NUTRITIONAL REQUIREMENTS OF *TREPONEMA DENTICOLA* AND *TREPONEMA VINCENTII*

by

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Dissertation submitted to the Graduate Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Anaerobic Microbiology

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June 1982

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ACKNOWLEDGEMENTS

I would like to express my gratitude and appreciation to my advisor, Dr R. M. Smibert, for all of his expert and invaluable advice, and for his sincere concern and patience during this investigation. My thanks also go to my research committee and to the faculty and staff of the Anaerobe Laboratory of Virginia Polytechnic Institute and State University. I would especially like to thank the members of Dr. Smibert's laboratory: Polly Cooper, Ella Beaver, Linda Stover, and Judy Cooke, who made me feel like a part of the group and who also added some much needed comic relief. Hugh George also deserves a special note of appreciation for his advice on certain procedures used with the treponemes.

I would like to thank the staff of the media preparation room, especially Phyllis Sparks and Margaret Vaught who helped to make most of the media used in this study.

Donya Stephens gets my special thanks and appreciation for her expert typing in preparing this manuscript.

Above all else, I wish to express my appreciation and love to my wife, Louise, who gave me the support and love that I needed to accomplish this goal.

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INTRODUCTION

The cultivable oral treponemes (Treponema denticola, Treponema vincentii, and Treponema scoliodontum) and the cultivable genital treponemes (Treponema phagedenis, Treponema refringens, and Treponema minutum) have been reported to require serum or ascitic fluid for growth (48,118,126,129). Serum albumin was the only serum fraction reported to replace serum for the growth of T. phagedenis (33,91,146). Serum globulin fractions could not support growth. The function of serum albumin for the growth of T. phagedenis was to detoxify lipids (111,146). Serum albumin has been shown to reversibly bind long chain fatty acids (45,138). Albumin has also been reported to be required for growth of the leptospires (68,73) by detoxifying required fatty acids (34).

When grown in a fatty acid-free medium that contained albumin, T. phagedenis was reported to require a pair of fatty acids, one saturated of at least 14 carbons and one unsaturated of at least 15 carbons with one, two, or three double bonds (67). Elaidic acid (trans-18:1) could substitute for this pair of fatty acids. A similar observation was made for the parasitic leptospires (73). The parasitic leptospires were reported to require a pair of fatty acids (one saturated and one unsaturated) of at least 15 carbons and albumin for growth. Elaidic acid could substitute for this pair of fatty acids.

The alpha globulin fraction of whole serum was reported to replace serum required for the growth of T. denticola (F. E. Austin, M.S. thesis, Virginia Polytechnic Institute and State University, Blacksburg, 1978, 57,123). Austin also reported that alpha globulin could replace

serum for the growth of T. vincentii. Beta globulin, gamma globulin, albumin, and several individual serum proteins were incapable of replacing serum for growth of these two oral treponemes. The purpose of this investigation was to identify the growth factor(s) supplied by alpha globulin for growth of T. denticola and T. vincentii.

Review of Literature

Classification of Spirochetes

Spirochetes are slender, flexuous, helically coiled gram-negative bacteria that range in size from 0.1 to 3.0 μm in diameter and 3.0 to 500 μm in length (127). The spirochetes include both aerobic and anaerobic bacteria that are free-living or saprophytic. Some spirochetes are pathogenic to man and animals. Spirochetes divide by transverse fission (58,60,127) and have deoxyribonucleic acid (DNA) with a guanine plus cytosine (G + C) content of 36 to 66 moles % (66,127). Spirochetes exhibit three types of motility (23,127): rapid rotation lengthwise about the helix, cell flexion, and locomotion along a helical path.

Spirochetes possess certain morphological characteristics that make them distinct from other bacteria. All spirochetes have a multilayered envelope or sheath as the outermost external cell layer (58,165, 171) which appears to be similar to the outer membrane of other gram-negative bacteria (120). The outer envelope surrounds the protoplasmic cylinder. The protoplasmic cylinder consists of the cytoplasm and nuclear regions surrounded by a cytoplasmic membrane-outer peptidoglycan layer complex. Periplasmic flagella are located in the periplasmic space located between the outer envelope and the protoplasmic cylinder. They resemble bacterial flagella in ultrastructure (58,65) and chemical composition (58,64). Periplasmic flagella are attached subterminally in the protoplasmic cylinder at each end of the cell and extend along the cell toward the opposite pole where they overlap one

another (126). Recent evidence supports the view that the periplasmic flagella play a role in spirochete motility (20,23,112).

