CULTIVATION OF LEPTOSPIRAE
I. NUTRITION OF LEPTOSPIRA CANICOLA

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ABSTRACT

STALHEIM, O. H. V. (University of Wisconsin, Madison), and J. B. WILSON. Cultivation of leptospires. I. Nutrition of Leptospira canicola. J. Bacteriol. 88:48-54. 1964.—The nutrition of Leptospira canicola was investigated by use of synthetic media of suitable ionic strength. At an incubation temperature of 30 C, the minimal components were calcium, iron, magnesium, and ammonium ions, thiamine, and a fatty acid source; barium and strontium replaced calcium. Aspartic acid, glutamic acid, or methionine stimulated the rate and amount of growth; the best growth occurred in medium containing additional amino acids. Additions of cyanocobalamin or biotin permitted growth at 37 C. The stimulatory effects of added cyanocobalamin, biotin, pyridoxine, pantothenate, lipoic acid, or nicotinic acid were additive at 37 C, but not at 30 C. Fatty acids containing 14, 16, 17, or 18 carbon atoms supported growth; linoleic and linolenic acids were toxic. Glycerol monooleate or trioleate, or Tween 40, 60, or 80 supported moderate to good growth; a mixture of monoolein and Tween 60, or Tweens 60 and 80 supported the best growth. Ten strains of L. canicola cultivated in a synthetic medium containing Tweens 60 and 80 attained cellular densities per ml of 10⁶ to 4.0 × 10⁶ organisms. L. canicola cells, suspended in medium containing oleic-1-C¹⁴ acid, incorporated label primarily into cellular lipids; a lesser amount was located in the protein fraction, and only trace amounts were found in the nucleic acid fraction. The rate of incorporation was not affected by added sodium acetate. L. canicola was found to have fatty acid decarboxylase activity.

Attempts to develop chemically defined media for the cultivation of pathogenic leptospires have culminated in media containing fractions of serum, or synthetic media capable of supporting the growth of “adapted” strains. Leptospiral nutritional requirements have been studied in medium supplemented with rabbit serum albumin (Schneiderman et al., 1953), rabbit serum globulin (Johnson and Wilson, 1960), oleic acid-albumin complex (Ellinghausen and McCullough, 1962), and gelatin (Woratz, 1957). Cationic and fatty acid requirements are demonstrable in medium containing deionized, extracted rabbit albumin (Johnson and Gary, 1963a, b). After a preliminary period of adaptation in medium containing 1% rabbit serum, several leptospirol serotypes have been cultivated in a synthetic medium (Vogel and Hutner, 1961), which we have used for further investigations into the nutritional requirements of Leptospira canicola. The formula of the medium has been modified, but maximal cellular densities are only one-tenth those of media containing rabbit serum or fractions of serum.

MATERIALS AND METHODS

The primary test organism was L. canicola strain Dog-L-Reinhard. Preparation of the synthetic medium 198E (Vogel and Hutner, 1961) was facilitated by the use of 100 × concentrations of classes of the components; amino acids were prepared in 33 × concentrations. Because medium 198E prepared with DL-amino acids supported poor growth during initial experiments, L-amino acids at one-half concentrations were used. Stock solutions were made in deionized, distilled water and stored at 5 C. Pyrex, screw-cap tubes, containing 10 ml of synthetic or experimental media at pH 7.4, were sterilized by autoclaving.

The inoculum consisted of that amount of a 7- to 10-day-old culture grown in synthetic medium which would give a final concentration of 10⁴ to 3 × 10⁵ organisms per ml when added to the test medium. To evaluate the effect of
vitamins and amino acids, inocula were from cultures which had been subcultured repeatedly in medium prepared without the material to be tested. Incubation was at 30 C, except in certain phases of studies on vitamin requirements when incubation was at 37 C.

Macroscopic evidence of leptospiral growth indicated at least a tenfold growth response. To evaluate accurately the growth response, microscopic counts were made of samples from duplicate tubes by use of a Petroff-Hauser bacterial counting chamber. The standard deviation of the counting procedure was 1.65 × 10^4. Counts were performed shortly after maximal cellular growth was attained, although cell numbers were stable for several days thereafter. The plate counting technique of Larson et al. (1959) was used to construct growth curves from which generation times were calculated.

