DEPENDENCY OF TREPONEMA MICRODENTIUM ON OTHER ORAL ORGANISMS FOR ISOBUTYRATE, POLYAMINES, AND A CONTROLLED OXIDATION-REDUCTION POTENTIAL

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Abstract

SOCRANSKY, S. S. (Forsyth Dental Center, Boston, Mass.), W. J. LOESCHE, C. HUBERSAK, AND J. B. MACDONALD. Dependency of Treponema microdentium on other oral organisms for isobutyrate, polyamines, and a controlled oxidation-reduction potential. J. Bacteriol. 88:200-209. 1964.—Strains of Treponema microdentium can be cultivated on a variety of autoclaved commercially available media in the presence of other oral organisms. Organisms supporting growth in these circumstances include a facultative diphtheroid accompanied by either a strain of Fusobacterium or a motile gram-negative anaerobic rod. Culture filtrates and lysates of these "supporting organisms" failed to substitute for growing organisms. Measurement of the oxidation-reduction potential of the test system demonstrated that the spirochetes grew in a narrow range of Eh (optim. -190 mV). The supporting organisms could be replaced by their filtrates when the Eh of the medium was poised in this range by a combination of reducing agents. Both filtrates contained a heat-labile factor required by the spirochete, which could be replaced by 5 μg/ml of cocarboxylase. Isobutyric acid, which could be detected in the fusiform filtrate, and putrescine which could be detected in the diphtheroid filtrate, replaced the spirochete’s remaining filtrate requirement. Maximal growth occurred when any of the following were incorporated into the medium: 2 μg/ml of sodium isobutyrate; 250 μg/ml of putrescine dihydrochloride; 200 μg/ml of spermidine phosphate, or 150 μg/ml of spermine tetrahydrochloride.

Oral spirochetes are usually cultivated on a complex Tyndallized veal heart infusion medium containing ascitic fluid (Hampp, 1943; Rosebury et al., 1951) or rabbit blood (Berger, 1958; Sohransky, Macdonald, and Sawyer, 1959). Attempts to simplify and define this medium have met with partial success. Hampp and Nevin (1959) cultivated Borrelia vincentii in a veal heart infusion medium in which five coenzymes and glucose-1-phosphate substituted for ascitic fluid enrichment. Later, the same investigators (Nevin and Hampp, 1959), using a simplified basal medium, demonstrated a requirement of B. vincentii for five coenzymes, oleic acid, carbon dioxide, and L-asparagine. In addition, small quantities of ascitic fluid were essential for growth.

Since enrichment substances such as ascitic fluid are difficult to obtain and variable in ability to support spirochetal growth, potential sources of the spirochetes’ growth requirements were looked for in their normal environment. One likely source of these factors appeared to be other organisms present in the oral cavity. Nevin, Hampp, and Duey (1960) demonstrated stimulation of growth of B. vincentii by an oral microaerophilic diphtheroid. The stimulation could be duplicated by the addition of cocarboxylase or acetyl phosphate to the medium. Neither of these compounds could be demonstrated in the diphtheroid or its culture filtrate. Neither the diphtheroid nor cocarboxylase replaced the requirement for ascitic fluid of B. vincentii.

Preliminary experiments in our laboratory revealed that oral spirochetes would grow abundantly on autoclaved commercially available media in the presence of other oral organisms. The media used did not require blood, ascitic fluid, or any other enrichment additive. The purpose of the studies reported here was to isolate and identify the organisms “supporting” spirochetal growth, and to investigate the nature of the growth-promoting substances involved.