Spirochetes are members of the order Spirochaetales which contains the following 5 genera: Borrelia, Cristispira, Leptospira, Spirochaeta, and Treponema (127). Major characteristics of the five recognized genera are listed in Table 1. The individual characteristics of these organisms have been reviewed extensively (19,22,23,58,65,126,130).

Nutrition and Metabolism of Spirochetes

Genus Spirochaeta

The organisms of the genus Spirochaeta are either strict anaerobes (S. stenostrepta, S. zuelzerae, and S. litoralis) or facultative anaerobes (S. aurantia) (22). The spirochaetae ferment glucose by the Embden-Meyerhof-Parnas pathway (22,65,126). Culture media generally contains carbohydrate and low concentrations of mineral salts, reducing agents, yeast extract, and peptones (65). Serum is not required and these organisms are capable of de novo synthesis of cellular fatty acids and polar lipids from glucose or acetate (76,100,101).

Genus Borrelia

The Borrelia species are named for the species of the arthropod host. Not much was known about the biochemical characteristics of the borreliae until these organisms were cultivated in vitro in 1971 by Kelly (80). Kelly's medium is suitable for cultivation of B. hermsi, B. parkeri, B. turicatae, and B. recurrentis (80,81). This medium contains glucose, yeast extract, peptones, N-acetylglucosamine, gelatin, pyruvate, inorganic salts, rabbit serum, and bovine serum albumin (BSA). They are grown under microaerophilic conditions. The borreliae

TABLE 1. Characteristics of the genera of the order Spirochaetales

Genus	Characteristics	References
Cristispira	0.5 - 3.0 μm by 30 - 150 μm; over 100 perisplasmic flagella that form crista during unfavorable conditions; widely distributed in intestines of marine and fresh water mollusks; not cultivated in vitro but thought to be facultative anaerobes; type species is C. pectinis.	85, 86
Borrelia	0.2 - 0.5 μm by 3 - 30 μm; 30-40 periplasmic flagella; parasitic or living on mucous membranes; cultivated in vitro under microaerophilic conditions; fermentative metabolism; some are pathogenic for man and animals causing relapsing fever; transmitted by blood sucking arthropods (lice and ticks); classification based on the arthropod vector; type species is B. anserina.	29, 38, 80, 81
Spirochaeta	0.2 - 0.75 μm by 5 - 500 μm; 2 periplasmic flagella; free-living in mud, sewage, and water that contains H_2S; obligate and facultative anaerobes; fermentative metabolism; cultivated in vitro except the type species S. plicatilis; G + C content 50-66 mol% (buoyant density).	21, 22, 130
Leptospira	0.1 μm by 6 - 20 μm; 2 periplasmic flagella; tightly coiled with one or both ends bent or hooked; free-living in soil and water or parasitic in man and animals; cultivated in vitro aerobically; respiratory metabolism; some strains pathogenic for man and animals; type species L. interrogans includes pathogenic and saprophytic serovars; G + C content 36-39 mol% (buoyant density and T_m).	156
Treponema	0.09 - 0.5 μm by 5 - 20 μm; 2 or more periplasmic flagella; found in the oral cavity, intestinal tract, and genital regions of man and animals; some species pathogenic for man and animals; non-pathogenic species cultivated in vitro anaerobically; pathogenic species not cultivated in vitro; fermentative metabolism using carbohydrates or amino acids; type species is T. pallidum; G + C content 32-50 mol% (T_m).	128, 130, 131

ferment glucose by the Embden-Meyerhof-Parnas pathway with formation of lactic acid (14,126). They require an exogenous supply of long chain fatty acids for growth (113). Fatty acids are supplied to the borreliae by the serum and albumin. These organisms metabolize lysophosphatidylcholine in the serum to yield fatty acids, choline, glycerol, and inorganic phosphate (81). The borreliae are incapable of chain elongation and beta-oxidation of fatty acids (92). Long chain fatty acids are incorporated unaltered into the cellular lipids.