The cationic growth requirements were investigated by use of medium prepared without the cation to be tested, to which were added different amounts of the test cation, or other cations substituted in equimolar amounts. Purines, pyrimidines, amino acids, and several ammonium salts were individually tested for their ability to support growth as the sole source of nitrogen in synthetic medium. Different concentrations of amino acids were added singly and in mixtures to medium containing (NH₄)₂SO₄ but no amino acids, to test their ability to stimulate the rate and amount of growth.

Different vitamins were added to vitamin-free synthetic medium to test their growth-supporting capability; they were also added to medium containing the essential vitamin, thiamine.

Lipids, including mono- and triglycerides of fatty acids, Tweens, and oils, were substituted singly or in combinations at different concentrations for the monoolein in synthetic medium. Lipids were dissolved in 95% ethanol and added to tubes of medium before autoclaving in 1% volumes; the Tweens were dispersed in water.

Egg yolk fat was prepared by mixing an equal volume of egg yolks with saturated NaCl solution and extracting the mixture with ether (Chargaff, 1942). Rabbit serum albumin was extracted as described by Johnson and Gary (1963a).

Radioactivity studies. Uptake of oleic acid by L. canicola cells was measured in the presence of oleic-1-C¹⁴ acid, 38 µe per mole (Volk Radiochemical Co., Skokie, Ill.). Leptospirae were sedimented from cultures in phosphate buffer-rabbit serum medium (Johnson and Wilson, 1960) by centrifugation (10,000 × g for 30 min), and were resuspended in synthetic medium containing 0.02 µe/ml of radioactive oleic acid substituted for monoolein. At intervals, 2-ml samples of the mixture were removed, diluted to 20 ml in a cold (5 C) 0.25% solution of extracted rabbit albumin in 0.02 n phosphate buffer (pH 7.4), and centrifuged. Additional washings were found unnecessary. The sedimented cells were transferred to copper planchets (1 in. in diameter) with about 1 ml of phosphate buffer and were dried at room temperature. The radioactivity was determined for the original medium before inoculation, the supernatant medium after the cells were removed by centrifugation, and the sedimented cells or cellular fractions. Cells were fractionated into lipid, nucleic acid, and protein fractions essentially as described by Roberts et al. (1957), except that 95% ethanol-ethyl ether (3:1, v/v) was used to extract the lipids.

The decarboxylation of fatty acid by L. canicola was measured by the method of Tabor (1962). An 18-ml amount of L. canicola culture was placed in a centrifuge tube; the organisms were sedimented, and the supernatant fluid was decanted. The leptospirae were resuspended in 10 ml of synthetic medium containing radioactive oleic acid substituted for monoolein, and were incubated at room temperature. At intervals, 0.9-ml samples were removed and spread on aluminum planchets. After the addition of 2 drops of 2 n HCl, the planchets were dried in the partial vacuum of an evacuated desiccator jar which contained a small beaker of 20% solution of KOH.

Radioactivity was determined with a Mylar window, gas-flow counter. Samples were examined at infinite thinness to eliminate self-absorption; all counts were corrected for background.

Results

Cationic requirements. No growth occurred in medium 198E without added Ca++, or in medium containing MgSO₄ or CoCl₂ substituted for CaCl₂. Ba++ or Sr++, in similar concentrations, replaced Ca++. No growth occurred in medium without added iron. Ferrous sulfate, ferrous ammonium sulfate, and ferric ammonium citrate, over a rather broad range of concentrations, were equally adequate sources.
When *L. canicola* was subcultured in media containing 20 or 50 mg per 100 ml of MgSO₄, poor growth occurred; growth did not occur in medium made without added MgSO₄. Medium containing 20 mg per 100 ml of Na₂HPO₄ substituted for 40 mg per 100 ml of MgSO₄ supported good growth during five serial subcultures. However, after the sixth subculture, the rate and amount of growth was decreased, and abnormalities in the morphology and motility of the organisms were observed. Approximately two-thirds of the organisms were elongated from three to six times and had very sluggish movements. Growth did not occur after a seventh subculture.

