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Uptake, Transbilayer Distribution, and Movement of Cholesterol in Growing *Mycoplasma capricolum* Cells†

Sanda Clejan, Robert Bittman,* and Shlomo Rottem*

ABSTRACT: The sterol-requiring mycoplasma, *M. capricolum,* was adapted to grow in a medium containing low fetal calf serum (FCS) concentrations, providing cells in which unesterified cholesterol comprised only about 3.6% by weight of the total membrane lipids. The native strain grown with 10% FCS contained a sixfold higher cholesterol concentration than the adapted strain. When an early exponential-phase culture of the adapted strain was transferred to a medium containing 10% FCS, cell growth was stimulated and the cells accumulated cholesterol into their cell membrane. Alteration of cell metabolism by treatment of the adapted culture with chloramphenicol, valinomycin, nonactin, or gramicidin at 37 °C, or transfer to 4 °C; resulted in an almost complete inhibition of growth and a partial inhibition of cholesterol uptake, suggesting that the adapted cells incorporate part of their cholesterol in a growth-dependent process and not only by a physical process involving adsorption of the sterol from the growth medium into the membrane. The rate of cholesterol translocation from the outer to the inner half of the lipid bilayer was monitored by stopped-flow kinetic measurements of the association of the polyene antibiotic, filipin, with free cholesterol in intact cells and isolated membranes. The cholesterol distribution in the two halves of the bilayer was almost invariant after only 1 h of incubation with medium containing 10% FCS despite a dramatic rise in cholesterol content, approaching that in the native strain. However, when growth was inhibited by chloramphenicol, valinomycin, nonactin, or gramicidin, cholesterol remained localized predominantly in the outer half of the bilayer. These studies establish that free cholesterol is translocated rapidly from the external surface of the bilayer of *growing M. capricolum* cells at 37 °C.

Mycoplasmas are unique among prokaryotes in requiring cholesterol for growth (Razin, 1975). Mycoplasmas lack the ability to synthesize cholesterol and, therefore, require an exogenous source of cholesterol, usually supplied by serum lipoproteins present in the growth medium (Slutzky et al., 1977). The cholesterol taken up from the lipoproteins or from any other exogenous source is incorporated exclusively into the cell membrane. There is no doubt that the sterol is first incorporated into the outer half of the lipid bilayer. Kinetic analysis of the association of the polyene antibiotic, filipin, with cholesterol in intact cells and isolated membranes of *Mycoplasma gallisepticum* showed that cholesterol is distributed symmetrically between the inner and outer halves of the lipid bilayer of intact *M. gallisepticum* cells (Bittman and Rottem, 1976). This suggests that translocation of the cholesterol from the outer to the inner half of the bilayer takes place. The presence of about 50% of the membrane cholesterol in the inner half of the lipid bilayer of *M. gallisepticum* has also been demonstrated by experiments measuring the exchange of labeled cholesterol between resting cells or isolated membranes with unlabeled cholesterol in high-density lipoprotein (Rottem et al., 1978). In these studies, the rate of movement of cholesterol from the inner to the outer half of the lipid bilayer was found to be very slow or nonexistent, resembling that recorded in influenza virus membranes (Lenard and Rothman, 1976). These findings imply that the translocation of cholesterol across the lipid bilayer may be much faster in growing cells than in resting cells. The ability to alter the cholesterol content of some mycoplasma species by adapting the cells to grow with very low cholesterol (Rottem et al., 1973) provided a most useful model for establishing the rate of cholesterol translocation in growing mycoplasma cells. In this report, we show that the rapid reaction between filipin and cholesterol in intact cells and isolated membranes of *M. capricolum* can be analyzed to lead to conclusions concerning the localization of cholesterol at intervals during growth stimulation. The advantages of using the cholesterol-depleted *M. capricolum* cells to define the factors controlling the uptake of cholesterol from the growth medium are discussed.

