The Effect of Coenzyme Leakage and Replacement on the Growth and Metabolism of Two *Fusobacteria*

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SUMMARY

Suspensions in water of two species of *Fusobacterium* leaked several coenzymes when incubated at normal growth temperatures. Chromatography of filtrates from these suspensions revealed the presence of NAD, NADP, FMN, tetrahydrofolic acid and, in one of the two, pyridoxal phosphate. Analyses of some enzymic activities in whole organisms demonstrated deficiencies in coenzymes: glutamate dehydrogenase was virtually inactive in the absence of added NAD; tryptophanase activities were diminished by washing but the extent differed between strains; histidase activity was not decreased by washing or suspension in water or saline. Both lag phase and doubling time increased markedly in severely washed organisms inoculated into fresh medium. Addition of appropriate coenzymes shortened the lag phase for both strains and shortened the doubling time in one.

INTRODUCTION

Bacteria may leak ultraviolet-absorbing material when treated with certain chemicals or heat (Kenis & Morita, 1968; Brown & Winsley, 1969; Allwood & Russell, 1970; Morris & Russell, 1970; Allwood & Hugo, 1971). An *Escherichia coli* lysine auxotroph also leaks a variety of nucleotide derivatives when grown in medium containing suboptimal concentrations of lysine (Lilly, Clarke & Meadow, 1963). Commonly, however, living organisms retain vital compounds when incubated at normal growth temperature. The classical exceptions to this are the Rickettsiae which lose vital coenzymes *in vitro* (Moulder, 1962).

Aqueous suspensions of *Fusobacterium polymorphum* and *F. fusiforme* leak several coenzymes. Supernatant fluids from suspensions of *F. polymorphum* contained FMN, 5,6,7,8-tetrahydrofolic acid (THFA), degradation products from THFA, NAD and NADP. In addition, reducing substances can be washed from the bacteria because growth of washed *F. polymorphum* under semi-anaerobic conditions occurs only when reducing substances are added to the growth medium, whereas unwashed cultures grew well without additional reducing substances (Coles, 1973).

Because amino acid metabolism plays an important role in the growth of fusobacteria (Loesche & Gibbons, 1968; Werner, Neuhaus & Hussels, 1971), we attempted to correlate coenzyme leakage with ability to maintain tryptophanase, glutamate dehydrogenase and histidase activities under a variety of conditions.

METHODS

Organisms. *Fusobacterium polymorphum* (ATCC10953) was obtained from the American Type Culture Collection. *Fusobacterium fusiforme* was obtained from the Communicable

Disease Center, U.S. Public Health Service, Atlanta, Georgia, U.S.A. Stock cultures of both organisms were maintained by growing at 37 °C for 18 h in Brewers modified thioglycolate medium (Fisher Scientific Co., Fair Lawn, New Jersey, U.S.A.).

Media. Modified Omata's medium (Coles, 1968) was used for growth studies on *F. polymorphum*. *Fusobacterium fusiforme* did not require adenine but did require thymine (125 mg/l), folic acid (1 mg/l) and nicotinic acid (1 mg/l). Therefore, Omata's medium was further modified by omitting adenine and including the required compounds. Screw cap tubes (16 × 150 mm) containing 10 ml medium were autoclaved (15 min at 121 °C), flushed with sterile 5 % CO₂ + 95 % N₂, cooled, inoculated with about 1 × 10⁸ organisms, and again flushed with 5 % CO₂ + 95 % N₂. Coenzymes of nucleotides, where used, were filter sterilized and added immediately before inoculation. Sterile H₂ was bubbled through all NAD, NADP and FMN solutions for 3 min immediately before their addition to the growth media. Coenzymes and nucleotides were added at a concentration of 100 µg/tube. Cultures were incubated anaerobically at 37 °C. Growth was measured by turbidity increase at 660 nm using a Coleman Model 44 Spectrophotometer (Ace Scientific Supply Co., Linden, New Jersey, U.S.A.).

