SHORT COMMUNICATION

Sterol Requirement for the Growth of *Treponema hyodysenteriae*

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The addition of cholesterol to a liquid medium containing bovine serum albumin (BSA) fraction V or acetone-delipidized BSA fraction V instead of serum stimulated the growth of *Treponema hyodysenteriae*, a serum-requiring spirochaete associated with swine dysentery. As little as $1.25 \mu\text{g}$ cholesterol ml^{-1} increased viable counts about 1000-fold. Sitosterol and cholestanol, but not pregnenolone, cholestenone or stigmasterol, produced a growth response comparable to that of cholesterol. The results suggest that *T. hyodysenteriae* requires a sterol for growth.

INTRODUCTION

Exogenous cholesterol is taken up by certain species of *Treponema*, but a specific requirement for sterols by any member of this genus has so far not been established (Meyer & Meyer, 1971). At least one species, *T. phagadenis*, can be grown in media supplemented only with delipidized bovine serum albumin (BSA) fraction V and fatty acids (Oyama *et al.*, 1953; Johnson & Eggebraten, 1971) and is therefore, presumably, independent of exogenous sterols. However, Rajkovic (1967) observed that the addition of cholesterol to a serum-free medium produced a fourfold increase in the total count of the Noguchi strain of *T. pallidum*, now known as *T. refringens* (Smibert, 1976*a*). Rajkovic (1967) suggested that cholesterol was an essential lipid for this organism, although the growth enhancement he observed was small considering that the initial count was 2×10^7 organisms ml^{-1} .

Observations in this laboratory on *T. hyodysenteriae*, a serum-requiring, pathogenic spirochaete which is associated with swine dysentery (Harris *et al.*, 1972; Smibert, 1976*b*), have shown that this organism is inhibited by digitonin, a phenomenon indicative of sterol-dependence in mycoplasmas (Freundt *et al.*, 1973). The investigations reported here were carried out to determine whether cholesterol is required for the growth of *T. hyodysenteriae*.

METHODS

Organisms. *Treponema hyodysenteriae* strains P18A and KF9, both virulent for pigs, were used (Lemcke *et al.*, 1979). Suspensions in *mist. dessicans* (Lemcke *et al.*, 1979) or washed suspensions harvested from rabbit serum broth (see below) and resuspended in phosphate-buffered saline, pH 7.3, containing bovine serum albumin (BSA) fraction V (Miles Laboratories, Stoke Poges, Bucks.; 1% w/v) were stored at -70°C and diluted in Trypticase soya broth (BBL) immediately before inoculation. The volume of inoculum was 0.05 ml per 2 ml medium. The final concentration of rabbit serum carried over from the *mist. dessicans* inoculum was therefore never more than 0.002% (v/v). The viable count of the inoculum used in each series of experiments was determined, in terms of colony-forming units (c.f.u.) ml^{-1} , on 5% (v/v) sheep blood agar (Lemcke *et al.*, 1979). The inoculum of P18A in experiments 1 to 6 (see below) varied between 1.25×10^8 and 5.6×10^8 c.f.u. ml^{-1} and in experiment 7 was 7.5×10^1 c.f.u. ml^{-1} . The inoculum of KF9 in experiment 6 was 4.0×10^2 c.f.u. ml^{-1} .

Media. The basal medium was Trypticase soya broth supplemented with glucose (0.25 %, w/v), cysteine. HCl (0.05 %, w/v), NaHCO_3 (0.4 % w/v) and Spectam Soluble [Abbott Laboratories – distributor Parke, Davis & Co., Pontypool, Gwent; $800 \mu\text{g ml}^{-1}$ (equivalent to spectinomycin at $400 \mu\text{g ml}^{-1}$)]. Rabbit serum broth was basal medium supplemented with rabbit serum (Flow Laboratories; 10 %, v/v). All media were dispensed in 2 ml amounts into 150×12 mm Pyrex tubes, sealed, inoculated and incubated under 10 % (v/v) CO_2 in N_2 (Lemcke *et al.*, 1979). Four tubes of each medium were inoculated and a fifth served as an uninoculated control.