Materials and Methods

Preparation of medium and inoculation of spirochetes. PPLO broth (BBL), without crystal violet or serum, was autoclaved at 15 psi for 15 min with 1.2% agar (Difco), and poured into
petri plates (6 by 1.5 cm); the plates were filled to within 2 mm of the rim. After the medium had cooled, wells were cut into the center of the agar medium by means of sterile glass tubing (8 mm in diameter), creating a “spirochete well plate” (Rosebury and Foley, 1941). Mixed spirochetes and oral bacteria, obtained from the gingival crevice area of man, were inoculated directly into the central well of the “spirochete well plates.” After 7 days of anaerobic incubation, the spirochetes grew out as a haze, away from other forms of bacteria which were retained in the central well of the well plate. Spirochetes free from other bacteria could then be collected by Pasteur pipette from the periphery of the spirochetal haze to the central wells of fresh well plates. Stock cultures of spirochetes were maintained in early experiments by the serial transfer of spirochetes with the other oral organisms growing in the central well. A pure strain of spirochetes was obtained for later experiments by serial transfer of single surface colonies in an anaerobic environment as previously described (Socransky et al., 1959).

The spirochetes investigated in this study were considered to be Treponema microdentium on the basis of size and morphology as revealed by electron microscopy (Listgarten, Loesche, and Socransky, 1963).

Cultivation of organisms other than spirochetes. Mixed oral organisms were maintained by serial transfer as mixed cultures in well plates or as confluent drops on heart infusion-blood agar plates. Pure cultures of organisms other than spirochetes were isolated on PPLO Agar and maintained on PPLO Agar or in PPLO broth. When two or more organisms were placed in the well, they were first combined by means of a “wheel plate” (Macdonald, Sutton, and Knoll, 1954) in which each organism was streaked from the periphery of the plate to a common central hub. A loopful of the organisms in the central hub was then placed in the well along with the spirochetal inoculum. In later experiments, broth cultures of the “supporting organisms” were combined and diluted to an optical density of 1.0 at 560 nm; 0.05 ml of this dilution was placed in the central well, along with the spirochetal inoculum.

Incubation. All plates and broth cultures excepting those involved in measuring oxidation-reduction potential were incubated anaerobically in Brewer jars in 95% H₂ and 5% CO₂ for 3 to 7 days at 35 to 37 C. The atmosphere used in measuring oxidation-reduction potential is described below.

Measurement of oxidation-reduction potential. An apparatus was constructed as shown in Fig. 1. Three platinum electrodes, consisting of 24-gauge platinum wire, were embedded in the base of a petri plate (6 by 1.5 cm) by an epoxy resin (Araldite; Ciba Pharmaceutical Products, Inc., Summit, N.J.). The leads soldered to these electrodes were Teflon-insulated 18-gauge braided lead in wires which could be autoclaved in situ. A saturated potassium chloride 2% agar bridge joined the medium in the plate to a saturated calomel electrode. The entire apparatus was placed in a Brewer jar or a desiccator with the leads from the platinum and calomel electrodes passing through a plasticine seal to a Beckman model G pH meter. The atmosphere in the jars was exchanged by evacuating the jar to 30 mm of mercury and filling it with nitrogen (high purity; Linde Co., New York, N.Y.) seven times. On the final exchange, 5% CO₂ was introduced with the nitrogen. In certain experiments, alkaline pyrogallol or glucose oxidase was activated in the jar after the atmosphere had been exchanged, to remove residual oxygen.

The electrodes were tested in the 95% N₂ and 5% CO₂ atmosphere by measuring the potential of known oxidation-reduction systems including 0.1 N and saturated calomel solutions, as well as potassium ferricyanide-ferrocyanide systems and ferrous-ferric chloride systems. The last two systems were measured in buffer solutions in the presence and absence of 1.2% agar. All test measurements were compared with those of a Beckman platinum electrode kept as a standard.

Finally, all experiments were repeated, in-
corporating 1 to 20 ppm of the following oxidation-reduction indicators in the medium: methylene blue, indigo carmine, phenosafranine, and neutral red.

