PARTIALLY DEFINED MEDIUM FOR THE CULTIVATION OF *BORRELIA VINCENTII*

T. A. NEVIN AND E. G. HAMPP[1]

National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland

Received for publication February 9, 1959

The recognition and evaluation of the physiological activities of *Borrelia vincentii* are dependent upon the formulation of a more reproducible medium for its cultivation. A defined medium, developed by Steinman *et al.* (1952) was adequate for the growth of the Reiter treponeme, but would not support the growth of several other varieties of spirochetes (Hampp, 1957).

More recently, *B. vincentii* has been cultivated in a veal heart infusion medium wherein five coenzymes and glucose-1-phosphate were substituted for the ascitic fluid enrichment (Hampp and Nevin, 1959). The infusion medium could not be used for more definitive studies, however, since erratic, submaximal growth occurred frequently when any one of the required factors was omitted. Thus a simple reproducible basal medium has been devised which, although requiring an ascitic fluid enrichment, is suitable for further experimentation. Moreover, requirements for several nutrilites have been established.

MATERIALS AND METHODS

The medium consisted of 1 per cent "Vitamin Free" Casamino acids (Difco),[2] 10 mg per cent L-tryptophan, 0.5 per cent K_2HPO_4, and 0.25 per cent glucose. A mineral supplement included $MgSO_4 \cdot 7H_2O$, 8 mg; $FeSO_4 \cdot 7H_2O$, 0.4 mg; and $MnCl_2$, 0.12 mg; per L of complete medium. Added also was a mixture of known vitamins containing riboflavin, biotin, thiamin-HCl, folic acid, niacin, niacinamide, calcium pantothenate, pyridoxine, pyridoxamine, and pyridoxal. Each was supplied at 1 μg per ml final concentration. These materials were combined, adjusted to pH 7.5, and brought to $\frac{2}{3}$ the desired final volume. Ten-ml samples were dispensed into 15 by 125 mm culture tubes, and sterilized in the autoclave (121 C, 15 min). In the present work, stainless steel culture tube caps were used during sterilization.

The addition of reduced glutathione at 1 mg per ml, and a mixture of purines and pyrimidines, including adenine, guanine, xanthine, hypoxanthine, uracil, cytosine, and thymine, at 5 μg per ml each final concentration, completed the basal medium used in the experiments. These last, as well as those required nutrilites subsequently determined, were sterilized by sintered glass filtration and added aseptically to the medium. Sterile distilled water was used to bring all tubes to a final volume of 15 ml. The steel caps were replaced by sterile rubber stoppers in order to maintain anaerobiosis during the 10-day incubation period.

The inoculum was grown in veal heart infusion medium enriched with dog ascitic fluid (Hampp and Nevin, 1959). The cells were collected by centrifugation, then resuspended in a 15-ml sample of the experimental medium. Subsequent inocula, approximately 4 to 7 $\times$ 10^6 cells per tube, have been grown in the partially defined medium.

Growth in the medium was estimated as turbidity when viewed in fluorescent light against a dark background. Thus, it has been profitable to interpret a number of experiments on the basis of growth *vs.* no growth when determining the need for certain compounds. Whenever necessary, direct cell counts were performed in a Petroff-Hauser chamber. The ascitic fluid enrichment was reduced in volume whenever practicable to identify additional nutrilites. All tests were conducted in quadruplicate.

RESULTS

During the first series of experiments, a 10 per cent by volume ascitic fluid enrichment was used as the source of required cofactors. Growth was poor in these experiments, averaging only 10 to 15 $\times$ 10^6 cells per ml. The addition of 1 μg per

[1] Senior Research Associate, American Dental Association at the National Institute of Dental Research.

[2] Casamino acids can be replaced by a mixture of known L-amino acids. As a matter of convenience, the less refined material is used routinely.

TABLE 1

Effect of fatty acids on growth and granule formation by Borrelia vincentii in a simplified basal medium

Fatty Acid*	Cell Count per Ml†	Granules‡
	× 10^6	
Arachidic	61	Numerous
Stearic	40	Numerous
Palmitic	34	Numerous
Myristic	57	Numerous
Lauric	51	Numerous
Adipic	57	Numerous
Vaccinic	47	Numerous
Oleic	60	Few
Linoleic	46	Numerous
Methyl ricinoleic	36	Numerous
None	31	Moderate

* Added at 0.1 μg per ml final concentration to the medium.

† Cell count includes granules.

‡ Numerous = over 15 per cent of cell count; moderate = 5 to 15 per cent; and few = less than 5 per cent.

ml of cocarboxylase to the medium roughly doubled the cell count. The same concentration of thiamin-HCl had no activity.