Medium supplements. Solutions of BSA fraction V, delipidized BSA fraction V prepared from this by acetone extraction (Razin & Rottem, 1963) and fatty acid-free BSA fraction V (Sigma), each at 5 % (w/v), were made up in 0.025 M-phosphate buffer, pH 6.9 (Ellinghausen & McCullough, 1965) and sterilized by filtration. A 15 % (w/v) solution of delipidized BSA fraction V was used where final concentrations higher than 0.5 % (w/v) were required. Cholesterol (5-cholesten-3 β -ol, puriss USP), cholestenone (4-cholesten-3-one, pure), pregnenolone (5-pregnen-3 β -ol-20-one, pure), sitosterol (pure NF), stigmasterol (5,22-cholestadien-24-ethyl-3 β -ol, pure) and cholestanol (5 α -cholestan-3 β -ol, pure) were obtained from Koch-Light. In experiments 1 to 4 (see below), a 2 % (w/v) ethanolic solution of cholesterol was diluted in warm ethanol and dispersed, by injection through a fine capillary, in basal medium held at 80 °C. The final concentration of ethanol did not exceed 0.05 % (v/v). In experiments 5 to 7, 2 % (w/v) stock solutions of cholesterol and other sterols were prepared in warm ethanol and injected into a sterile 2 % (v/v) aqueous solution of Tween 80 (Difco) held at 80 °C in a water bath and stirred. The resulting opalescent suspensions contained 0.4 % (w/v) sterol, 20 % (v/v) ethanol and 1.6 % (v/v) Tween 80, and were stable on cooling. Further dilutions were made in 1.6 % (v/v) Tween 80, so that the final concentration of Tween 80 in the medium was never more than 0.016 % (v/v). The maximum concentration of ethanol was 0.2 % (v/v). The sterol dilutions were warmed and injected through a fine capillary into basal medium supplemented with delipidized BSA fraction V held at 37 °C. In all experiments, ethanol and/or Tween 80 alone were added to control media to give the same concentrations as those present in sterol-containing media.

Growth studies. The growth of *T. hyodysenteriae* was studied in basal medium supplemented with (1) 0.5 % (w/v) BSA fraction V or fatty acid-free BSA fraction V and $10 \mu\text{g cholesterol ml}^{-1}$, (2) a series of seven concentrations of cholesterol between 1.5 and $100 \mu\text{g ml}^{-1}$, (3) 0.5 % (w/v) BSA fraction V or delipidized BSA fraction V and a series of seven concentrations of cholesterol between 1.5 and $100 \mu\text{g ml}^{-1}$, (4) $10 \mu\text{g cholesterol ml}^{-1}$ and a series of five concentrations of delipidized BSA fraction V between 0.25 and 1.5 % (w/v), (5) 0.5 % (w/v) delipidized BSA fraction V and either a series of eight concentrations of pregnenolone between 0.125 and $10 \mu\text{g ml}^{-1}$ or a series of ten concentrations of sitosterol, cholestanol, cholestenone or stigmasterol between 0.125 and $40 \mu\text{g ml}^{-1}$ or $10 \mu\text{g cholesterol ml}^{-1}$ as control, (6) 0.5 % (w/v) delipidized BSA fraction V and a series of six concentrations of cholesterol between 1.25 and $40 \mu\text{g ml}^{-1}$ and (7) 0.5 % (w/v) delipidized BSA fraction V and either cholesterol, pregnenolone, sitosterol, cholestanol, cholestenone or stigmasterol at $10 \mu\text{g ml}^{-1}$. Unsupplemented basal medium and/or medium without the particular substrate under test together with rabbit serum broth were included as controls in each series of experiments. At least two experiments were carried out in each series. *Treponema hyodysenteriae* P18A was used throughout as test organism together with KF9 in experiment 6. The inoculum was derived from *mist. dessicans* suspensions in experiments 1 to 4 and from washed suspensions in experiments 5 to 7; thus, in the latter experiments, sterols were completely eliminated from the inoculum.