Preparation of filtrates and lysates of supporting organisms. The organism to be tested was inoculated into PPLO broth with or without 0.1% glucose and incubated anaerobically for 2, 4, 6, and 9 days at 35°C. The cultures were centrifuged at 10,000 × g for 30 min, and the supernatant fluid was removed and sterilized by passage through an ultrathin glass filter (Corning Glass Works, Corning, N.Y.). The packed organisms were suspended in fresh PPLO broth with and without 200 μg/ml of L-cysteine and sonically disrupted in a 9-kc sonic oscillator for 1 hr. The resultant suspension was resuspended to the original volume of the culture in PPLO broth and sterilized by filtration or by heating to 100°C for 20 min. Packed, nonviable organisms were prepared by heating broth cultures of the organisms for 30 min at 80°C and centrifuging to concentrate the organisms.

In later experiments, the fusiform and diphtheroid cultures were grown anaerobically for 4 days in PPLO broth supplemented with 0.1% glucose. The cultures were centrifuged at 10,000 × g, and the supernatant fluids were filter-sterilized through 0.45-μ Millipore filters or autoclaved at 10 psi for 10 min.

Assay of filtrate activity. The basal medium for the assay of filtrate activity consisted of PPLO broth supplemented with 1,000 μg/ml of glucose, 800 μg/ml of L-cysteine, 400 μg/ml of nicotinamide, and 5 μg/ml of cocarboxylase. The cysteine and cocarboxylase were filter-sterilized and added aseptically to the remainder of the medium which had been previously autoclaved at 10 psi for 10 min. For growth and routine maintenance of spirochetes, the basal medium was supplemented with 5% diphtheroid culture filtrate and 5% fusiform culture filtrate.

Two assay systems were used. In the first, the basal medium supplemented with 1.2% agar (Difco) and the substances being assayed was poured into petri plates (6 by 1.5 cm) and the spirochetes were inoculated into the central "well." Results were considered positive when a typical spirochetal haze was observed after incubation, and negative when there was no visible indication of growth.

In the second assay system, the spirochetes were inoculated into the basal medium supplemented with the substances being assayed and changes in culture optical density at 470 mμ were determined by use of a Beckman model B spectrophotometer. It was recognized in early experiments that spirochetes exhibit a marked carry-over of nutrients, even after washing with basal media. To overcome this difficulty, the spirochetes were serially transferred for five passages in duplicate tubes of the test medium before being assayed.

Preliminary characterization of the active factors in the filtrates. To partially characterize the active factors in the culture filtrates, samples of the filtrates were subjected to the following procedures: (i) heating to 56, 80, and 100°C for 30 min at pH 7.0; (ii) autoclaving at 121°C for 15 min; (iii) extraction three times with 3 volumes of ether at pH 2.5, 7.0, and 10.5; (iv) steam distillation at pH 2.5, 7.0, and 10.5; and (v) dialysis against 0.067 M phosphate buffer (pH 7.0).

Determination of isobutyrate and putrescine uptake. T. microdentium was grown in the basal medium supplemented with 0.5 μg/ml of sodium isobutyrate for two successive passages and then transferred to a medium containing 2 μg/ml of sodium isobutyrate-1-C14. The organisms were grown anaerobically for 3 days and harvested by centrifugation. Radioactivity was determined in the culture supernatant, and also in a sample of the cells after collection on a Millipore filter. The filter was cemented to a planchet for counting. The remaining cells were then washed three times with 0.067 M phosphate buffer (pH 7.0) and suspended in a 1% solution of unlabeled sodium isobutyrate for 24 hr at 4°C. Radioactivity was again determined in the cells and the suspending medium. The cells were reharvested by centrifugation, suspended in distilled water, and sonically disrupted for 1 hr in a 9-kc Raytheon sonic oscillator. A sample of this suspension was steam-distilled at pH 2.0, and another sample was subjected to column chromatography (Wisman and Irvin, 1957).

