

GROWTH ENHANCEMENT OF THE REITER TREPONEME BY FATTY ACIDS^{1,2}

DAVID A. POWER AND MICHAEL J. PELCZAR, JR.

Department of Microbiology, University of Maryland, College Park, Maryland

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Interest in the Reiter strain of *Treponema pallidum*, a nonpathogenic, cultivatable, anaerobic spirochete, has been stimulated by the demonstration that the organism possesses four antigens; one, a thermolabile protein, being group specific for treponemes (D'Alessandro *et al.*, 1949). This antigen has recently been employed in a Reiter protein complement fixation test for syphilis (Cannefax and Garson, 1957). Studies comparing the results of the Reiter protein complement fixation tests, the *T. pallidum* complement fixation test, and the *T. pallidum* immobilization test on syphilitic sera show, in general, that the Reiter protein complement fixation test antigen has a specificity and sensitivity comparable to the *T. pallidum* complement fixation and *T. pallidum* immobilization test antigens (D'Alessandro and Dardanoni, 1953; Cannefax and Garson, 1957; Rein *et al.*, 1957; De Bruijn, 1957; De Bruijn and Bekker, 1957; Miller *et al.*, 1958). Of the three tests mentioned above (Reiter protein complement fixation, *T. pallidum* complement fixation, and *T. pallidum* immobilization), the antigen for the Reiter protein complement fixation test is most readily available since the organism from which it is extracted can be conveniently cultivated in large quantities.

Several media have been used in the past for the cultivation of the Reiter treponeme (Reiter, 1926; Little and SubbaRow, 1945; Whiteley and Frazier, 1948; Gelperin, 1949; Rose and Morton, 1952; Gastinel *et al.*, 1956). All are complex in composition, generally consisting of peptone, tissue extracts or serum, a reducing agent, such as thioglycolate, and other supplements. Extensive studies on the nutritional requirements of

the Reiter treponeme by Steinman *et al.* (1952, 1953, 1954) have revealed that the organism will grow in a medium consisting of 13 amino acids, 1 pyrimidine, 3 vitamins, glucose, inorganic salts, and crystallized serum albumin. The function of the crystallized serum albumin, according to these investigators, is to act as a detoxifying carrier for an essential lipid; it could be replaced by defatted albumin and certain fatty acids, but by neither alone (Oyama *et al.*, 1953).

Media of the thioglycolate broth type composition, when supplemented with 10 per cent inactivated sheep serum, will support good growth of this organism. Spirolate broth (BBL) which consists of trypticase, yeast extract, glucose, sodium chloride, l-cysteine, and sodium thioglycolate has been formulated specifically for this purpose.

Relatively few of the studies on this organism have been concerned with the quantitative measurement of growth, particularly with respect to the correlation of results obtainable by direct counting, protein nitrogen determination, cellular weight, and turbidimetric techniques. This investigation was therefore undertaken to perform such determinations as well as to investigate the possibility of increasing the maximum cell crop presently obtainable in commercially available media. Furthermore, in view of the lipid requirement of the organism, and since it contains a large amount of alcohol-ether extractable material (Oyama *et al.*, 1953), the effect of fatty acids on the growth of the Reiter treponeme was studied.

EXPERIMENTAL METHODS

The Reiter treponeme employed in this study was cultivated and maintained in spirolate broth supplemented with 10 per cent (v/v) inactivated sheep serum. (The medium, spirolate broth plus 10 per cent inactivated sheep serum, is referred to throughout this paper as the "reference" medium.) Initial quantitative growth measurements, cell crop, and growth curve determina-

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tions, were performed with this medium; and later it served as the control for evaluation of several fatty acid supplements. The medium was dispensed in 20-ml amounts into screw capped test tubes of 30-ml capacity. The inoculum for each tube consisted of approximately 0.05 ml of a 7-day culture of the organism grown in the "reference" medium.

The fatty acid supplements investigated for their influence on total cell crop were water dispersible TEM (tartaric acid esters of monoglycerides) compounds³ (TEM 4C, the diacetyl tartaric acid ester of cottonseed oil monoglycerides; TEM 4S, the diacetyl tartaric acid ester of soybean oil monoglycerides; TEM 4T, the diacetyl tartaric acid ester of tallow monoglycerides) and ether soluble pure palmitic, stearic, oleic, and linoleic acids.⁴ These supplements were incorporated into the "reference" medium in amounts ranging from 0.025 mg to 0.50 mg per ml of final medium. When mixtures of fatty acids were used, they were mixed in equal amounts by volume, to give a final total fatty acid content of 0.20 mg per ml of medium.

Quantitative measurements of growth from all media were performed after 7 days incubation at 37 C. It had been previously determined from growth curve studies that the maximum cell crop was reached in 5 to 7 days. The system of analysis for cell crop determination included: turbidimetric measurement using the Bausch and Lomb Spectronic 20, at a wave length of 650 m μ ; direct microscopic counts, using the Petroff-Hauser bacteria counter; and dry weight and nitrogen determinations, as described by Pelczar *et al.* (1955).