Materials and Methods

Growth of the Organism and Isolation of Membranes. Mycoplasma capricolum (California kid) was grown in

† From the Department of Chemistry, Queens College of the City University of New York, Flushing, New York 11367 (S.C. and R.B.), and the Biomembrane Research Laboratory, Department of Clinical Microbiology, the Hebrew University-Hadassah Medical School, Jerusalem, Israel (S.R.). *Received June 6, 1978.* This work was supported in part by grants from the City University of New York Faculty Research Award Program, Biomedical Research Support Grant No. RR 07064, and The United States-Israel Binational Science Foundation (BSF).

TABLE I: Growth and Lipid Composition of Native and Adapted *M. capricolum* Cells.

strain	% FCS in medium	A_{640}[a]	phospholipid (μg/mg of cell protein)	free cholesterol[b] (μg/mg of cell protein)	free cholesterol/phospholipid[c] (mol/mol)
native	10.00	0.21	80.2	42.3	1.02
adapted	1.25	0.12	50.0	7.5	0.29
adapted	0.62	0.10	48.0	6.2	0.25

[a] A_{640} was measured after 15 h of growth. [b] The content of free cholesterol was calculated based on a free cholesterol to cholesterol ester molar ratio of 1.1, which was determined colorimetrically as described under Materials and Methods. An average molecular weight of 626 was assumed for the cholesterol esters. [c] An average molecular weight of 750 was assumed for the phospholipids.

200–400-mL volumes of a modified Edward medium (Razin and Rottem, 1975) containing 0.6–10% of fetal calf serum (FCS,[1] Grand Island Biological Co.), 1% fatty-acid-poor bovine serum albumin (Sigma Chemical Co.), and 25 μg/mL each of palmitic and oleic acids (Sigma Chemical Co.). To test for cell leakiness, in some experiments 0.1 μCi/mL [*6-methyl*-^{3}H]thymidine (10 Ci/mmol, New England Nuclear Corp.) was added. Growth was determined by measuring the absorbance of the culture at 640 nm. The organisms were harvested after 15–20 h of incubation by centrifugation at 12 000*g* for 10 min at 4 °C. The cells were washed once in 5 mL of 0.25 M NaCl solution, treated at 37 °C for 10 min with deoxyribonuclease (50 μg/mL, Sigma Chemical Co.) in the presence of 20 mM $MgCl_2$, and then washed once and resuspended in 4 mL of 0.25 M NaCl solution. Membranes were prepared from portions of the diluted cell suspensions by ultrasonic disruption for four 15-s periods at 0 °C in a Branson Model S-110 sonicator. The absorbance values of membrane suspensions at 500 nm were 80–90% lower than those of cell suspensions containing the equivalent mass per milliliter.

Protein and Lipid Analysis. Protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard. Lipids were extracted in chloroform–methanol (9:1, v/v). The solvent was evaporated under nitrogen, and the dried lipid was dissolved in 1 mL of glacial acetic acid. The total cholesterol concentration in the extracted lipids was measured colorimetrically (Zlatkis and Zak, 1969). Unesterified cholesterol was separated from esterified cholesterol by thin-layer chromatography on silica gel G plates using benzene–ethyl acetate (5:1, v:v). The free and esterified cholesterol were scraped from the plates and extracted, and their relative concentrations were determined as described previously (Bittman and Rottem, 1976). Phospholipid phosphorus was measured by the method of Taussky and Shorr (1953).