To test for the incorporation of putrescine, T. microdentium was grown in the basal medium supplemented with 50 μg/ml of putrescine dihydrochloride for three successive passages and then inoculated into a medium containing 100 μg/ml of putrescine-1,4-C14 dihydrochloride. After 3 days of anaerobic incubation, the cells
were harvested and washed three times with 0.067 M phosphate buffer (pH 7.0); activity was determined in the cells and in the supernatant washing fluid.

RESULTS

Demonstration of the required role of other oral organisms. Gingival debris from eight individuals, containing spirochetes and other bacteria, was inoculated into PPLO Agar well plates as described above and incubated anaerobically. Spirochetes from all eight individuals grew out as large hazes and were serially transferred to fresh plates with and without other oral organisms. In all instances, spirochetes grew when inoculated with the other oral organisms but did not grow in the absence of these organisms. Spirochetes from seven of the eight sources were maintained for eight passages before being discarded; spirochetes from the other individual were maintained for 72 passages until a known combination of organisms replaced the mixed oral flora. In every passage, the ability of the spirochetes to grow on the basal medium without other organisms was tested with uniformly negative results.

In the presence of other oral organisms, the spirochetes grew equally well in autoclaved or filter-sterilized basal media, or in different basal media, such as Trypticase Soy Agar (BBL), Trypticase with 5% yeast extract (Difco), or Brain Heart Infusion Agar (Difco) with and without 10% horse blood. Spirochetes isolated from any of the eight individuals could be maintained by the mixed bacteria from the other seven individuals.

Identification of organisms capable of supporting spirochetal growth. A group of 16 organisms which had been isolated and characterized by Macdonald et al. (1954) during studies of mixed anaerobic infections was demonstrated to support spirochetal growth. Although all 16 strains together supported spirochetal growth, none of the 16 strains individually was effective. Strains were systematically deleted from the mixture until two minimal effective combinations were identified. The first consisted of a facultative diphtheroid (JB3B) with a fusiform (JF3), the second consisted of the diphtheroid (JB3B) with an anaerobic, motile, gram-negative rod (KSO). Since the fusiform was easier to grow than the motile gram-negative rod, the combination of the diphtheroid and fusiform was used in succeeding experiments. These two organisms could support spirochetes freshly isolated from the oral cavity.

Attempts to replace the diphtheroid or the fusiform, or both, by strains of facultative streptococci, lactobacilli, other “diphtheroids” (both facultative and anaerobic), staphylococci, Veillonella, and other fusiforms, failed. However, certain strains of peptostreptococci could replace the fusiform. No organism tested could replace the diphtheroid.

Attempts to replace the fusiform and diphtheroid by their filtrates, sonic lysates, and heat-killed cells. Culture “filtrates” and culture “lysates” of the fusiform and diphtheroid were added to the basal medium in concentrations varying from 0.001 to 100% of the final medium in an attempt to substitute for one or both of the required viable organisms. In addition, the fusiform filtrate was prepared from cultures grown in PPLO broth containing up to 50% of the diphtheroid filtrate and vice versa. Filtrates and lysates were also prepared from cultures in which the two organisms were grown together in broth or in agar.

Finally, heat-killed fusiforms and diphtheroids were placed in the wells of some of the test media in concentrations approximately equal to the final concentrations the viable organisms attained in the central well.

In no instance could either organism be replaced by its culture supernatant, culture lysate, heat-killed cells, or any combination of the above prepared in varying concentrations or with varied periods of incubation.

Separation of living supporting organisms from spirochetes. Since the lysates, heat-killed organisms, and culture supernatants of the supporting organisms failed to support growth, it was necessary to determine whether the supporting organisms could be physically separated from the spirochetes and still support growth.

In one experiment, three recessed wells were placed 1.5 cm apart in the agar medium contained in a petri plate (15 by 1.5 cm). Spirochetes were inoculated into the central well, the diphtheroid was inoculated into one outside well, and the fusiform was inoculated into the opposite well. Typical spirochetal hazes resulted in this experiment, but only after a 7-day incubation period. The ability of the spirochetes to grow in
this experiment demonstrated that the supporting organisms would maintain spirochetal growth while separated from the spirochetes and also from each other.