RESULTS

The cell crop of Reiter treponeme harvested from the "reference" medium, under the conditions described, may be summarized as follows:

Optical density	= 0.36	} per ml of medium
Cell count	= 340 $\times 10^6$	
Dry weight	= 280 μ g	
Bacterial nitrogen	= 15 μ g	

³ Obtained from Dr. Mary S. Shorb, Department of Poultry Husbandry, University of Maryland. Originally supplied by Hachmeister, Inc., Pittsburgh, 30, Pennsylvania.

⁴ These fatty acids were generously donated by the Proctor and Gamble Company, Cincinnati, Ohio, through the courtesy of Dr. Leo F. Judge.

TABLE 1
Enhancement of growth of the Reiter treponeme in "reference" medium supplemented with TEM 4T†*

Supplement TEM 4T	Optical Density	Cell Count/ml	Bacterial Nitrogen
mg/ml	650 m μ	$\times 10^6$	μ g/ml
0	0.37	360	15.5
0.025	0.44	450	20.3
0.10	0.62	820	34.8
0.25	0.68	1320	46.1
0.50	0.52	600	23.7

* BBL spiroplate broth plus 10 per cent inactivated sheep serum.

† Diacetyl tartaric acid ester of tallow monoglycerides.

Omission of the serum or its replacement with lipoidal substances alone resulted in failure of the medium to support growth of the treponeme. However, supplementation of the "reference" medium with the monoglyceride tartaric acid ester substances revealed that these materials were capable of increasing the cell crop. The greatest effect was noted with TEM 4T. Accordingly, the optimum concentration of TEM 4T consistent with maximum cell crop was determined. These results are shown in table 1. Repetition of such an experiment on several occasions has shown that maximum growth enhancement is obtained at a level of between 0.20 to 0.25 mg of TEM 4T per ml of medium; the cell crop was increased 2- to 3-fold.

Since TEM 4T consists essentially of palmitic, stearic, oleic, and linoleic acids (Dugan, 1957), the effect of these fatty acids on cell crop yield was investigated. The "reference" medium was supplemented with these fatty acids individually and in various combinations and concentrations, and the cell crop obtainable from media with each of these variations was determined. The results are shown in figures 1 and 2. With the exception of linoleic acid, each of the fatty acids employed individually resulted in an improvement of cell crop beyond that of the "reference" medium. However, in all instances, the total cell crop was below that produced in the spiroplate-serum-TEM 4T medium ("reference" medium plus TEM 4T).

When the fatty acids were incorporated into the medium in various combinations, it was

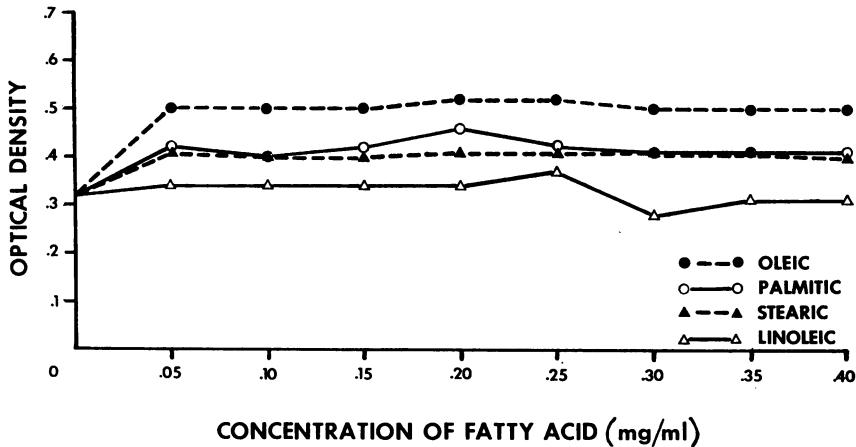


Figure 1. Growth response of the Reiter treponeme in spiroate-serum broth ("reference" medium) supplemented with individual fatty acids.

