RESPONSE OF *Treponema pallidum* TO CERTAIN NUTRILITES*†

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Studies of the nutrition of *Treponema pallidum* are based on maintaining motility under *in vitro* experimental conditions (Boak, Fawcett, and Carpenter, 1949; Kimm, Allen, Morton, and Morgan, 1960; Nelson, 1948; Nelson and Steinman, 1948; Weber, 1960). Although this method does not always yield the clear-cut results of the growth-no growth experiments usual in studies of the nutrition of cultured spirochaetes, or of *Eubacteriales*, it is an effective indicator of viability. In its present application, the response of the virulent organism to certain factors which are required for the growth of several cultured spirochaetes has been investigated. In addition, certain precedent observations have been confirmed.

Material and Methods

After propagation in rabbit testes, actively motile (90–100 per cent.) *Treponema pallida* were extracted from the minced tissue by shaking in a filter-sterilized† menstrium of the following composition:

Casamino acids (Difco) 0.5 per cent.;
K₂HPO₄ 0.5 per cent.;
Reduced glutathione 0.1 per cent.;
L-tryptophane 0.01 per cent.;
MgSO₄ · 7H₂O 0.8 per cent.;
MnCl₂ 0.012 mg. per cent.;
FeSO₄ · 7H₂O 0.04 mg. per cent.

Carbon dioxide was supplied as NaHCO₃ (0.025 per cent.), and the final pH was 7.2. The bulk of the testicular debris was removed by centrifugation at 2,000 r.p.m. for 10 minutes. Thereafter, sufficient sterile menstrium was added to ensure 15×10^6 to 25×10^6 cells per ml. in each test mixture at the final concentration. The suspensions were then transferred to glass Wheaton bottles fitted with rubber sleeve caps. The bottles had been flushed with Forming gas (90 per cent. N₂; 10 per cent. H₂) three times, then autoclaved at 121°C. for 15 minutes.

The materials to be tested were dissolved in aliquots of the menstrium, sterilized by filtration, and then dispensed into Wheaton bottles prepared as above. These

solutions were prepared so that in a 5-ml. test volume, vitamins and coenzymes would be supplied at 1 µg. per ml., oleic acid at 0.5 µg. per ml., purines and pyrimidines at 5 µg. per ml., and glucose at 5 µg. per ml. Yeast extract (0.5 per cent.) was used as a source of vitamins and other factors in early experiments.

The suspensions of spirochaetes and the test solutions were "gassed" into an anaerobic chamber (a germ-free isolator, Reyniers and Son, Chicago, Ill.) by means of a double-dooredclave. An atmosphere of Forming gas was maintained in the chamber, and a palladium catalyst aided in reducing the small amounts of oxygen inadvertently admitted during passage through theclave. The experiments were assembled in Wheaton bottles, prepared as described, or in sterile culture tubes fitted with stainless-steel caps. Either was used with equal facility in the chamber. A small sample (0.1 ml.) of the test suspension was withdrawn by syringe for determining motility. Under conditions of continuous anaerobiosis (Rosebury and Reynolds, 1964; Socransky, Macdonald, and Sawyer, 1959), each mixture could be sampled repeatedly without introducing extraneous oxygen. The samples were passed out of the chamber through theclave, which served as an anaerobic lock, after which the test suspensions were placed inside theclave for incubation. Theclave is jacketed; thus its internal temperature can be controlled by circulating water of suitable temperature through the jacket.

The percentage motility was estimated as the number of unequivocally motile spirochaetes per 100 counted.

When the effect of several different gases or mixtures of gases was investigated, Torbal anaerobic jars (Torsion Balance Co., Clifton, N.J.) were used. Air was replaced by flushing with the gas, at 3–5 P.S.I. valve-diaphragm pressure for 10 minutes. When hydrogen was not in the gas mixture, a 30 × 110-mm. tube, containing 10 ml. of a 10 per cent. solution of pyrogallol in 2.5 N NaOH, was placed in the jar to absorb residual oxygen.