In a second experiment, the fusiform and diphtheroid were inoculated onto the surface of a 100 mg Millipore filter, placed on the surface of a PPILO Agar plate. The two organisms were allowed to grow anaerobically for 2 to 4 days and then removed along with the filter. Spirochetes were inoculated into the underlying agar medium, and the plates were reincubated anaerobically for 4 to 6 additional days. Examination of the plates showed that the spirochetes grew on these plates without the presence of the supporting organisms, but not on control plates which had been preincubated anaerobically without the supporting organisms.

Measurement of pH in growing cultures. The failure of filtrates and lysates of the supporting organisms to support spirochetal growth, and yet the ability of the spirochetes to grow when separated from the supporting organisms under certain conditions, suggested the possibility that the supporting organisms were altering the physicochemical environment of the medium in addition to, or instead of, producing chemical "growth factors."

To investigate changes in pH during growth of spirochetes with the supporting organisms, pH indicators (bromothymol blue, phenol red) were incorporated in the medium in concentrations of 5 to 25 ppm. In addition, the pH values of the above cultures were periodically read by means of a glass electrode. These measurements demonstrated that pH remained consistently between 7.2 and 7.6.

Measurement of Eh in growing cultures. All electrodes selected for this experiment had a range within ±5 mv of the arbitrarily selected standard electrode when tested against the four different oxidation-reduction systems. A range of 10 mv is not excessive when one considers the variations inherent in a system consisting of a complex medium on which are growing three distinct groups of microorganisms.

It was recognized that residual oxygen might markedly affect the oxidation-reduction measurements in our system. However, it was shown that the same measurements were attained after the simple nitrogen-exchange technique as when traces of residual oxygen were removed by activating glucose oxidase or alkaline pyrogallol in the jar.

Figure 2 shows the Eh attained by the electrode in the central well of the test plate, plotted against the time of incubation in hours. Each of the curves represents the mean of four separate experiments. The spirochetes grew only when inoculated with the diphtheroid and fusiform. In all experiments where there was spirochetal growth, the Eh readings in the central well were between −185 and −220 mv. In two additional experiments, the spirochetes failed to grow. In one instance the Eh was above this range, and in the other instance the Eh was below this range. The diphtheroid growing alone produced an Eh of −90 to −110 mv. The fusiform growing alone produced an Eh of −240 to −280 mv. The Eh of unoinoculated media was −50 to −70 mv. The readings of the second and third electrodes are not plotted. The Eh of the second electrode placed 1 cm from the well remained at −50 to −70 mv until the spirochetal haze approached and then gradually dropped to about −185 mv. The third electrode was placed at the periphery of the plate as a control, and its Eh reading remained consistently between −50 and −70 mv. Eh indicators incorporated into the medium served as visual confirmation of the above results.

Replacement of the viable supporting organisms. Since the uninoculated medium had an Eh 120 to 150 mv higher than the range in which spirochetal growth occurred, attempts were made to
lower the Eh of the medium with a combination of reducing agents. A medium with the following composition was shown to have the desired Eh: PPLO broth (BBL), 1.2% agar (Difco), 1.0 mg/ml of glucose, 800 μg/ml of L-cysteine, and 400 μg/ml of nicotinamide, along with 5% diphtheroid filtrate and 5% fusiform filtrate. The PPLO base with 1.2% agar was sterilized by autoclaving at 15 psi for 15 min. The remaining constituents were filter-sterilized and added aseptically to the basal medium before the medium was poured. This medium attained an Eh of approximately −180 mv, without associated organisms. The medium produced abundant growth of spirochetes (Fig. 3). Deletion of either or both filtrates or the reducing agents resulted in questionable growth of the spirochetes or no growth.

The complete medium was capable of supporting spirochetes freshly isolated from the human mouth and would also support excellent growth of spirochetes (10⁶ organisms per ml) in agar-free media.