Organisms used for studies on amino acid degradation were grown similarly in the medium used for the growth studies, with the following changes. For bacteria induced for tryptophanase (EC.4.2.1.-) the medium contained L-tryptophan (200 mg/l). For the histidase (L-histidine ammonia lyase, EC. 4.3.1.3) study, the tryptophan concentration was decreased to 50 mg/l and L-histidine (200 mg/l) was added to the medium. For the glutamate dehydrogenase [L-glutamate:NAD oxido-reductase (deaminating), EC. 1.4.1.2], the tryptophan concentration was 50 mg/l and no histidine or glutamic acid was added beyond the amount contained in the Casamino acids.

Analysis of enzyme activity. For each assay, one half of the appropriate culture was washed four times with equal volumes of water at 4 °C. The washed bacteria were suspended to a protein concentration of approximately 1 to 1.5 mg/ml. The other half of the culture was centrifuged and resuspended in distilled water, without further washing, to the same concentration. Both washed and unwashed organisms were assayed for the appropriate enzymic activity.

For determination of tryptophanase activity the method of Freundlich & Lichstein (1960) was used. Glutamate dehydrogenase activity was determined by following the release of ammonium ion from glutamic acid using Nessler's reagent (Umbreit, Burris & Stauffer, 1957). Tubes for this assay contained 200 µmol potassium phosphate buffer pH 7.5; 100 µmol sodium glutamate pH 7.5; washed or unwashed bacteria corresponding to 200 µg protein; 20 µg NAD, where specified; and water to a final volume of 2 ml. The reaction was stopped by addition of 0.2 ml of 10 % (w/v) TCA. All assays were performed at 37 °C. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

The method of Cornell & Lien (1970) was used to determine histidase activity, except that Mg²⁺ was substituted for the Zn²⁺ because Zn²⁺ ions were found to inhibit histidase in both organisms whereas Mg²⁺ stimulated the reactions.

Preparation and chromatographic analysis of filtrates from aqueous suspensions. A bacterial culture (4 l) grown anaerobically at 37 °C for 18 h (mid-exponential phase) was centrifuged at 20 000 g. The pellet and containers were rinsed with distilled water at 4 °C. The bacteria were suspended in distilled water to a concentration of 5 × 10⁹ organisms/ml and incubated at 37 °C in stoppered flasks with no more than 5 mm air space. They were gently shaken for 3 h. The suspensions were centrifuged at 20 000 g and the supernatant fluids filtered through a sterile 0.2 µm membrane filter (Ace). The filtrates were then lyophilized and reconstituted to

Fig. 1. Absorption spectra of filtrates from *Fusobacterium polymorphum* suspensions. Organisms were harvested from the mid-exponential phase of growth, centrifuged and resuspended in water (zero time), then incubated at 37 °C for 3 h. A, zero time filtrate; B, 3 h filtrate.

1/50 their original volume. The filtrates were frozen and kept in the dark for chromatographic analysis and for use as supplements for both enzyme assays and the growth experiments. Identification of coenzymes in the filtrates was made by one and two dimensional thin-layer chromatography on Bakerflex silica gel IB-F sheets (J. T. Baker Chemical Co., Phillipsburg, New Jersey, U.S.A.) The solvent systems used were ammonia-methanol (3:7, v/v) as used by Nuttall & Bush (1971), and water-saturated butanol. After development (the solvent migration was 150 mm at 25 °C), the sheets were air-dried and examined under 260 nm and 366 nm u.v. light.

RESULTS

Supernatant fluids from aqueous suspensions of both organisms absorbed u.v. light. Without decrease in turbidity of the suspensions, the u.v. absorption increased during 3 h of incubation at 37 °C (Fig. 1). The absorption peak was sharp at 260 nm, indicating that there was no large release of protein, as would be expected from lysis.