Assessment of growth. In experiments 1 to 5, the amount of growth after 6 to 8 d at 37 °C relative to the uninoculated control tubes was estimated in four replicate tubes by visual assessment of turbidity, by the fall in pH and by titration of the acid formed during growth against 0.01 M-NaOH. Since the fall in pH and the amounts of acid produced by *T. hyodysenteriae* were relatively small, even in rabbit serum broth, viable counts on duplicate cultures were done in experiments 6 and 7 as the finite method of assessing the growth response of this species to sterols. The viable counts were performed in experiment 6 after incubation at 37 °C for 4 d in the case of P18A and for 7 d, since growth is slower, in the case of KF9. In experiment 7, the count was performed after 7 d.

RESULTS

Growth response to different preparations of BSA fraction V and cholesterol (experiments 1 to 4). No growth of P18A was detected in unsupplemented basal medium or in basal medium supplemented with either 0.5 % (w/v) BSA fraction V or fatty acid-free BSA fraction V alone (experiment 1). The addition of $10 \mu\text{g cholesterol ml}^{-1}$ to basal medium containing either preparation of BSA fraction V stimulated growth, although not to the level obtained in rabbit serum broth (Table 1). However, cholesterol alone failed to stimulate growth unless the concentration was $50 \mu\text{g ml}^{-1}$ or more as indicated by a fall in pH of

Table 1. *Growth of Treponema hyodysenteriae* strain P18A in basal medium with various added supplements, in particular cholesterol

Four tubes of each medium were monitored in each of two experiments
after inoculation with 1.25×10^8 c.f.u. ml⁻¹.

Addition(s) to basal medium	Growth after 6 d at 37 °C		
	Turbidity*	Decrease in pH (units)†	Titrateable acidity‡
No addition	0	0	0
0.5% BSA fraction V	0	0	0
0.5% BSA fraction V + 10 µg cholesterol ml ⁻¹	3+	0.5	2.0
0.5% fatty acid-free BSA fraction V	0	0	0
0.5% fatty acid-free BSA fraction V + 10 µg cholesterol ml ⁻¹	2+	0.3	1.0
10% rabbit serum	4+	1.0	3.4

* 0, None; + to 4+, degree of turbidity.

† Calculated from the pH of the uninoculated control tube incubated for the same period.

‡ Volume (ml) of 0.01 M-NaOH required to raise the pH of 2 ml culture to that of the uninoculated control.

0.15 to 0.2 units and the development of acidity neutralized by 0.5 to 0.7 ml 0.01 M-NaOH per 2 ml culture (experiment 2).

The growth response of P18A to supplementation of basal medium with cholesterol and BSA fraction V or delipidized BSA fraction V revealed that as little as 1.5 µg cholesterol ml⁻¹ stimulated growth in the presence of 0.5% (w/v) of either preparation of BSA fraction V, with optimal growth occurring at cholesterol concentrations between 6.25 and 25 µg ml⁻¹ (experiment 3). The fall in pH was slightly greater (0.25 to 0.4 units) in basal medium supplemented with BSA fraction V than in that with delipidized BSA fraction V (0.3 to 0.35 units). In contrast, in media containing 10 µg cholesterol ml⁻¹, growth was similar at all concentrations of delipidized BSA fraction V between 0.25 and 1.5% (w/v), falls in pH of 0.25 to 0.3 units (equivalent to 0.7 to 1.0 ml 0.01 M-NaOH per 2 ml culture) being recorded (experiment 4).

In all these experiments a fall in pH of 0.7 to 1.0 units (equivalent to 2.1 to 3.0 ml 0.01 M-NaOH per 2 ml culture) occurred in the control cultures growing in rabbit serum broth.