Requirement for filtrates. The spirochete’s requirement for the filtrates differed in agar and broth (Table 1). In agar both filtrates were required for growth, whereas in broth either filtrate would support growth but not to the extent of both filtrates together. When tested singly, concentrations of either filtrate above 5% did not increase the culture turbidity and became inhibitory at concentrations greater than 10%. Since the broth assay method allowed a more accurate quantitative measurement of growth, as well as allowing one filtrate to be examined at a time, it was used in further experiments.

Requirement of Treponema microdentium for coenzyme A. It was noted that the filtrates of both supporting organisms contained a heat-labile factor which could be replaced by coenzyme A, as suggested by Steinman, Oyama, and Schulze (1954) and Nevin et al. (1960) (Table 2). T. microdentium would grow in the basal medium without added coenzyme A in the presence of either the filter-sterilized diphtheroid filtrate or the filter-sterilized fusiform filtrate. When both filtrates were heat-inactivated, spirochetal growth was restored by the addition of 5 μg/ml of coenzyme A to the medium.

**Table 1. Requirement of Treponema microdentium for culture filtrates**

<table>
<thead>
<tr>
<th>Method of assay</th>
<th>Composition of medium</th>
<th>Percentage of maximal growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>In agar</td>
<td>Basal medium</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Basal medium + 5%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>diphtheroid filtrate</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Basal medium + 5% fus-</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>form filtrate</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Basal medium + 5% fus-</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>form and 5% diphtheroid</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>filtrates</td>
<td>0</td>
</tr>
<tr>
<td>In broth</td>
<td>Basal medium</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Basal medium + 5%</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>diphtheroid filtrate</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Basal medium + 5% fus-</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>form filtrate</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Basal medium + 5% diph-</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>throid and 5% fusiform</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>filtrates</td>
<td>100</td>
</tr>
</tbody>
</table>

*FIG. 3. Spirochetal haze after 5 days of incubation in basal medium supplemented with nicotinamide, cysteine, and culture supernatant fluids of the diphtheroid and fusiform.*
2. Requirement of Treponema microdentium for cocarboxylase.

<table>
<thead>
<tr>
<th>Composition of medium</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal medium*</td>
<td>0</td>
</tr>
<tr>
<td>Basal medium* + filter-sterilized diphtheroid filtrate and autoclaved fusiform filtrate</td>
<td>+</td>
</tr>
<tr>
<td>Basal medium* + filter-sterilized fusiform filtrate and autoclaved diphtheroid filtrate</td>
<td>+</td>
</tr>
<tr>
<td>Basal medium* + autoclaved diphtheroid and fusiform filtrates</td>
<td>0</td>
</tr>
<tr>
<td>Basal medium + autoclaved diphtheroid and fusiform filtrates + 5 μg/ml of cocarboxylase</td>
<td>+</td>
</tr>
</tbody>
</table>

* Without cocarboxylase.

FIG. 4. Effect of concentration of sodium isobutyrate on the growth of Treponema microdentium.

FIG. 5. Effect of concentration of polyamine on the growth of Treponema microdentium. Basal medium supplemented with O, spermine tetrahydrochloride; Δ, spermidine dihydrochloride; ●, putrescine dihydrochloride.

pH 7.0, dialyzable, soluble in ether at pH 2.5, and somewhat soluble in ether at pH 7.0, but not at pH 10.5. It could be steam-distilled at pH 2.5 but remained in the residue at pH 7.0 and 10.5.

The active factor present in the diphtheroid filtrate was also heat-stable at pH 7.0 and was dialyzable, but was not ether-soluble at pH 2.5 and 7.0 and was only slightly soluble at pH 10.5. It could not be steam-distilled at any pH.