Kinetic Measurements. Filipin complex (lot no. 8393-DEG-11-8) was obtained from the Upjohn Co. and was purified as described previously (Bittman and Rottem, 1976). Stock solutions of filipin were prepared in dimethylformamide. The final concentration of filipin, after mixing with cells or membranes, was 16 μM, and the final concentration of dimethylformamide was 0.3% (v/v). Initial rates of filipin–cholesterol association were measured at 358 nm at 10 °C with a stopped-flow spectrophotometer (Durrum Instrument Corp.) equipped with a Tektronix storage oscilloscope and a Polaroid camera. A signal of 1.6 V corresponded to 100% transmittance when buffer was in the stopped-flow cuvette. Cells or membrane preparations were diluted with 0.40 M sucrose–10 mM sodium phosphate–20 mM $MgCl_2$ solution (pH 7.0) to give a total cholesterol concentration ranging from about 1 to 4 μM. At least six measurements of the initial rates of filipin binding were made at each cholesterol concentration. The initial rates of disappearance of free filipin and the second-order rate constants for filipin–cholesterol association were calculated as described previously (Bittman and Rottem, 1976). The pressure used to charge the air actuator assembly was reduced to 30 psi to minimize cell shearing. No transmittance changes were detected after the initial 100- to 150-ms disturbance period had elapsed when cells or membranes were mixed with buffer solution in the stopped-flow apparatus.

Chloramphenicol and Ionophore Treatment. Chloramphenicol, valinomycin, or nonactin (all obtained from Sigma Chemical Co.) and gramicidin (Mann Research Laboratories) were added to the growing cultures as a solid (chloramphenicol) or as an ethanolic solution (valinomycin, nonactin, and gramicidin). Ethanol did not exceed a final concentration of 0.05% (v/v). Serum was added to the growth medium after the time intervals indicated in Tables III and IV. The leakage of K^+ from the valinomycin-, nonactin-, and gramicidin-treated cells was determined by monitoring the K^+ concentration in the supernatant from the 12 000*g* centrifugation. A Model 303 Perkin-Elmer atomic absorption spectrometer equipped with an automatic null recorder was used to measure K^+. The extent of ionophore-mediated K^+ efflux after 30 min of incubation with FCS and valinomycin (10 μM) or nonactin (10 μM) was approximately 30% of the total K^+ content of the cells and about 42% when treated with 3 μM gramicidin. The ionophore-mediated K^+ efflux did not exceed 65% after 4 h of incubation. The total trapped K^+ concentration, determined by lysis with 1-propanol (1:1, v/v) and comparison with standards in the same medium, ranged from 130 to 165 mM. To determine whether the chloramphenicol- and ionophore-treated cells remained intact, the percent efflux of [^{3}H]thymidine-labeled components and of NADH oxidase into the supernatant obtained after centrifugation at 12 000*g* for 20 min was determined. Total [^{3}H]thymidine-labeled components and NADH oxidase activity were found by disrupting intact cells by prolonged sonication. Furthermore, no transmittance changes at 450 nm were observed after the initial rapid disturbance period when filipin was mixed with ionophore-treated cells in the stopped-flow apparatus. Filipin does not absorb at this wavelength, but turbidity changes corresponding to membrane disruption could have been detected. The initial rate of filipin–cholesterol association in ionophore-treated cells remained first order in each reactant, and the second-order rate constants were similar in magnitude to those in untreated cells. The viability of the cells, as determined by the colony-count technique (Butler and Knight, 1960), was reduced by approximately three orders of magnitude by incubation with 10 μM valinomycin for 3 h at 37 °C.

Results

Adaptation. Serial passages of the sterol-requiring mycoplasma, *M. capricolum*, in a modified Edward medium con-

[1] Abbreviation used: FCS, fetal calf serum.

TABLE II: Second-Order Rate Constants for Association of Filipin and Free Cholesterol in Intact Cells and Isolated Membranes of the *M. capricolum* Adapted Strain Transferred to a Cholesterol-Rich Medium.[a]

time of incubation in 10% FCS (h)	A_{640}	free cholesterol (μg/mg of membr protein)	k_{cells}[b] (M^{-1} s^{-1})	k_{membr}[b] (M^{-1} s^{-1})	k_{cells}/k_{membr}[c]
0	0.08	33	5.0×10^4	7.1×10^4	0.73 ± 0.08 (14)
1	0.16	44	4.3×10^4	8.3×10^4	0.53 ± 0.04 (9)
2	0.24	92	4.0×10^4	8.8×10^4	0.45 ± 0.06 (16)
4	0.26	127	4.4×10^4	9.1×10^4	0.45 ± 0.07 (16)

