SUBSTITUTION OF KNOWN COMPOUNDS FOR ASCITIC FLUID IN THE CULTIVATION OF *BORRELIA VINCENTII*

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The isolation and cultivation of the various members of the family Treponemataceae on artificial culture media have been complicated first by the need of an adequate base medium for growth, and secondly by the absolute requirement of the microorganisms for ascitic fluid, blood, plasma, serum, or serum products (Noguchi, 1912; Kast and Kolmer, 1940; Rosebury and Foley, 1942; Wichelhausen and Wichelhausen, 1942; Hampp, 1943, 1954; Little and SubbaRow, 1945; Fitzgerald and Hampp, 1952). Ascitic fluid is in most instances superior to all other materials used for this purpose, particularly for primary isolation of the spirochaetes (Hampp, 1943). Once the microorganisms are obtained in pure culture, many of the serum products may be substituted for ascitic fluid with success for maintenance of the strains.

The lack of knowledge of the nature of the indispensable factors present in such complex biological materials has been a deterrent in the elucidation of the nutritional requirements of this little understood group of organisms. Little and SubbaRow (1945) have reported that serum albumin is apparently the material contained in ascitic fluid, blood, plasma, and serum which is essential for the growth of the Reiter treponeme. In addition, Whiteley and Frazier (1948), Eagle and Steinman (1948), and Oyama *et al.* (1953) also concluded that serum albumin is a required factor for the growth of this avirulent strain of *Treponema pallidum* and they attempted to elucidate its mode of action in defined media.

The present report is concerned with the partial definition of the nutrition of a strain of *Borrelia vincentii*, designated as N9 (Hampp, 1947*a*, *b*, *c*; Hampp *et al.*, 1948; Fitzgerald and Hampp, 1952), which has permitted the cultivation of this organism in an infusion medium without natural enrichment.

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MATERIALS AND METHODS

The basal medium employed in this study has been described previously (Hampp *et al.*, 1948; Fitzgerald and Hampp, 1952), and is essentially a veal heart infusion broth containing thiopeptone (BBL). For this study, the medium was sterilized by autoclaving at 121 C for 15 min rather than by tyndallization and the precipitate formed by this treatment was allowed to settle out. Prior to use, the clear broth was separated from the precipitate, appropriate quantities were heated in a boiling water bath to remove the residual oxygen, and enough filter-sterilized glutathione was added to provide a final concentration of 1 mg per ml.

Aqueous solutions of the several coenzymes, sugar phosphates, and other metabolites tested were prepared. Mixtures, containing portions of all but one of the test substances, were sterilized by passage through Corning sintered glass filters, and dispensed into sterilized 15 by 125 mm culture tubes. Fresh solutions of test materials were prepared for each experiment. A requisite factor could then be identified by the failure of the spirochaete to attain maximal growth in the basal medium without the addition of that factor. All experiments were conducted in duplicate. Positive controls were effected by the addition of all the test supplements to the basal medium. Growth never developed in the absence of additives. In addition, cultures containing ascitic fluid as the only additive were used as growth controls where required. For purposes of this work, maximal growth was considered to be equal to the number of cells (80 to 90 $\times 10^6$ cells per ml) that developed from a standard inoculum in the basal medium supplemented with ascitic fluid.

The original inoculum was grown in the described basal medium enriched with canine ascitic fluid (10 per cent by volume), and the cells thus obtained were harvested by centrif-

ugation. After resuspension in fresh basal medium, the number of cells was estimated by direct counting in a Petroff-Hauser chamber and an average of 35.26×10^6 cells was introduced into each tube of the first test series. Each tube was then filled with sterile test medium to give a final volume of approximately 15 ml, leaving only sufficient space to insert a sterile rubber stopper. This served to mix the inoculum and additives while maintaining suitably reduced conditions without the use of special anaerobic equipment. Thereafter, in the serial growth tests each experiment provided the inoculum for the next experiment and 0.5-ml volumes were used for this purpose. The incubation period was 10 days at 35 C.

EXPERIMENTAL RESULTS

Preliminary experiments with ascitic fluid revealed that the material essential for the growth for *B. vincentii* was insoluble in acetone. It remained in solution, however, on acidification of the fluid and precipitation of proteins at pH 4.5 to 5.0. Additional qualitative tests, based on the method of Fiske and SubbaRow (1925), suggested the presence of a fairly high concentration of organic phosphates. It was decided, therefore, to substitute a number of coenzymes and phosphorylated intermediates for the usual ascitic fluid enrichment of the basal medium. A list of the compounds tested is presented in table 1.

Maximal growth of *B. vincentii* (80 to 90×10^6 cells per ml) was attained when cocarboxylase, codecarboxylase, adenosine triphosphate, diphosphopyridine nucleotide, coenzyme A, and glucose-1-phosphate were added to the basal medium in the concentrations indicated in table 2. The cultures were maintained under these conditions through 12 successive transfers without evidence of diminution in cell counts, indicating that such subcultures could be continued indefinitely. The cell counts thus obtained are comparable to those when ascitic fluid was employed as the sole enrichment for the basic medium. This is the first time to the knowledge of the authors that a pure culture of a spirochaete has been grown with maximal cell yield in a medium without ascitic fluid or other natural supplements.

In the course of these experiments it was not unusual for some growth of the microorganism to occur in the absence of a single required factor; however, such growth was erratic and usually not more than half maximal. These data are summarized in table 2.