Replacement of the filtrates with known compounds. A variety of acids, including formic, acetic, propionic, butyric, isobutyric, valeric, isovaleric, hexanoic, lactic, pyruvic, succinic, and oleic, were converted to their sodium salts and added to the basal medium in concentrations of 0.1, 0.5, 1.0, 3.0, and 10.0 μg/ml in attempts to support spirochetal growth. Only sodium isobutyrate was capable of supporting spirochetal growth; it was required at a level of 2 μg/ml for maximal growth (Fig. 4).

In another series of experiments, the polyamines, spermine tetrahydrochloride, spermidine phosphate, and putrescine dihydrochloride, were assayed in attempts to support spirochetal growth. All three polyamines were able to support spirochetal growth, with spermine being most effective and putrescine least effective (Fig. 5). The optimal concentrations or spermine tetrahydrochloride, spermidine phosphate, and putrescine dihydrochloride were 150, 200, and 250 μg/ml, respectively. The combination of any one of the polyamines with sodium isobutyrate did not enhance spirochetal growth.

Although sodium isobutyrate or one of the polyamines could support spirochetal growth for extended passages in broth, these compounds alone or in combination failed to support growth in Difco agar (Table 3).

Since it was possible that the agar was binding one or both of the required factors, sodium isobutyrate and spermine tetrahydrochloride were added in concentrations up to 1,000 times those required for growth in broth. In other experiments, the agar was presoaked in 1% concentrations of the growth factors before use or purified by the method of Grabar (1959). The results of these experiments were uniformly negative. Hardy, Lee, and Nell (1963) demonstrated that anaerobic spirochetes would grow as surface or subsurface colonies in the presence of lowered concentrations of Ionagar No. 2. (Consolidated Laboratories Inc., Chicago Heights, Ill.). Replacement of the 1.2% Difco agar in the test medium with 0.7% Ionagar No. 2 was
successful in overcoming the apparent agar inhibition.

Determination of the presence of isobutyrate and putrescine in culture filtrates. The filtrates of the two supporting organisms were acidified, placed on Celite-sucrose columns, and eluted as described by Wiseman and Irvin (1957). All fractions were collected and assayed for ability to support spirochetal growth. Only a single fraction, from the fusiform filtrate, was capable of supporting spirochetal growth. This fraction migrated with authentic isobutyric acid and was assumed to be isobutyric acid.

Polyamines in the filtrates of the supporting organisms were extracted from alkaline solution into t-butanol by the method of Rosenthal and Tabor (1956). Samples of the extracts were spotted on Whatman no. 4 chromatographic paper and developed in three solvent systems: (i) phenol, (ii) n-butanol-acetic acid-water (40:10:50), and (iii) m-cresol-acetic acid-water (50:2:48) (Brenner and Kenten, 1951). Putrescine was identified in the diphtheroid filtrate on the basis of having the same RF as authentic putrescine in the three solvent systems employed. No attempt was made to elute the “putrescine” spots to test growth-supporting ability.

Incorporation of isobutyrate and putrescine. The C14-labeled sodium isobutyrate was taken up by the spirochetes and incorporated in the cells (Table 4). Approximately two-thirds of the radioactivity of the medium was picked up by the organisms. This activity was not removed by washing or by exchange with unlabeled sodium isobutyrate. No radioactivity was detected in the distillate after steam distillation or in the isobutyric acid band after column chromatography. These results suggest that the labeled isobutyrate was taken up by the cells and converted to some form other than isobutyrate.

The C14 label of putrescine either was not taken up by the cells or was easily removed by washing (Table 5). No attempt was made to determine whether the putrescine was altered during growth of the organisms.

Discussion

Several investigators have noted the stimulatory or supportive effect of oral organisms on the growth of oral spirochetes (Wichelhausen and Wichelhausen, 1942; Nevin et al., 1960). With the exception of Nevin et al. (1960), no attempt has been made to study this relationship. These investigators demonstrated that an oral micro-aerophilic diphtheroid was stimulatory to growth of B. vincentii and that the stimulatory effect of the diphtheroid could be duplicated by the use of coenzyme A or acetyl phosphate. In the present investigation, neither acetyl phosphate nor coenzyme A alone would replace either of the filtrates required for spirochetal growth.