Examination of the cultures under the dark-field microscope revealed numerous granules and morphologically atypical cells. Since no lipids had been added to the medium of first approximation, a series of common fatty acids were screened for activity. Each was supplied at 0.1 μg per ml final concentration. Among the several fatty acids which improved growth, only oleic acid was functional also in reducing granule formation. These experiments are summarized in table 1. It should be noted that 50 to 60 × 10^6 cells per ml is about maximal growth in the test system. Although it is perhaps questionable, in this instance granules were counted as spirochetes, since insofar as it is known, each granule was derived from a single spirochete (Hampp *et al.*, 1948). It was later determined that the oleic acid concentration could be increased to 0.5 μg per ml without deleterious effect. Thus fatty acid concentration would not be a limiting factor in further experimentation.

Upon the addition of thiamin pyrophosphate and oleic acid, the medium proved suitable for further nutritional studies. To this end, a single vitamin was omitted from each of a series of media. Subcultivation of the spirochete in such deficient media demonstrated requirements for riboflavin, biotin, and folic acid, since growth beyond the second transfer did not occur in the absence of any of these three compounds. Dihydrofolic acid will substitute for folic acid, but tetrahydrofolic acid, citrovorum factor, and *p*-aminobenzoic acid are not utilized.

No further growth requirements could be determined in the test system as it was constituted. Therefore, the volume of ascitic fluid was reduced from 1.5 to 0.2 ml per tube (1.33 per cent by volume). At the reduced level no visible turbidity occurred during a 10-day incubation period. Supplementation of the medium with certain of the coenzymes which enhanced growth in a veal heart infusion medium (Hampp and Nevin, 1959) demonstrated a need for adenosine triphosphate. Inspection of table 2 will show that at a limiting level of ascitic fluid, 0.2 ml per tube, neither the riboside, the monophosphate, nor the diphosphate would substitute for the triphosphate. Microscopic examination of those tubes in which no visible turbidity developed revealed a few motile cells but continued incubation produced no change in the outcome. At an increased level of ascitic fluid, 0.5 ml per tube, there was no evidence of an adenosine triphosphate requirement.

A requirement for coenzyme A was established also. In this instance, direct cell counts were necessary for differentiation since, as is manifest in table 3, either pantothenate or pantetheine

TABLE 2

Comparison of adenosine and its several phosphates in the partial replacement of ascitic fluid for the growth of Borrelia vincentii in a simplified medium

Compound Omitted from Medium	Growth*	
	1.33% Ascitic fluid	3.33% Ascitic fluid
Adenosine	+	+
Adenosine monophosphate	+	+
Adenosine diphosphate	+	+
Adenosine triphosphate	−	+
None	+	+
All	−	+

* + = growth; − = no growth.

TABLE 3

Relative effectiveness of molar equivalents of coenzyme A, pantetheine, and calcium pantothenate on the growth of Borrelia vincentii in a partially defined medium

Compound Added to Medium	Cell Count per Ml
	× 10^6
Coenzyme A	55
Pantetheine	31
Calcium pantothenate	32
None	6*

* There were no motile cells in the control.

can be used by the spirochete. The precursors, added at 0.45 mμmoles per ml, permitted only about ½ the amount of growth as was obtained with a like amount of coenzyme A. No significant growth occurred unless one of the three was supplied. Adenosine triphosphate was included in the medium at the μg per ml level.

The cell crop obtained (55 × 10^6 per ml) when adenosine triphosphate and coenzyme A were added to medium containing 0.2 ml of ascitic fluid was comparable to that previously obtained by adding 1.5 ml of ascitic fluid per tube. Therefore, the level of ascitic fluid was reduced further, to 0.05 ml per tube (0.3 per cent). At this level perceptible growth (10 to 15 × 10^6 cells per ml) still occurred.

In view of the observations of Steinman *et al.* (1953) on the need of the Reiter treponeme for NH_4^+ and of a saprophytic treponeme for CO_2 (Steinman *et al.*, 1954), *B. vincentii* was tested for similar requirements. Equimolar amounts of urea, L-arginine, L-glutamine, and NH_4^+ (as ammonium acetate) were inactive. L-Asparagine, however, increased the cell count to about 22 × 10^6 cells per ml when supplied at 4 mg per 15 ml of medium. The addition of 4 mg of $NaHCO_3$ per tube as a source of CO_2 to media containing L-asparagine brought the yield up to 45 to 50 × 10^6 cells per ml, just slightly less than maximum.