The potassium requirement was investigated by use of a medium containing 5 mg per 100 ml of Na₂HPO₄ substituted for 10 mg per 100 ml of KH₂PO₄. During ten serial subcultures, no decrease in growth occurred; a potassium requirement was not demonstrated.

**Nitrogen requirement.** The nitrogen requirement was satisfied by (NH₄)₂SO₄; 25 mg per 100 ml was the optimal concentration as the sole nitrogen source, and moderate growth (10³ organisms per ml) occurred during ten serial subcultures. Urea replaced (NH₄)₂SO₄. One of several amino acids, purines, or pyrimidines supported similar amounts of growth, although the responses to organic nitrogen sources were delayed 4 to 6 days compared with (NH₄)₂SO₄. The following amino acids (µg/ml) supported growth: L-aspartic acid, 50 to 250; L-asparagine, 50 to 100; L-glutamic acid, 50 to 500; glycine, 3; L-isoleucine, 10; L-leucine, 10; L-methionine, 5 to 50; L-phenylalanine, 5 to 50; L-tryptophan, 10; ornithine, 50; creatinine, 50; sarcosine, 50; L-tyrosine, 50; glutamine, 10; glutathione, 10; and cysteine, 10. Growth was not supported by: DL-alanine, 30 to 100; L-histidine, 125 to 250; L-proline, 20; DL-serine, 10 to 50; DL-valine, 5 to 50; and L-tyrosine, 10 to 50. The following purines and pyrimidines at concentrations of 50 and 250 µg/ml also served as sole nitrogen sources: uracil, thymine, cytosine, uridine, guanylic acid, 5-nitouracil, 2-amino pyridine, 6-methyl uracil, and pyridine 3-sulfonic acid. Adenine and 2-cytidilic acid did not support growth.

**Amino acid requirements.** The ability of amino acids to stimulate the growth of *L. canicola* when added to synthetic medium containing (NH₄)₂SO₄, but no amino acids, was investigated. Additions of L-aspartic acid (125 to 2,500 µg/ml), L-glutamic acid (50 to 250 µg/ml), or L-methionine (5 to 10 µg/ml), singly or in combinations at the lower concentration of each, stimulated the rate and amount (2 × 10⁶ organisms per ml) of growth.

The following amino acids were inhibitory at the lowest concentration tested (µg/ml): alanine, 50; asparagine, 50; serine, 10; tyrosine, 10; tryptophan, 10; and valine, 50. The following were nonstimulatory at lower concentrations, but inhibitory at the following concentrations (µg/ml): glycine, 100; histidine, 250; leucine, 100; proline, 200; isoleucine, 200; and phenylalanine, 500. When the growth of *L. canicola* was compared in medium containing various mixtures of stimulatory and nonstimulatory amino acids, the best growth (3 × 10⁶ organisms per ml) occurred in medium containing a mixture as given in Table 3.

**Vitamin requirements.** *L. canicola* could not be cultivated beyond two subcultures in synthetic medium lacking thiamine. The following vitamins, singly or in various combinations, did not support growth in the absence of thiamine: B₁₂, biotin, p-aminobenzoic acid, calcium pantothenate, pyridoxine, folic acid, cholesterol, nicotinic acid, inositol, ascorbic acid, riboflavin, and menadione.

Moderate growth (10³ organisms per ml) occurred in medium containing thiamine as the only vitamin (minimal medium) during ten transfers; the lag phase of growth was 9 to 13 days compared with 4 days in complete medium. When vitamins were added singly (0.002 µg/ml) to minimal medium, growth was stimulated slightly (2 × 10⁶ organisms per ml) by nicotinic acid, pantothenate, pyridoxine, and lipoic acid; B₁₂ (0.0002 µg/ml) or biotin (0.02 µg/ml) supported growth equal to that in complete medium (3 × 10⁶ organisms per ml) during six subcultures. The effects of the stimulatory vitamins were not additive when incubated at 30°C. When cultures were incubated at 37°C, *L. canicola* did not grow in minimal medium. Good growth occurred in minimal medium supplemented with either B₁₂ or biotin, but the best growth occurred in complete medium (Table 3). Additions of CoC₂ (0.4 µg/ml) to minimal medium prepared without cobalt stimulated the amount, but not the rate of growth at an incubation temperature of 30°C, and supported growth at 37°C. The amount of growth equalled that in minimal medium supple-
mented with B₁₂ during four subcultures. Methionine did not spare the B₁₂ requirement.