Genus Leptospira

There is only one recognized species, L. interrogans, which is divided into 2 groups: the biflexa complex which is composed of the free-living and saprophytic serovars, and the parasitic complex which includes the pathogenic serovars. The leptospires are aerobic bacteria with simple nutritional requirements. Long chain fatty acids are used as the sole source of carbon and energy (55,72,73). The fatty acids are catabolized by beta-oxidation to form acetate and CO_2 (22,126,156). Two carbon fragments, formed by beta-oxidation of fatty acids, are further oxidized via a tricarboxylic acid cycle (11,55). The leptospires cannot use carbohydrates or amino acids as energy sources (34,65). They can unsaturate long chain fatty acids, but cannot effect chain elongate (55,72,150) and de novo synthesis of long chain fatty acids (150). These organisms have lipase activity (126).

The leptospires are cultivated in a medium supplemented with serum or albumin plus Tween 80 (polysorbitan monooleate) (14,34, 35,69,70,162, 164). Thiamine is required for growth (34,141,164). The active factor in serum is albumin (74). The globulin fractions of serum do not

support growth. Long chain fatty acids in the serum are required for growth of the leptospires. Albumin acts to bind and detoxify these fatty acids (35,54,68,140,141). Lipid-free albumin does not support growth. Neither glycerol, inulin, coenzyme A, short chain fatty acids, nor synthetic peptides can substitute for the albumin requirement (126). Albumin and long chain fatty acids can replace the serum requirement (35,126).

The biflexa complex of saprophytic leptospires can be cultivated in a medium that contains albumin and either a saturated or an unsaturated fatty acid with 15 or 16 carbons. The parasitic leptospires require an unsaturated fatty acid with 16 or more carbons (70). Tween compounds can replace these fatty acids (70,126). The leptospires have recently been cultivated in protein-free media containing Tween compounds that have been treated by either charcoal (16) or anion exchange chromatography (142).

Genus Treponema

The members of the genus Treponema are divided into 2 groups, the non-cultivable pathogens and those treponemes cultivated in vitro. The non-cultivable pathogenic species are T. pallidum, T. pertenue, T. carateum, and T. paraluis-cuniculi which cause venereal and congenital syphilis, yaws, pinta, and rabbit syphilis respectively. These organisms are propagated in laboratory animals. Very limited success has been achieved growing T. pallidum in cell tissue cultures or in vitro (39,59,75,121). The second group contains host-associated treponemes that are found in the oral cavity, genital regions, and intestinal tract of man and animals, and in the bovine rumen. The oral species

are T. denticola, T. vincentii, T. oralis, T. macrodentium, and T. scoliodontum. The genital species are T. phagedenis, T. refringens, and T. minutum (128,131). The intestinal species include the causative agent of swine dysentery, T. hyodysenteriae (51, 61), and the recently described non-pathogen, T. innocens (82). Treponema succinifaciens (127) and T. bryantii (143) are found in the bovine rumen. These host-associated species have been cultivated in vitro.

The cultivable treponemes require short chain or long chain fatty acids for growth. Short chain fatty acids are supplied by adding either rumen fluid or an artificial mixture of fatty acids to a complex medium. Long chain fatty acids are supplied by the addition of 10% (vol/vol) animal serum. Serum contains mainly palmitic (C 16:0), stearic (C 18:0), oleic (C 18:1), and linoleic (C 18:2) acids (43). Serum lipids adhere to the treponeme cell surface (126). The cultivable treponemes have a fermentative metabolism and produce short chain fatty acids and alcohols as end products. Treponema, Borrelia, Leptospira, and Spirochaeta have high cellular concentrations of lipids (18-20% of the dry weight of the cell) (71,76,93,99,101,126,130).

Intestinal and rumen treponemes. Some intestinal treponemes require serum for growth. Treponema hyodysenteriae is a large treponeme found on the intestinal mucosa of pigs and it is the only pathogenic treponeme to be cultivated in vitro (87,88,137). Treponema hyodysenteriae and the non-pathogenic T. innocens are grown in a medium supplemented with 10% fetal calf serum (82). The serum supplies cholesterol which is required for growth of these 2 treponemes (88,99). Treponema

strains PR-7 (132), PF-23, and PF-58 (94), isolated from swine intestines, require rumen fluid for growth. Isobutyrate and valerate can replace rumen fluid for growth of these treponemal strains. The rumen treponemes, T. succinifaciens and T. bryantii, also require short chain fatty acids found in rumen fluid. Carbohydrates are fermented and serve as the sole source of energy for these intestinal and rumen treponemes (27,132,143).