Results and Discussion

Oleic acid and cocarboxylase, which are required for cultivating certain nonvirulent spirochaetes (Nevin and Hampp, 1959; Oyama, Steinman, and Eagle, 1953; Steinman, Oyama, and Schulze, 1954), also prolonged the motility of *T. pallidum* in yeast extract-supplemented menstria. As is indicated in Table I, when 0.5 µg. per ml. oleic acid was added to the menstrium, about 30 per cent. of the

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† Trade names are used for identification only and do not represent an endorsement by the Public Health Service or the U.S. Department of Health, Education, and Welfare.

‡ Ultra-fine sintered glass filters, Corning Glass Works, Corning, N.Y.

spirochaetes were still motile after 164 hours. A similar level of motility was observed when 1 µg. per ml. of cocarboxylase was added instead of the oleate. No additive effect was obtained when both compounds were added. Efforts to substitute other fatty acids for oleate suggested that butyrate would probably be satisfactory. There were relatively few motile spirochaetes in the unsupplemented menstruum. Both oleate and cocarboxylase were added to the menstrua used in further experiments. That the need for oleate did not appear in previous investigations was probably a result of the use of albumin (Nelson and Steinman, 1948) or high serum levels (Boak and others, 1949; Weber, 1960) in the menstrua. It is also probable that cocarboxylase was supplied by the tissue fluids.

TABLE I
EFFECT OF GROWTH-PROMOTING SUBSTANCES ON
THE MOTILITY OF *T. PALLIDUM*

Base Menstruum ³ with Yeast Extract	Percentage Motile ¹	
	96 hrs ²	164 hrs ²
None		8
Cocarboxylase (µg./ml.)		36
Oleate (0.5 µg./ml.)		32
Butyrate (0.5 µg./ml.)		28
Oleate + Cocarboxylase		31
Butyrate + Cocarboxylase		35
Base menstruum ^{3,4} with B vitamins, cocarboxylase, and oleate		
Glucose (5 µg./ml.)	14	
Adenine (5 µg./ml.)	11	
Adenine + Glucose	49	

¹ Initial motility was 95–100 per cent.

² Incubated at 30° C. in Forming gas.

³ Composition in text.

⁴ Yeast extract was omitted from this menstruum.

Simplification of the menstruum seemed a suitable method of identifying the additional compounds required; a mixture of "B" vitamins was therefore substituted for the yeast extract. The mixture included: pantothenic acid, riboflavin, niacin, niacinamide, pyridoxine, pyridoxamine, pyridoxal, folic acid, and biotin. As may be seen in Table I, it was necessary to add glucose (Bryant, 1952; Kimm, Allen, Morton, and Morgan, 1962) and adenine to the vitamin-supplemented menstruum in order to sustain satisfactory levels of motility. If either compound was omitted from the menstruum, few motile spirochaetes were found after 96 hrs' incubation. There was no evidence that pyrimidine was required. The probability that pyruvic acid may be substituted for glucose (Nelson, 1948; Weber, 1960) was not investigated. The vitamin supplement, glucose, and adenine replaced yeast extract in following experiments.

In studies of the nutrition of *Borrelia vincentii* (Hampp and Nevin, 1959; Nevin and Hampp, 1959), the need for a coenzyme supplement for the cultivation of this organism in a simplified medium was revealed by reducing the amount of added ascitic fluid from 10 to 0.33 per cent. Thus it seemed probable that further simplification of the suspending menstruum for *T. pallidum* might be effected by reducing the amount of testicular tissue fluid ordinarily present in the suspensions. Therefore, after the testicular debris had been removed, the suspensions were re-centrifuged at 10,000 r.p.m. for 15 minutes. The supernatant was decanted, and the spirochaetes were re-suspended in fresh sterile menstruum. Although preliminary experiments with re-suspended cells had not indicated any measurable response to its inclusion, it was decided to add a coenzyme supplement such as that required by *B. vincentii* to the menstruum.

The changes in spirochaetal motility resulting from the reduction of the tissue fluid content of the menstruum are summarized in Table II. About 22 per cent. of the re-suspended spirochaetes were motile initially (1 to 2 hrs), the proportion decreasing to about 14 per cent. after 72 hrs' incubation. However, when 0.1 ml. of the tissue fluid-rich supernatant (unfractionated control) was included, 97 per cent. were motile initially, and 82 per cent. after 72 hours. An equivalent amount of an acetone-soluble, ether-insoluble, Molisch-positive fraction of the tissue fluid supernatant, presumably a carbohydrate (Rice and Nelson, 1951), caused a resumption of motility from 31 per cent. initially to 70 per cent. after 72 hours. The enhancement of spirochaetal motility by tissue fluids (Nelson, 1948) or fractions thereof (Rice and Nelson, 1951) is thus confirmed.