Growth response to delipidized BSA fraction V and other sterols (experiment 5). Of the sterols pregnenolone, sitosterol, cholestanol, cholestenone and stigmasterol added to basal medium supplemented with 0.5% (w/v) delipidized BSA fraction V, only sitosterol at a concentration of 10 µg ml⁻¹ or more and cholestanol at a concentration of 5 µg ml⁻¹ or more stimulated the growth of P18A. The fall in pH (0.3 units) was the same as that produced by growth in basal medium supplemented with delipidized BSA fraction V and 10 µg cholesterol ml⁻¹. Again the fall in pH in rabbit serum broth was 0.7 units.

Effect on the viable count of P18A and of KF9 of delipidized BSA fraction V and cholesterol (experiment 6). The results with both P18A and KF9 essentially confirmed those obtained in previous experiments with P18A (Fig. 1). However, in the medium without cholesterol, viable counts revealed slight growth which was not detected in experiments where the method of assessing growth was less rigorous. The addition of as little as 1.25 µg cholesterol ml⁻¹ increased the viable counts by about 1000-fold. There was little difference in the amount of growth obtained with cholesterol at concentrations between 1.25 and 40 µg ml⁻¹. The viable counts for both P18A and KF9 were five- to tenfold lower in the cholesterol-containing media than in rabbit serum broth.

Fig. 1. Effect of cholesterol on the viable count of *Treponema hyodysenteriae* strains P18A and KF9 growing in basal medium supplemented with delipidized bovine serum albumin fraction V. (a) Strain P18A; inoculum 1.3×10^8 c.f.u. ml^{-1} ; growth assayed after incubation at 37°C for 4 d. (b) Strain KF9; inoculum 4.0×10^3 c.f.u. ml^{-1} ; growth assayed after incubation at 37°C for 7 d. [RSB, Basal medium supplemented with 10% (v/v) rabbit serum.]

Effect on the viable count of P18A of delipidized BSA fraction V and other sterols (experiment 7). Viable counts of P18A in media containing cholesterol, sitosterol or cholestanol at $10 \mu\text{g ml}^{-1}$ were 2.7×10^5 , 8.8×10^5 and 1.4×10^6 c.f.u. ml^{-1} , respectively. These results confirmed those obtained in the exploratory experiment (5) which suggested that sitosterol and cholestanol as well as cholesterol stimulated the growth of *T. hyodysenteriae*. Counts in media containing pregnenolone, cholestenone, stigmasterol or no added sterol were 6.8×10^3 , 7.3×10^4 , 3.6×10^3 and 1.7×10^3 c.f.u. ml^{-1} , respectively. With the exception of cholestenone, these counts represent a small increase over the inoculum, although there was no visual evidence of growth.

DISCUSSION

The poor response to pregnenolone suggests that the entire sterol molecule, including the aliphatic side-chain, is necessary if a sterol is to support the growth of *T. hyodysenteriae* effectively. The absence of growth in the medium containing cholestenone implies that a hydroxy group is required at position 3. Of the sterols with a 3-hydroxy group, cholestanol and sitosterol produced a response comparable to that of cholesterol, but stigmasterol did not, possibly because of an increased degree of unsaturation in its molecule. Overall, the pattern of sterol-requirement was similar to that found in mycoplasmas (Edward & Fitzgerald, 1951; Smith & Lynn, 1958; Rodwell, 1963).

The results presented here suggest that *T. hyodysenteriae* requires sterol for growth. Since cholesterol was effective at a concentration of $1.25 \mu\text{g ml}^{-1}$, it seems unlikely that an impurity could account for its activity. Currently, mycoplasmas are the only prokaryotes recognized as having a specific requirement for sterol (Razin, 1978). Our results substantiate the earlier suggestion of Meyer & Meyer (1971) that serum-dependent treponemes might require sterol for growth. Since the outer envelope of treponemes consists of a triple-layered membrane (Hovind-Hougen, 1976), cholesterol may be important in maintaining the structural integrity and fluidity of this envelope, as it is in the limiting membrane of mycoplasmas and eukaryotic cells (Razin & Rottem, 1978). The structural features of the sterols active for *T. hyodysenteriae* also suggest that their function in this spirochaete is similar to that in mycoplasmas. It remains to be determined whether other serum-requiring treponemes are also sterol-dependent.

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