Chromatography of the concentrated filtrates demonstrated several u.v.-absorbing and fluorescent compounds. Of those, NAD, NADP and FMN were readily identifiable by R_F , absorption and fluorescent characteristics in filtrates from both organisms. No pyridoxal phosphate was detected in the *F. polymorphum* filtrates, but it was readily detected in the filtrate from the *F. fusiforme* suspension. Two other spots were noted on all chromatograms. Both showed bright blue fluorescence when excited at 366 nm. One was identical in R_F and

Table 1. *Tryptophanase activity of F. polymorphum and F. fusiforme*

Tubes contained L-tryptophan pH 8 (10 μ mol) and phosphate buffer pH 8 (200 μ mol), plus additions of pyridoxal phosphate or concentrated filtrates or concentrated washings where indicated, and bacteria and water to 2 ml. The reaction was stopped after 1 h incubation at 37 °C by rapid freezing at -70 °C. Tubes were kept at -70 °C until assayed for indole.

Age of culture (h)	Activity (nmol indole produced/mg protein/h)					
	No wash			Washed 4 times		
	No additions	Pyridoxal phosphate*	+ Conc. washings†	No additions	Pyridoxal phosphate*	+ Conc. washings†
<i>F. polymorphum</i>						
42 (late exponential phase)	117	150	110	119	115	113
60 (stationary phase)	251	294	254	122	254	197
90 (late stationary phase)	30	244	170	13	200	168
			+ Conc. filtrate‡			+ Conc. filtrate‡
<i>F. fusiforme</i>						
21 (early exponential phase)	33	100	89	5	112	104
48 (late exponential phase)	41	180	163	11	107	98
96 (late stationary phase)	70	190	165	47	236	180

* Pyridoxal phosphate added (10 μ g/ml reaction mixture). All values have appropriate zero time values subtracted.

† Concentrated ($\times 50$) washings (0.5 ml/tube) from suspension of *F. polymorphum* that had been grown for 60 h.

‡ Concentrated filtrate from *F. fusiforme* suspension used for chromatogram.

fluorescence with tetrahydrofolic acid. The other was identical with tetrahydrofolate which had been exposed to air for 24 h or reacted with H_2O_2 for 5 min.

The enzymic activities tested correlate well with the coenzymes detected in the filtrates. For instance, *F. polymorphum* was able to maintain tryptophanase activity of both washed and unwashed suspensions until it reached the stationary phase (Table 1). This is in agreement with the finding that no pyridoxal phosphate was detected in the filtrates from suspensions of mid-exponential phase bacteria. At about 24 h after the stationary phase was reached, however, even the unwashed bacteria lost tryptophanase activity. In all cases where activity was lost, it could be restored by the addition of pyridoxal phosphate to the reaction mixture. The activity of the late stationary phase organisms could also be restored partially by addition of concentrated washing from these late stationary phase bacteria (Table 1).

Fusobacterium fusiforme, however, required exogenous pyridoxal phosphate for maximum tryptophanase activity under all conditions tested (Table 1). Unwashed organisms from all stages of growth were stimulated three- to fourfold in the assay by coenzyme addition. Washing of the suspensions further decreased the unstimulated activity. Coenzyme additions to the washed bacteria restored the activity to the levels obtained using unwashed organisms with added coenzyme. Further, the addition of concentrated filtrates from suspensions of *F. fusiforme* restored tryptophanase activity. Pyridoxine, pyridoxamine, pyridoxal and pyridoxamine phosphate were all unable to restore tryptophanase activity in either organism. The stimulation of tryptophanase in whole bacteria by pyridoxal phosphate suggests an

Table 2. Glutamate dehydrogenase activity of *F. polymorphum* and *F. fusiforme*.

Tubes contained L-glutamate pH 7.5 (100 μ mol) and phosphate buffer pH 7.5 (200 μ mol), plus additions of NAD or concentrated filtrates where indicated, and cells and water to 2 ml. The reaction was stopped after 1 h incubation at 37 °C by addition of 0.2 ml of 10 % (w/v) trichloroacetic acid.