In addition, the relatively high average cell counts obtained when diphosphopyridine nucleotide, adenosine triphosphate, or glucose-1-phosphate was omitted from the medium are more suggestive of stimulatory effects, rather than absolute requirements for these materials.

In an effort to determine the applicability of these findings for other strains of spirochaetes, preliminary experiments were conducted on the growth of additional strains of oral spirochaetes in the basal medium free of ascitic fluid but supplemented with the six substances indicated in table 2. Four strains of the oral treponemes identified as FM, MRB, JH, and N39 (Hampp, 1946, 1947*b*, 1951; Hampp *et al.*, 1948; Fitzgerald and Hampp, 1952); three additional strains of *B. vincentii*, designated as N19, SCN, and EH (Hampp, 1947*a*, *b*; Hampp *et al.*, 1948; Fitzgerald and Hampp, 1952); and three strains of *Borrelia buccalis*, denoted as GT2, PBB, and NH1 (Hampp, 1954), were employed for this purpose. All organisms grew well through two transfers under these conditions, with the exception of the

TABLE 1

Compounds screened as substitutes for ascitic fluid in nutrition of Borrelia vincentii

Coenzymes	Phosphorylated Intermediates	Miscellaneous
Cocarboxylase	Glucose-1-PO_4	Deoxyribonucleic acid
Codecarboxylase	Glucose-6-PO_4	Ribonucleic acid
Coenzyme A	Mannose-6-PO_4	Pyruvic acid
Adenosine triphosphate	Galactose-6-PO_4	Lecithin
Adenosine diphosphate	Ribose-5-PO_4	Glycogen
Diphosphopyridine nucleotide	Fructose-1-6-diPO_4	Glucose
Triphosphopyridine nucleotide	Phosphoglyceric acid	
Uridine monophosphate		

TABLE 2

Comparison of cell crops obtained in absence of given growth factors with those in which all required factors were provided

Factor Omitted from Basal Medium	Final Conc	Cell Crop per ml × 10⁶*	
		Avg	Range
	μg/ml		
Coenzyme A	0.3	31.4	0–55
Pyridoxal phosphate	1.0	25.8	7–50
Diphosphopyridine nucleotide	0.1	52.75	37–62
Adenosine triphosphate	1.0	46.5	41–50
Cocarboxylase	1.0	22.8	0–46
Glucose-1-phosphate	33.3	66.3	7–71
Control with all above factors present		88.8	78–92
Control with all above factors omitted		5.0†	4–6

* Includes 12 experiments in duplicate.

† These organisms were for the most part nonmotile.

strains of *B. buccalis*, which did not survive. The latter finding was not unexpected, since these microorganisms are more fastidious than the other oral spirochaetes, requiring for growth both a larger amount of ascitic fluid and a serum supplement (Hampp, 1954).

DISCUSSION

The requirement for five coenzymes and a phosphorylated sugar by *B. vincentii* in order to reach maximal growth may explain in part the difficulty heretofore experienced in isolation and cultivation of these organisms (Hampp, 1945). Further the complex nutritional pattern exhibited may be a guide to a better understanding of the obligate parasitism which is usual among the Treponemataceae.

The need for cocarboxylase and coenzyme A is not peculiar to *B. vincentii*. Steinman *et al.* (1954*a*) reported a similar requirement by a saprophytic treponeme, which also required citrovorum factor. There are no reports known to the authors which suggest the need for exogenous diphosphopyridine nucleotide (DPN) and codecarboxylase in spirochaetal nutrition. Barban (1954) has, however, demonstrated a DPN-dependent glutamic dehydrogenase and a pyridoxal phosphate dependent transaminase (Barban, 1956) in cell-free extracts of the Reiter treponeme. In connection with this last, Steinman *et al.* (1954*b*) were unable to show any requirement for vitamin B_6 for growth of the Reiter organism, whereas in the present work it was found that *B. vincentii* must be supplied with codecarboxylase. The varied cofactor requisites exhibited by these spirochaetes may, upon further investigation, prove valuable in designing a taxonomic system for the Treponemataceae. This is further exemplified by the finding that three strains of *B. buccalis* did not survive in the basal medium containing known compounds sufficient for *B. vincentii* strain N9, whereas strains of oral treponemes and additional strains of *B. vincentii* reached maximal growth.

The role of either adenosine triphosphate or glucose-1-phosphate in the nutrition of *B. vincentii* is not clear. Adenosine triphosphate is required in the nutrition of pleuropneumonialike organisms (Smith, 1955) but there are no prior reports known to the authors of similar requirements by other bacteria. With respect to glucose-1-phosphate, the organism does not utilize glycogen in the test system, even when supplied with glucose-1-phosphate. A glucose requirement is also not evidenced.

From these findings it is apparent that the three types of oral spirochaetes studied differ among themselves in their nutritional requirements. Further studies employing a wide range of cultivable spirochaetes will be required before the nature of the essential factors in complex biological materials can be unequivocally established.

SUMMARY

The ascitic fluid supplement normally required for the growth of *Borrelia vincentii* in a veal heart infusion medium can be replaced by a mixture of five coenzymes (cocarboxylase, codecarboxylase, coenzyme A, adenosine triphosphate, and diphosphopyridine nucleotide) and glucose-1-phosphate. The organism reached maximal growth through 12 successive transfers in this medium. Further, four strains of oral treponemes and three additional strains of *B. vincentii* reached maximal growth through two transfers in the same medium; however, three strains of *Borrelia buccalis* did not survive under these conditions.

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