The two “supporting organisms” in this investigation appear to control the oxidation-
reduction potential of the medium as well as providing the spirochetes with "growth factors" consisting of putrescine, isobutyrate, and a heat-labile component which could be replaced by cocarboxylase. The addition of these "growth factors" as well as reducing agents to PPLO broth provides a medium for the cultivation of *T. microdentium* which is less complex and more reproducible than the ascetic fluid- or rabbit serum-containing media previously employed. The failure of *T. microdentium* to grow in the above medium in the presence of Difco agar can be explained by the inhibitory effect of agar as described by Hardy et al. (1963). The fact that spirochetes will grow in the basal medium supplemented with both the fusiform and diphtheroid filtrates in the presence of Difco agar suggests the possibility that another factor is present in these filtrates which overcomes the inhibitory effect of the agar.

Several investigators have recognized the requirement of anaerobic spirochetes for a lowered oxidation-reduction potential. For example, Hardy et al. (1963) stressed the importance of adding reducing agents to the medium, to cultivate anaerobic spirochetes on solid media. The observations reported in this investigation suggest that the spirochetes may require a narrow range of Eh for the initiation of growth. In two of six Eh experiments, *T. microdentium* failed to grow in the presence of the supporting organisms. In one instance, the Eh in the central well was 70 mv higher than the range in which spirochetal growth took place, and in the other instance the Eh was 60 mv lower than this range. In addition, *T. microdentium* would not grow in the presence of the living diphtheroid and a filtrate of the fusiform, or in the presence of the living fusiform and a filtrate of the diphtheroid. In these experiments, the spirochetes had a source of both "chemical growth factors"; however, the Eh of the medium was probably outside the −185 to −220 mv range in which spirochetal growth was shown to take place, since the Eh produced by the diphtheroid growing alone was −90 to −110 mv, whereas the Eh produced by the fusiform growing alone was −240 to −280 mv. These observations contrast with findings with other anaerobes which have been found to grow over a fairly wide range of Eh.

A requirement for isobutyric acid is not unique to *T. microdentium*, since several other organisms, particularly rumen bacteria, have been shown to have a similar requirement (Bryant and Doetsch 1955; Allison, Bryant, and Doetsch, 1958; Bryant and Robinson, 1961). Wegner and Foster (1960) isolated a *Borrelia* strain from the rumen, which required both a branched-chain and a straight-chain fatty acid. Many of the organisms isolated from the rumen could utilize either isobutyric or isovaleric acid. However, isovaleric acid will not substitute for isobutyric acid as a growth factor for *T. microdentium*.

The role of isobutyric acid in the metabolism of *T. microdentium* has not been elucidated. Allison et al. (1962a, b) demonstrated that, in rumen bacteria, fatty acids function at least in part to provide a carbon skeleton for the biosynthesis of branched-chain amino acids and lipids. The lipid component included 14-carbon to 17-carbon branched-chain fatty acids and aldehydes.

An unusual finding in this study was the ability of polyamines to replace isobutyric acid as a growth factor for *T. microdentium*. Bacteria have been shown to require polyamines for growth (Herbst and Snell, 1949; Martin, Pelczar, and Hansen, 1952; Sneath 1955). In addition, polyamines have been shown to have stabilizing effects on bacteria (Mager, 1955), protoplasts (Mager, 1959), and mitochondria (Tabor, 1960). The high concentration of the polyamines required for the growth of *T. microdentium* suggests that they act by stabilizing the organisms rather than by providing some essential metabolite. Some substantiation for this hypothesis is provided by the failure of labeled putrescine to be incorporated into the organisms. The available data suggest the possibility that isobutyric acid is incorporated into a structural site responsible for cell integrity. In the presence of a suboptimal concentration of isobutyrate, the polyamines are necessary to maintain cellular structure.

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