Age of culture (h)	Activity (μ mol NH produced/mg protein/h)					
	No additions	+ NAD*	+ Conc. filtrate†	No additions	+ NAD*	+ Conc. filtrate
<i>F. polymorphum</i>						
24	1	6	5	0	4	2
48	0	15	10	0	11	8
72	0	26	11	0	25	11
<i>F. fusiforme</i>						
24	0.4	3	2	0.5	3	2
48	1	4	4	0	3	3
72	0.6	4	4	0	4	3

* Added to a final concentration of 10 μ g/ml. All values have appropriate zero time values subtracted.

† Concentrated ($\times 50$) filtrate from either organism (0.1 ml/tube).

Fig. 2. Effect of cofactors and AMP on growth of washed *F. polymorphum*. ●, Unwashed control; ▲, washed bacteria with concentrated filtrate (0.1 ml/tube); ■, washed bacteria with known additions (10 μ g each of NAD, NADP, FMN and AMP/ml medium); ○, washed organisms, no addition. Organisms were harvested during the mid-exponential phase of growth and suspended in water (4 °C). The unwashed control was inoculated into fresh medium. The rest of the suspension was washed 4 times in water (4 °C) and inoculated. All tubes were inoculated with 1×10^6 organisms/ml.

Fig. 3. Effect of cofactors and AMP on growth of washed *F. fusiforme*. ●, Unwashed control; ▲, washed bacteria with concentrated filtrate from *F. fusiforme* resting suspension (0.1 ml per tube); ■, washed bacteria with known additions (10 µg each of NAD, NADP, FMN, AMP and pyridoxal phosphate/ml medium); ○, washed organisms, no additions. Organisms were harvested during the mid-exponential phase of growth and suspended in water (4 °C). The unwashed control was inoculated into fresh medium. The rest of the suspension was washed 4 times in water (4 °C) and inoculated. All tubes were inoculated with 1×10^5 organisms/ml.

unusual membrane permeability in these fusobacteria since phosphorylated compounds are not easily taken up by bacteria.

The presence of NAD in all filtrates of both organisms suggested that NAD-requiring enzymes might show suboptimal activity. Accordingly, the glutamate dehydrogenase activity of each organism was examined, and was found to require exogenous NAD for maximum activity (Table 2). This requirement was seen in washed and unwashed organisms from all stages of growth. Also, the concentrated filtrates used for chromatography from both organisms enhanced the glutamate dehydrogenase activity for each organism.

Although exogenous reducing substances are required for growth of washed *F. polymorphum* under semi-anaerobic conditions (Coles, 1973), leakage of reduced glutathione (GSH) was not reflected by an appreciable decrease in histidase activity for either organism at any stage of growth.

To determine the effects of coenzyme leakage on growth of the organisms, 4 times washed and unwashed bacteria were inoculated (10^6 organisms/10 ml medium) and incubated anaerobically with and without various coenzymes. Both washed and unwashed inocula of *F. polymorphum* grew in the unsupplemented medium (Fig. 2). Washing, however, nearly

doubled the lag phase (from 18 h for the unwashed culture to 32 h for the washed) and increased the doubling time from 5.5 to 9.5 h.

Addition of coenzymes, or the concentrated filtrates used for chromatographic analysis and as supplements for the enzyme assays, shortened both the lag and the doubling time. Addition of single compounds of the mixture or omission of any of the compounds from the mixture decreased the growth stimulation.

The results of growth studies with *F. fusiforme* (Fig. 3) were quite different from those with *F. polymorphum*. While washing increased the lag from 10 to 18 h, the doubling time was the same for both washed and unwashed inocula. The addition of sterile concentrated filtrates to media inoculated with washed bacteria shortened the lag phase to approximately 13 h and also decreased the doubling time to less than that obtained with unsupplemented medium inoculated with washed or unwashed bacteria. The concentrated filtrate from *F. polymorphum* did not show this effect on *F. fusiforme* unless pyridoxal phosphate was added with it. The addition of the known cofactor mixture also decreased the lag but only slightly decreased the doubling time. As with *F. polymorphum*, addition of single supplements to the medium or omission of one of the compounds from the complete mixture resulted in little stimulation of growth. One difficulty with the growth studies on *F. fusiforme* was that the cultures often exhibited a logarithmic decrease in turbidity at about 50 h (the beginnings of which can be seen in Fig. 3). This decrease remains unexplained; however, we have detected the release of bacteriophage from mitomycin C-treated cultures of this organism. We are currently investigating the possibility that spontaneous induction of this phage caused the sudden drop in culture turbidity.