An inoculum as small as 3 to 4 × 10^5 cells per ml has survived up to 4 weeks at 35 C in the basal medium supplemented with all of the required factors, but in the absence of ascitic fluid. Thereafter, the addition of 1 drop of ascitic fluid enabled the organism to reach visible turbidity in 4 or 5 days, and maximal growth in 10 days.

DISCUSSION

The present work and that of Eagle and Steinman (1948), Steinman and Eagle (1950), and Oyama *et al.* (1953) suggest that the spirochetes, although more exacting nutritionally, probably do not differ greatly from other microorganisms in their fundamental metabolism.

In common with such organisms as *Corynebacterium diphtheriae* (Cohen *et al.*, 1941), *Mycobacterium tuberculosis* (Dubos and Davis, 1946), and the Reiter treponeme (Oyama *et al.*, 1953) among others, *B. vincentii* requires oleic acid for growth in a simplified medium. Oleic acid does not, however, substitute for ascitic fluid in the system used. Neither is there evidence of toxicity at the level used, since small inocula survived for several weeks in the presence of oleate without added protein. These observations are somewhat different from those reported by Oyama *et al.* (1953) wherein it was reported that crystalline bovine albumin had as its primary function the supplying of an essential lipid or when further purified, the detoxification of added excess oleic acid.

SUMMARY

A relatively simple and reproducible basal medium has been devised for the cultivation of *Borrelia vincentii*. Required additions to the medium include riboflavin, biotin, folic acid, cocarboxylase, oleic acid, and an ascitic fluid factor not yet identified. Maximal growth approximates 50 × 10^6 cells per ml.

The volume of ascitic fluid required for maximal growth has been reduced from 10 per cent to 0.3 per cent by the substitution of coenzyme A, adenosine triphosphate, CO_2 (as $NaHCO_3$), and L-asparagine.

Small inocula (3 to 4 × 10^5 cells per ml) have survived up to 4 weeks in the medium without added ascitic fluid.

REFERENCES

COHEN, S., SNYDER, J. C., AND MUELLER, J. H. 1941 Factors concerning the growth of *Corynebacterium diphtheriae* from minute inocula. J. Bacteriol., **41**, 581–591.

DUBOS, R. J. AND DAVIS, B. D. 1946 Factors affecting the growth of tubercle bacilli in liquid media. J. Exptl. Med., **83**, 409–423.

EAGLE, H. AND STEINMAN, H. G. 1948 The nutritional requirements of treponemata. I. Arginine, acetic acid, sulfur-containing com-

pounds, and serum albumin as essential growth-promoting factors for the Reiter treponeme. J. Bacteriol., **56,** 163–176.

HAMPP, E. G. 1957 Investigations of the nutritional requirements of the oral spirochetes. Preprinted abstracts, 35th Gen. meeting, Intern. Assoc. Dental Research.

HAMPP, E. G. AND NEVIN, T. A. 1959 The substitution of known compounds for ascitic fluid in the cultivation of *Borrelia vincentii*. J. Bacteriol., **77,** 800–803.

HAMPP, E. G., SCOTT, D. B., AND WYCKOFF, R. W. G. 1948 Morphologic characteristics of certain strains of oral spirochetes and *Treponema pallidum* as revealed by the electron microscope. J. Bacteriol., **56,** 755–769.

OYAMA, V. I., STEINMAN, H. G., AND EAGLE, H. 1953 The nutritional requirements of treponemata. V. A detoxified lipid as the essential growth factor supplied by crystallized bovine albumin. J. Bacteriol., **65,** 609–616.

STEINMAN, H. G. AND EAGLE, H. 1950 Nutritional requirements of treponemata. II. Pantothenic acid, glutamine and phenylalanine as additional growth-promoting factors for the Reiter treponeme. J. Bacteriol., **60,** 57–68.

STEINMAN, G. H., EAGLE, H., AND OYAMA, V. I. 1952 The nutritional requirements of treponemata. III. A defined medium for the cultivation of the Reiter treponeme. J. Bacteriol., **64,** 265–269.

STEINMAN, H. G., EAGLE, H., AND OYAMA, V. I. 1953 Nutritional requirements of treponemata. IV. The total nitrogen requirement of the Reiter treponeme. J. Biol. Chem., **200,** 775–785.

STEINMAN, H. G., OYAMA, V. I., AND SCHULZE, H. O. 1954 Carbon dioxide cocarboxylase, citrovorum factor and coenzyme A as essential growth factors for a saprophytic treponeme (S-69). J. Biol. Chem., **211,** 327–335.