethyl-5-deaza-FMN; the spectrum of F_{420} is identical to that of synthetic 8-hydroxy-5-deazariboflavin but is shifted 3 to 6 nm (Christopher Walsh, personal communication). It appears likely that this shift may be due to the 7-CH_3 group rather than to a contribution from the side chain.

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Ca^{2+} Binding to Porcine Pancreatic Phospholipase A_2 and Its Function in Enzyme–Lipid Interaction†

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ABSTRACT: In addition to the Ca^{2+} ion bound in the active site, porcine pancreatic phospholipase A_2 has been assumed to possess a second metal-ion binding locus, also specific for Ca^{2+}, which enables the enzyme to interact with organized lipid-water interfaces at alkaline pH [van Dam-Mieras, M. C. E., Slotboom, A. J., Pieterson, W. A., and de Haas, G. H. (1975), *Biochemistry 14,* 5387-5394]. Because this interaction in the absence of Ca^{2+} is governed by the pK of the α-NH_3^+ group of the N-terminal L-Ala[1] residue, the binding of this latter Ca^{2+} ion and its effect on the pK of the α-NH_3^+ group were studied. Titration studies of protons released during tryptic activation of the zymogen and of the ^{13}C chemical shift of a ^{13}C-enriched N-terminal L-Ala[1] residue in phospholipase A_2 showed that, specifically, Ca^{2+} ions increase the pK of the α-NH_3^+ group from 8.4 to 9.3. The pK values of the α-NH_3^+ groups of [D-Ala[1]]phospholipase A_2 and DL-Ala^{-1}-phospholipase A_2, however, decreased considerably upon the addition of Ca^{2+}. Ultraviolet difference spectroscopy suggested that the second Ca^{2+} ion binds close to the single Trp[3] residue and a $K_D \simeq 20$ mM was estimated at pH 7.5. In the presence of micelles of the substrate analogue *n*-hexadecylphosphorylcholine, it was found by equilibrium dialysis that at pH 8 phospholipase A_2 binds two Ca^{2+} ions/mol of enzyme, whereas 1-bromo-2-octanone-inhibited phospholipase A_2 binds only one Ca^{2+} ion/mol of protein. Similar experiments at pH 6 revealed binding of only one Ca^{2+} ion/mol of native phospholipase A_2, whereas no Ca^{2+} binding could be measured for 1-bromo-2-octanone-inhibited phospholipase A_2. Moreover, a strong synergistic effect of the micellar lipid on the binding of both Ca^{2+} ions was observed. Finally, the observations from microcalorimetry that at pH 10 1-bromo-2-octanone-inhibited phospholipase A_2, lacking the first Ca^{2+}-binding site, only binds to micelles in the presence of Ca^{2+} are in good agreement with the existence of a second Ca^{2+} binding site on phospholipase A_2.

Phospholipases A_2 (EC 3.1.1.4) from different origins catalyze the specific hydrolysis of fatty acid ester bonds at the 2 position of 3-*sn*-phosphoglycerides. The pancreatic enzyme shows an absolute requirement for Ca^{2+} ions which bind in a 1:1 molar ratio to the protein. Although Ba^{2+} and Sr^{2+} bind with the same affinity, these metal ions are pure competitive inhibitors and no hydrolytic activity is found. Mg^{2+} ions do not bind at all. The unusually high specificity of phospholipase A_2 for Ca^{2+} points to a specific function of this metal ion in the catalytic event. Spectroscopic evidence has been reported for the perturbation of histidine and tyrosine residues upon Ca^{2+} binding (Pieterson et al., 1974a). Active-site-directed irreversible inhibitors such as the halo ketones *p*-bromophenacyl

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