TABLE II
EFFECT OF FRACTIONS OF RABBIT TESTICULAR
FLUID ON THE MAINTENANCE OF MOTILITY
OF VIRULENT *T. PALLIDUM*

Fraction in Base Menstruum ² with "B" Vitamins and Coenzyme Supplement ³	Percentage Motile	
	1–2 hrs	72 hrs ¹
Acetone-soluble Ether-soluble	34	28
Acetone-soluble Ether-insoluble	31	70
Acetone-insoluble	86	10
Unfractionated control	97	82
None added	22	14

¹ Incubated at 30° C., in Forming gas

² Composition in text.

³ Nevin and Hampp (1959).

The initial activity of acetone-insoluble fractions of tissue fluid and of unfractionated samples may indicate other nutrilites (e.g. phospholipids) or may reflect a residuum of the acetone-soluble, ether-

TABLE III
RESUMPTION OF MOTILITY BY *T. PALLIDUM* IN HUMAN SERUM AND AIR, AND SUSTENANCE
OF MOTILITY IN VARIOUS GASES OR MIXTURES

Percentage Motile		Gas						
		Air	CO ₂	N ₂ CO ₂	N ₂	N ₂ H ₂	H ₂	He
Initial (within 5 min.)	Serum added Control, no serum	94 0						
4 hrs (1)	Serum added Control, no serum	34 0	46 0	97 0	95 0	89 0	93 0	93 0

(1) Incubated at room temperature.

insoluble fraction, and the need for a carrier similar to the protein needed for the assimilation of fatty acids (Oyama and others, 1953). These possibilities were not studied further. Acetone-soluble, ether-soluble fractions of tissue fluid did not appear to enhance motility.

Resumed motility such as that indicated in Table II has been observed at varying intervals in fifteen of 65 different experiments, without relationship to the known nutritives tested. There was no evidence of an increase in cell numbers in any of these experiments; thus it seemed possible that the spirochaetes could be immobilized and could then be caused to resume motility under controlled conditions. Therefore, spirochaetes were extracted into 0.033 M K₂HPO₄ (pH 7.3) and washed once therein. Washing in this manner rendered the cells nonmotile. Upon adding 0.2 ml. heated (56°C.; 30 min.) human serum to 0.3 ml. of cell suspension in air at room temperature, over 90 per cent. of the cells became actively motile within 5–10 minutes. These data are summarized in Table III, in which it is also apparent that resumed motility can be maintained for at least 4 hours under anaerobic conditions. These results were obtained in several gases or mixtures of gases. Spirochaetes in suspension without added serum did not resume motility. Since there was no "carry-over", it is probable that sustained motility is dependent upon a nutritive of animal origin which the spirochaete uses readily but cannot accumulate.

Summary

- (1) The addition of oleic acid or butyric acid, cocarboxylase, and adenine to a base menstruum supplemented with "B" vitamins enabled *Treponema pallidum* to remain motile for extended periods under *in vitro* conditions.
- (2) The requirements of this organism for tissue fluids and for glucose, defined by other investigators, have been confirmed.
- (3) Over 90 per cent. of freshly-extracted spiro-

chaetes, which had been rendered nonmotile by washing and re-suspending in phosphate buffer, resumed active motility within 5 minutes when heated human serum was added to the suspensions.

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La réaction du *Treponema pallidum* à certains "nutrilites"

RÉSUMÉ

- (1) L'addition de l'acide oléique ou butyrique, de co-carboxylase et d'adénine à une base menstruum additionnée de vitamines "B" avait permis au *Treponema pallidum* de rester mobile pendant de longues périodes sous des conditions *in vitro*.
- (2) Les exigences de cet organisme pour les liquides des tissus et pour le glucose, décrites par d'autres chercheurs, ont été confirmées.
- (3) Plus de 90 pour cent des spirochètes récemment extraits qui avaient été rendus immobiles par le lavage et la résuspension dans une solution tampon de phosphate avaient repris leur mobilité en cinq minutes quand du sérum humain chauffé avait été ajouté aux suspensions.