[a] The initial rates of filipin-free cholesterol association were measured at 10 °C at various cholesterol concentrations in intact cells and isolated membranes obtained from at least nine different cell cultures, each incubated with medium containing 10% FCS for the indicated period of time. The number of cell cultures investigated is indicated in parentheses in the last column. [b] A representative example of second-order rate constants analyzed from one culture. [c] Average ratio of second-order rate constants. Error limits of the kinetic data are standard errors of the mean.

taining bovine serum albumin, palmitic and oleic acids, and decreasing concentrations of FCS were made in order to adapt the cells to grow in a cholesterol-poor medium. The FCS concentration was decreased from 10 to 0.62% by growing the cells in media containing one-half of the FCS concentration of the previous medium. At each FCS concentration two passages were made. Cells adapted to a cholesterol-poor medium containing 0.62% FCS grew more slowly than cells maintained in a cholesterol-rich medium containing 10% FCS, and their cultures reached the stationary phase of growth at a lower turbidity than cultures of the native strain. The native strain grown with 10% FCS contained about six times more cholesterol and had a cholesterol to phospholipid molar ratio fourfold higher than the adapted cells (Table I). Poor growth was obtained in a medium containing 0.3% FCS. Since the kinetic experiments require membranes with a cholesterol content sufficient to produce a change in absorbance when complexed with filipin, we carried out experiments with the adapted strain grown with 1.25% FCS.

Transfer to Cholesterol-Rich Medium. When an early logarithmic culture of *M. capricolum* was transferred to the cholesterol-rich medium (containing 10% FCS), the growth rate was markedly enhanced (Figure 1). This was accompanied by a marked increase in the free cholesterol content from about 28 μg/mg of membrane protein at zero time to about 127 μg/mg of membrane protein after 5 h of growth (Figure 1, Table II). The free to esterified cholesterol molar ratio was 1.1, and it was maintained throughout the experiment. When the culture was transferred to cholesterol-rich medium, but maintained at 4 °C, neither growth nor an increase in cholesterol content occurred. In order to examine whether active cell growth is necessary for the incorporation of cholesterol into the cell membrane, chloramphenicol (100 μg/mL) or the ionophores valinomycin (10 μM), nonactin (10 μM), or gramicidin (3 μM) were added to the adapted cells, and the cells were transferred to the cholesterol-rich medium. Cell growth and protein synthesis at 37 °C were completely inhibited under these conditions, but cholesterol accumulation into the cell membrane was only partially inhibited (Table III). The inhibitory effect of chloramphenicol on cholesterol incorporation was more pronounced when the inhibitor was added to the cells 30 min before the transfer to the cholesterol-rich medium. Phospholipid biosynthesis was, however, inhibited by varying degrees. The order of inhibition was valinomycin > nonactin > gramicidin > chloramphenicol, resulting in the increase in phospholipid to protein ratio of membranes from the treated cells (Table III). Since the leakage of [^{3}H]thymidine-labeled components and NADH oxidase from chloramphenicol-,

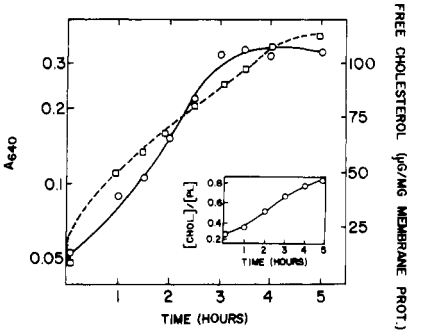

FIGURE 1: Time course of stimulation of cell growth (○) and increase in membrane free cholesterol content (□) upon transfer of adapted *M. capricolum* cells to medium containing 10% FCS. The inset shows the increase in the free cholesterol/lipid phosphorus molar ratio.