Fatty acid requirements. *L. canicola* did not grow in medium lacking a source of fatty acids. Growth occurred in medium containing a rather narrow range of lipids (Fig. 1); only long-chain fatty acids containing 14, 16, 17, or 18 carbon atoms supported growth. The following fatty acids (1 to 4 mg per 100 ml) did not support growth: propionic, butyric, caproic, caprylic, capric, undecanoic, lauric, arachidic, ricinoleic, linoleic, linolenic, or arachidonic. The triglycerides of the following fatty acids did not support growth: butyric, caproic, caprylic, capric, aluric, myristic, palmitic, or stearic; neither did the methyl esters of palmitic acid or oleic acid, glycerol, ethanol, cholesterol, acetic acid (2 mg per 100 ml), or acetic acid plus 2 mg per 100 ml of caproic, caprylic, capric, or lauric acids. Olive oil, peanut oil, coconut oil, TEM-4T, egg yolk fat, or egg lecithin (2 mg per 100 ml) supported moderate to good growth.

*L. canicola* organisms were incubated in medium containing oleic-1-C₁₄ acid for 72 hr when approximately 88% of the cellular radioactivity was located in the lipid fraction, 12% in the protein fraction, and only traces in the nucleic acids (Table 1). The uptake of label was not affected by the presence of sodium acetate (Fig. 2). Decarboxylation occurred when cells were resuspended in medium containing oleic-1-C₁₄ acid; a maximum of 26% of the label was volatilized during an incubation period of 90 min (Table 2).

![FIG. 1. Growth response of *Leptospira canicola* in synthetic medium containing different concentrations of fatty acids, Tweens, monoolein, and triolein.](image)

![FIG. 2. Incorporation of label from medium containing oleic-1-C₁₄ acid by *Leptospira canicola* and the failure of added sodium acetate (●) to affect the rate of incorporation.](image)

<table>
<thead>
<tr>
<th>Table 1. Radioactivity of fractions of <em>Leptospira canicola</em> incubated in synthetic medium containing oleic-1-C₁₄ acid*</th>
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<tbody>
<tr>
<td>Fraction</td>
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<tr>
<td>---------</td>
</tr>
<tr>
<td>Alcohol-ether-soluble</td>
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<tr>
<td>Hot trichloroacetic acid-soluble</td>
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<tr>
<td>Protein residue</td>
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*Leptospirae were sedimented from 300 ml of culture, resuspended in synthetic medium containing 0.02 μg/ml of oleic-1-C₁₄ acid substituted for monoolein, and incubated at 30°C for 72 hr. The cells were fractionated as described in the text.*
given in Table 3. With the exceptions of MnSO₄, CuSO₄, and ZnSO₄, which were not tested, each component was essential or growth-stimulating. The rate or amount of growth in TSM was not increased by the addition of soluble starch, dextran, polyvinylpyrrolidone, or methyl cellulose (0.1, 0.5, or 1.0 mg/ml); NaHCO₃ (0.05 mg/ml); pyruvic acid (0.05 or 0.5 mg/ml); or by gassing the tubes after inoculation with 5% CO₂ in air.

The smallest inoculum tested that initiated growth in TSM was 50 organisms per ml; the amount of growth was equal to that obtained with larger inocula, but the lag phase of growth was increased 3 days for each tenfold decrease in inoculum. The generation time was 14.6 hr. Media at pH values of 6.4, 6.8, 7.2, 7.6, 8.0, and 8.4 supported similar rates and amounts of growth; the pH values dropped slightly (0.2 to 0.9) during growth.

Ten laboratory strains of *L. canicola* in phosphate buffer-rabbit serum medium were inoculated into TSM and serially subcultured; 1 ml of inoculum was added to 10 ml of medium. After the first subculture, maximal cellular densities ranged from 10⁷ to 4 x 10⁷ leptospires per ml during six subcultures.