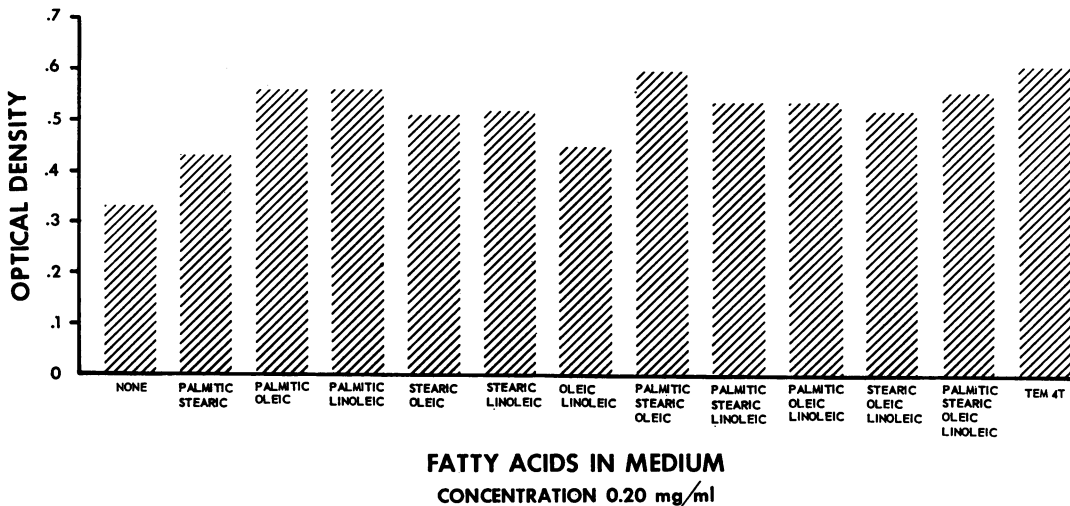


Figure 2. Growth response of the Reiter treponeme in spiroate-serum broth ("reference" medium) supplemented with mixtures of fatty acids at a concentration of 0.20 mg per ml of medium.

found that a mixture consisting of equal parts of palmitic, stearic, and oleic acids and totaling 0.20 mg total fatty acid per ml of medium, supported a cell crop comparable to that obtained with the TEM 4T supplement. In no instance did the total cell crop yield from the fatty acid supplemented "reference" medium exceed that previously found for the TEM 4T supplemented "reference" medium.

DISCUSSION

Current interest in methods for obtaining a large cell crop of the Reiter treponeme has resulted from the use of a protein antigen from this

organism in a complement fixation test for syphilis.

Growth of the Reiter treponeme in a thioglycolate broth type medium supplemented with 10 per cent sheep serum results in a cell crop of approximately 340×10^6 organisms per ml. This crop can be increased 2- to 3-fold by the addition of monoglyceride tartaric acid ester compounds, TEM 4T giving the best results. The growth enhancement property of these compounds may be attributed to their fatty acid content.

It was demonstrated that some fatty acids, namely palmitic, stearic, and oleic, have a stimulatory effect on growth of the Reiter treponeme.

Although both saturated and unsaturated fatty acids proved stimulatory, the maximum level of growth was obtained with a mixture of these acids, indicating that the organism has a requirement for both saturated and unsaturated fatty acids. A mixture of palmitic, stearic, and oleic acids provided a level of growth comparable to that obtained with TEM 4T and 2 to 3 times that obtained without the fatty acid supplement.

Since the monoglyceride tartaric acid ester compounds contain a high proportion of hydrophilic groups, which makes them readily dispersible in water, they are more conveniently incorporated into media than the ether soluble fatty acids.

The essential nature of certain fatty acids for the Reiter treponeme was shown by Oyama *et al.* (1953) who demonstrated that the serum requirement could be replaced by defatted serum albumin plus specific higher fatty acids. The enhancement of growth by additional fatty acid supplements as observed in this study would suggest some quantitative or qualitative deficiencies of serum in fully satisfying the requirements of the Reiter treponeme. It is of interest to note that the monoglyceride tartaric acid ester compounds, as well as higher fatty acids, have recently been involved in the replacement of serum for the cultivation of the protozoan *Trichomonas gallinae* (Shorb and Lund, 1958).

During this investigation, some variation in total cell crop was observed between sera of different lots, even though the medium had been supplemented with fatty acids. The reason for this discrepancy is not known at present.

Turbidimetric, nitrogen, and dry weight determinations were considered to be the most accurate methods for the quantitative measurement of growth. Optical density determinations give an indication of total cellular material; they can be correlated with direct measurement of growth and are the most convenient to perform. Direct microscopic counts are tedious and time consuming to perform; and, furthermore, some variations were observed between this method and the other techniques for measurement of growth due to the differences in individual cell sizes during the various growth phases.

Frequently, in old cultures especially, these organisms exhibit balloon-like forms when multiplication becomes untenable (Gelperin, 1949).

The organism retains its normal morphology when grown in the monoglyceride tartaric acid ester or fatty acid supplemented media.

No attempt was made to determine the essential nutrients supplied by the serum, other than to demonstrate that it could not be replaced by any of the fatty acids either individually or in combinations.

SUMMARY

Growth of the Reiter treponeme in a spirochete broth plus 10 per cent inactivated sheep serum medium supplemented with TEM 4T (the diacetyl tartaric acid ester of tallow monoglycerides) at a concentration of 0.20 to 0.25 mg per ml results in a cell crop 2 to 3 times as large as that obtained without the fatty acid supplement. The TEM 4T can be replaced by a mixture of palmitic, stearic, and oleic acids, in equal amounts, and at a total fatty acid concentration of 0.20 to 0.25 mg per ml of the medium.

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