valinomycin-, and nonactin-treated cells was only about 10% higher than that from untreated cells and about 15% higher in gramicidin-treated cells, the permeability barrier was retained largely intact. (The extent of lysis appears to depend on the concentration and source of the serum.) These experiments demonstrate that cholesterol uptake, which was previously shown in *Acholeplasma laidlawii* to be a physical process (Gershfeld et al., 1974; Razin et al., 1974), is affected in *M. capricolum* by cell growth. As will be shown below, in cells where growth is inhibited, cholesterol translocation from the outer half to the inner half of the bilayer is much slower than in growing cells, resulting in the accumulation of the sterol in the outer half of the bilayer and a decrease in the total unesterified cholesterol content of the cell membrane.

Distribution of Cholesterol between the Inner and Outer Halves of the Bilayer. The distribution of free cholesterol in the two halves of the lipid bilayer of the adapted cells was probed at various time intervals after the transfer to the cholesterol-rich medium (Table II). Approximately three-fourths of the free cholesterol was estimated to be localized in the outer half of the bilayer in cells that were isolated immediately after transfer to 10% FCS. After 1 h of incubation, cholesterol was distributed about equally between the two halves of the bilayer, and at longer times of incubation roughly 55% of the total free cholesterol was present in the inner half of the bilayer. In the chloramphenicol- or ionophore-treated cells, 66–92% of the free cholesterol remained localized in the outer half of the bilayer (Table IV).

Discussion

The present study demonstrates that the cholesterol content

TABLE III: Effects of Chloramphenicol, Valinomycin, Nonactin, and Gramicidin on the Membrane Composition of Adapted *M. capricolum* Cells upon Transfer to Cholesterol-Rich Medium.

treatment	time of incubation (h)	A_{640}	membr protein[a] (mg)	membr phospholipids (μg/mg of membr protein)	free cholesterol (μg/mg of membr protein)	free cholesterol/ phospholipid (mol/mol)
no inhibitor	0	0.08	1.8	302	33	0.21
	1	0.16	3.1	309	44	0.28
	2	0.24	4.2	297	92	0.60
	4	0.26	4.6	325	127	0.76
chloramphenicol[b]	1	0.13	2.0	358	37	0.20
	4	0.14	2.3	469	74	0.30
chloramphenicol[c]	1	0.10	1.9	365	35	0.19
	4	0.11	2.0	477	52	0.21
valinomycin[b]	1	0.14	2.1	300	38	0.25
	4	0.17	2.2	307	78	0.49
nonactin[b]	2	0.12	2.2	374	69	0.36
	4	0.13	2.4	390	80	0.40
gramicidin[b]	1	0.11	1.9	342	35	0.20
	4	0.13	2.2	458	72	0.31

[a] Cells were grown in 250 mL of medium. [b] The final concentrations of chloramphenicol, valinomycin, nonactin, and gramicidin were 100 μg/mL, 10 μM, 10 μM, and 3 μM, respectively. The cultures were transferred to media containing 10% FCS immediately after the addition of the inhibitor. [c] Added 30 min before FCS.

TABLE IV: Effect of Growth Temperature, Chloramphenicol, Valinomycin, Nonactin, and Gramicidin on Cholesterol Incorporation and Transbilayer Distribution Upon Transfer to Cholesterol-Rich Medium.[a]

expt	temp (°C)	free cholesterol (μg/mg of membr protein)	k_{cells}/k_{membr}	cholesterol distribution (μg/mg of member protein)	
				outer half	inner half
no inhibitor	37	127.0	0.45	57.2	69.8
	4	25.7	0.70	17.9	7.8
chloramphenicol	37	57.5	0.66	39.1	17.4
valinomycin	37	79.7	0.90	71.5	8.2
nonactin	37	81.5	0.78	63.1	18.4
gramicidin	37	71.3	0.92	65.5	5.8