The supernatant medium of a 6-day-old *L. canicola* culture in TSM was tested for its effect on the growth of *L. canicola*. After the organisms were removed by centrifugation, the supernatant fluid was concentrated to 25% of the original volume by pervaporation, sterilized by filtration, and added to TSM in a 1% concentration. In medium supplemented with cultural supernatant fluid, the amount of growth was one-half that on the controls.

**Discussion**

Yanagawa and Wilson (1962) classified leptospires into two types on the basis of their ability to grow in a boiled rabbit serum medium. Certain leptospires, including *L. pomona*, did not grow, but *L. canicola* and others grew. Using the best

<table>
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<th>Component</th>
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<th>Amt</th>
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<tr>
<td>KH₂PO₄</td>
<td>10.0</td>
<td>Pyridoxine HCl</td>
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<tr>
<td>MgSO₄·7H₂O</td>
<td>40.0</td>
<td>Hydroxyethylenediamine-tetraacetic acid</td>
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<tr>
<td>CaCl₂·2H₂O</td>
<td>1.4</td>
<td>(NH₄)₂SO₄</td>
<td>25.0</td>
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<td>Ferric ammonium citrate</td>
<td>0.55</td>
<td>Tween 60</td>
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</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>0.045</td>
<td>Tween 80</td>
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<tr>
<td>ZnSO₄·7H₂O</td>
<td>0.66</td>
<td>L-Aspartate</td>
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<tr>
<td>CuSO₄·5H₂O</td>
<td>0.04</td>
<td>L-Asparagine</td>
<td>3.0</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>0.04</td>
<td>L-Glutamate</td>
<td>3.0</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.04</td>
<td>Glycine</td>
<td>0.3</td>
</tr>
<tr>
<td>B₁₂</td>
<td>0.00002</td>
<td>L-Histidine</td>
<td>3.0</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.02</td>
<td>L-Isoleucine</td>
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<td>Nicotinamide</td>
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<td>DL-Calcium pantothenate</td>
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<td>L-Methionine</td>
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<tr>
<td>L-Phenylalanine</td>
<td>0.6</td>
<td>L-Proline</td>
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* Amounts are given in mg per 100 ml of deionized, distilled water. The medium was adjusted to pH 7.3 to 7.4 and sterilized at 121 C for 20 min.
synthetic leptospiral growth medium reported up to now, we have investigated the nutrition of a less fastidious species of Leptospira, *L. canicola*.

Leptospiral growth requires a medium with suitable ionic strength. Johnson and Gary (1963b) recently showed that additions of Mg\(^{++}\) and Ca\(^{++}\) are required for the growth of *L. pomona* in medium containing NaHPO\(_4\), NH\(_4\)Cl, thiamine, and deionized rabbit serum, and that further additions of K\(^+\) increase the amount of growth. Attempts to replace these ions were not reported. We find that considerably higher concentrations of these ions are required for growth of *L. canicola* in a synthetic medium containing a chelating agent (Vogel and Hutner, 1961). Equimolar concentrations of Na\(^+\), substituted for Mg\(^{++}\) or K\(^+\), also support growth of *L. canicola*, but small amounts of Mg\(^{++}\) (about 1.7 \(\times\) 10\(^{-10}\) M) are required for cell division. We found that Sr\(^{++}\) or Ba\(^{++}\) will replace the Ca\(^{++}\) growth requirement; these ions may be acting as fatty acid acceptors or precipitants. In the absence of hydroxyethylenediaminetetraacetic acid (Chel 41; Geigy Chemical Corp., Yonkers, N.Y.), salts precipitate from synthetic media, but some growth occurs.