Several other compounds not seen in chromatograms of filtrates were tested for growth stimulation. These include GSH, several nucleotides, thiamin pyrophosphate, tetrahydrofolate, dihydrofolate, methyltetrahydrofolate and FAD. Of these, only AMP, when added to the coenzyme mixtures, was stimulatory for both organisms. Preliminary evidence indicates that AMP stimulates glucose utilization in whole *F. polymorphum* organisms.

DISCUSSION

Suspensions of *F. polymorphum* and *F. fusiforme* leak several coenzymes under various conditions. The loss is reflected in both the level of certain enzymic activities and the pattern of growth in semisynthetic medium. The variation in leakage rates depended on both the species and the coenzyme studied.

Histidase activity did not show appreciable decline at any stage of growth by washing in either organism. Purified rat liver histidase also retains considerable activity unless treated with EDTA for 17 h (Cornell & Lien, 1970). It is therefore likely that the cofactors, GSH and Mg^{2+} , are bound tightly to the enzyme and would not be washed from the whole organisms by water. Thus, reducing substances which influence growth can be washed from *F. polymorphum* (Coles, 1973) without affecting the histidase activity.

The intent of the growth studies was not to demonstrate obligatory growth requirements but rather to show that, when available (as in damaged host tissues), certain coenzymes or other compounds stimulated growth. Since, however, growth is the sum total of all vital processes, it is likely that such stimulation would be seen only when all the deficient factors are present. This was true for both organisms examined. The bacteria must be able to synthesize the factors studied, otherwise they would not grow in the medium used. They are also capable of utilizing exogenous supplies of preformed NAD, NADP, FMN, AMP and, in the case of *F. fusiforme*, pyridoxal phosphate during growth. An example of the stimulation

of fusobacteria by damage to host tissue has been presented by Kaufman *et al.* (1972), who demonstrated that the pathogenicity of *F. fusiforme* was enhanced by co-injection of enzymically active extracts of *Bacteroides melaninogenicus*. Heated extracts without proteolytic activity were unable to enhance the lesions caused by the fusobacteria.

These data help to explain two phenomena noted for the growth and behaviour of fusobacteria. Difficulty in creating a synthetic medium for growth of washed bacteria or definition of growth requirements of fusobacteria have been encountered (Omata, 1953, 1959; Coles, 1968; Loesche & Gibbons, 1968; Hadi & Russell, 1970). Loss of NAD and pyridoxal phosphate by the organisms may explain why Hadi & Russell (1970) found that nicotinic acid and pyridoxine stimulated growth of *Fusobacterium nucleatum*.

The leakage of coenzymes from fusobacteria may also play a role in the apparent synergism seen between fusobacteria and species of oral *Borrelia* in acute ulcerative gingivitis. The oral *Borrelia* require unidentified growth factors (Nevin & Hampp, 1959) which could be supplied by materials leaked from fusobacteria (especially in localized ulcers enclosed by a pseudomembrane). Conversely, it is also possible for *Borrelia* or damaged tissue to supply fusobacteria with the coenzymes which they require for maximum activity.

It is also important to determine which coenzymes leak from other fusobacteria. Co-enzyme leakage varies not only with the species tested but also with the condition of the organism. Thus, synergistic effects noted with various borrelia would depend on the *Fusobacterium* used, its phase of growth and the possible requirements of the strain of *Borrelia* present in the lesion. The reason why Nevin, Hampp & Duey (1960) failed to demonstrate stimulation of *Borrelia* growth by fusobacteria may well have been related to the species and condition of the fusobacteria used.

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