[a] The data were obtained after a 4-h incubation in medium containing 10% FCS. Essentially identical data were obtained when inhibitor-treated cultures were incubated for 2 h. FCS was added to the medium (10% final concentration) immediately after the addition of chloramphenicol (100 μg/mL), valinomycin (10 μM), nonactin (10 μM), or gramicidin (3 μM). Similar rate ratios were found when chloramphenicol was added 15 to 45 min prior to FCS. [b] The cholesterol distribution (in μg/mg of membrane protein) was calculated from $k_{cells}/k_{membr} \times$ free cholesterol content, which equals the cholesterol content in the outer half of the lipid bilayer.

of adapted *M. capricolum* cells increased by more than twofold between the first and third hours of incubation in FCS-rich medium and approached the cholesterol content of the native strain. The increase in cholesterol content appears more pronounced when expressed as micrograms of cholesterol per milligram of membrane protein rather than as micrograms of cholesterol per milligram of phospholipid because the cells incorporate phosphatidylcholine and sphingomyelin from the FCS-rich medium up to the amount of 35% of the total lipid phosphorus, increasing the phospholipid to protein ratio of the membranes (Z. Gross and S. Rottem, unpublished data). The influence of the exogenous lipid components on cholesterol uptake and translocation remains to be established. The distribution of free cholesterol in the two halves of the bilayer was almost invariant after the first hour of incubation at 37 °C, despite the dramatic increase in cholesterol content, indicating that the rate of translocation of free cholesterol in growing *M. capricolum* is very rapid. Indeed, at a low temperature (4 °C) where growth rate was decreased dramatically, cholesterol uptake and translocation were inhibited. One cannot exclude the possibility that such inhibition arose because membrane lipid may be in the gel state at that temperature, as was found in the adapted strain of the closely related *M. mycoides* subspecies *capri* (Rottem et al., 1973). The localization of cholesterol predominantly in the outer half of the bilayer in chloramphenicol-, valinomycin-, nonactin-, and gramicidin-treated cells (Table IV) indicates that translocation is facilitated in actively growing cells where a membrane potential gradient is maintained and/or macromolecular synthesis takes place. We wish to emphasize that in these experiments the translocation is from the external surface, which is in contact with the cholesterol of the medium, to the internal surface, which is initially low in cholesterol content. In contrast, the inner to outer transbilayer movement of cholesterol in resting *M. gallisepticum* cells is an extremely slow process, having a half-time of at least 18 days at 37 °C (Rottem et al., 1978). Although a direct comparison cannot be made since different organisms were used, these experiments suggest that the rate of transbilayer cholesterol movement may depend on a number of factors, e.g., active cell growth, direction of movement, sterol concentration gradient in each half of the lipid bilayer, and others. However, in red blood cells, where no growth or macromolecular synthesis occurs, the rate of cholesterol translocation appears to be very high (Lange et al., 1977; Kirby and Green, 1977).

Isolated membranes of *Acholeplasma laidlawii* incorporate cholesterol by a physical adsorption process (Gershfeld et al., 1974; Razin et al., 1974). However, the results that we report

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*†

L. Dudley Eirich,‡ Godfried D. Vogels, and Ralph S. Wolfe*

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

† From the Department of Microbiology, University of Illinois, Urbana, Illinois 61801, and the Department of Microbiology, University of Nijmegen, Nijmegen, The Netherlands. *Received July 26, 1978.* This work was supported by National Science Foundation Grant PCM 76-02652. High-resolution and FD mass spectra were obtained under a grant from the National Cancer Institute (CA 11388).

‡ Recipient of a research fellowship (1976–1977) from the Faculty of Science, University of Nijmegen, Nijmegen, The Netherlands. Present address: Battelle Pacific Northwest Laboratories, Richland, Wash. 99352.

[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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