*L. canicola* utilizes nitrogen from inorganic and organic sources. The results of our studies on amino acids as sources of nitrogen compare favorably with the results of leptospiral transamination studies by Markovetz and Larson (1959) with two minor exceptions: we found that valine, at the concentrations used, did not support growth, whereas Markovetz and Larson (1959) found a slight transamination reaction; and glycine supported slight growth although transamination was not demonstrated. The results of the two investigations agree in that aspartic acid, glutamic acid, isoleucine, leucine, methionine, phenylalanine, and tryptophan serve as sources of nitrogen for leptospiral growth, whereas serine, histidine, tyrosine, and proline do not support growth. The results of our studies on growth stimulation when amino acids are included in a synthetic medium containing (NH\(_4\))\(_2\)SO\(_4\), confirm and extend the earlier reports of Schneiderman et al. (1953), Gerhardt and Ball (1959), and Vogel and Hutner (1961). In addition to the previous reports of stimulation by aspartic and glutamic acids, we find stimulation by methionine. The best growth occurs in medium containing a mixture of stimulatory and non-stimulatory L-amino acids. Growth inhibition by asparagine probably reflects an inhibitory concentration of ammonia due to hydrolysis of asparagine during autoclaving.

The demonstration of vitamin requirements for leptospiral growth is difficult in medium containing serum or serum fractions. When Johnson and Gary (1962) added several vitamins to medium supplemented with dialyzed rabbit serum, only thiamine stimulated the growth of *L. pomona*. Lack of stimulation by added B\(_12\) could be due to the use of inocula from medium containing rabbit serum, which is a rich source of B\(_12\) (Mollin and Ross, 1952). The report by Ellinghausen and McCullough (1962) that B\(_12\) is required for the cultivation of leptospires in medium supplemented with oleic-albumin complex (Difco), which is prepared from bovine serum fraction V, reflects the fact that B\(_12\) is bound mainly to serum globulins and little or none is bound to serum albumin (Mendelsohn et al., 1958).

Our results substantiate the reports that thiamine is an essential growth factor for *L. canicola* (Schneiderman et al., 1953), and that additions of nicotinic acid or pantothenate stimulate the rate of growth slightly (Vogel and Hutner, 1961). We found that the latter vitamins, and pyridoxine and lipoic acid as well, stimulate the rate and amount of growth slightly, but that thiamine plus either B\(_12\) or biotin supports maximal growth in synthetic medium at an incubation temperature of 30 C. The vitamin requirements are more strict when cultures are incubated at 37 C; thiamine alone does not support growth, and the best growth occurs in medium containing all the stimulatory vitamins.

Cobalt replaces B\(_12\), although the lag phase of growth is more than doubled. Because the lag phase is not decreased during subculture in medium containing thiamine and cobalt but no B\(_12\), apparently *L. canicola* does not store B\(_12\) or overproduce it with elimination into the medium in stimulatory amounts. Recently, cobalt was shown to be a growth factor for several members of the genus *Rhizobium* (Lowe and Evans, 1962).

As suggested by Johnson and Gary (1963a), fatty acids probably are the main source of energy and carbon for leptospires, because *L. canicola* grows in medium containing fatty acid as the sole source of carbon. Contrary to the report of Vogel and Hutner (1961), fatty acid methyl esters did not support growth in our studies, and a mixture
of Tweens 60 and 80 supported the best growth. By adjusting the concentrations of Mg++ and Ca++, certain fatty acids were recently found to replace albumin in the cultivation of mammalian cells (Ham, 1963).

The initial step in utilization of oleic acid by leptospirae appears to be decarboxylation. Label is volatilized from both palmitic-\(^{14}C\) and \(^{2}C^{14}\) acid by \(L.\) \(pomona\) (Johnson and Gary, 1963a), and four leptospiral serotypes incorporate label when incubated in the presence of \(C^{14}O_{2}\) (Yanagawa, Hiramune, and Fujita, 1963). Further degradation may follow, as indicated by the identification of several volatile acids in the supernatant medium from cultures of \(L.\) \(icterohaemorrhagiae\) (Fulton and Spooner, 1956), as well as our observations that the pH value of the medium drops slightly but consistently during leptospiral growth and that the supernatant medium of cultures is growth inhibitory.

Although the rate and amount of growth of \(L.\) \(canicola\) in synthetic medium does not equal that in medium supplemented with serum or fractions of serum, information on the requirements for the cultivation of a less fastidious \(Leptospira\) provides a basis for studies on the growth of more fastidious leptospirae.

Acknowledgments

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Literature Cited