Genital treponemes. Most work on the genital treponemes has been done using the Reiter and Kazan 5 strains of T. phagedenis. Prior to the early 1970's the Reiter strain was thought to be a non-pathogenic strain of T. pallidum, but recent DNA/DNA homology studies show that T. phagedenis and T. pallidum are 2 distinct species (102).

The cultivable genital treponemes, T. phagedenis, T. refringens, and T. minutum, are grown in media containing serum (114,118,129). Bovine serum albumin, but not serum globulin fractions (91), can replace serum for growth of T. phagedenis (3,33,91,11). The albumin serves as a detoxifying carrier of long chain fatty acids which are toxic to these organisms at concentrations needed for growth (111,146). The fatty acid requirement is satisfied by adding a pair of fatty acids (one saturated of at least 14 carbons and the other unsaturated of at least 15 carbons with 1, 2 or 3 double bonds) to a complex medium containing lipid-poor albumin (67,71). This pair of fatty acids can be replaced by elaidic acid (trans C18:1Δ9).

The cellular lipids of the genital treponemes reflect those of the medium. When grown in a medium that contained serum, the cellular fatty acids were those found in serum (3,101,159,166). When grown in a

medium containing albumin and the required pair of fatty acids, these acids were incorporated unaltered into the cellular lipids of T. phagedenis (67,71). Elaidic acid, when used, also was incorporated intact into the cellular lipids. Radioactively labeled glucose or acetate carbon is not incorporated into the cellular fatty acids (101). The genital treponemes seem incapable of any fatty acid synthetic ability. They can neither synthesize fatty acids de novo, alter the chain length, saturate, nor unsaturate long chain fatty acids (3,67,71,101).

The Reiter strain of T. phagedenis ferments amino acids and has transamination and decarboxylation activities (6,7,147). Peptidase activity also has been detected (152). Glucose is utilized via the Embden-Meyerhof-Parnas pathway as an energy source (H. A. George, Ph.D. thesis, Virginia Polytechnic Institute and State University, Blacksburg, 1980) and glucose carbons are incorporated into non-lipid cellular material (3). Thiamine also is required for growth of T. phagedenis (91,146,148).

The non-cultivable pathogen, T. pallidum, has been the focus of many metabolic and nutritional studies in an attempt to cultivate this organism in vitro. It is propagated in rabbits or primates. The effects of certain compounds and physical conditions on the retention of motility and virulence of T. pallidum in vitro have been studied. Treponema pallidum was thought to be a strict anaerobe like the other treponemes, but recent reports indicate that low concentrations of oxygen stimulate physiological activities of this treponeme (10,15,39,42,46,96,121). Treponema pallidum cells, extracted from rabbit testes, consume oxygen at a rate equivalent to that of the aerobic leptospires (26). From

glucose T. pallidum cells produce greater amounts of acetate and CO_2 aerobically than anaerobically (22,106,122). Glucose carbons are incorporated into cellular materials, including lipids (8,9). Treponema pallidum cells incorporate amino acids into proteins. This incorporation is inhibited by erythromycin (12,13). This indicates that T. pallidum can synthesize proteins.

Prolonged survival and virulence of T. pallidum cells are achieved by the addition of serum to the cell suspensions (40,42). Sandok et al. (121) reported an increase in cell numbers in a medium containing serum that lacked mammalian cells. Serum proteins, including albumin and globulin proteins, attach to the T. pallidum cells (1). Limited success has been achieved in growing T. pallidum in mammalian cell culture systems under reduced oxygen tensions (39,75).

Motility of T. pallidum cells is prolonged by the addition of BSA and long chain fatty acids (97). Fatty acids alone are inhibitory. The fatty acids required for motility retention are similar to those required for growth of T. phagedenis, a saturated and an unsaturated long chain fatty acid.

The cellular fatty acids of T. pallidum reflect those of serum, tissue, or the added fatty acid when the cells are incubated with these materials (98,122,159). Treponema pallidum cells cannot beta-oxidize long chain fatty acids. They incorporate fatty acids unaltered into their cellular lipids. One difference between the lipids of T. pallidum and the lipids of other treponemes is that monogalactosyl diglyceride is detected in other treponemes (93,101), but not in T. pallidum (98).

The pathogenicity of T. pallidum may be associated with its ability to attach to host cell surfaces. Attachment of T. pallidum cells to either cultured mammalian cells or to host cells prolongs motility (2,41,52). The outer membrane proteins of T. pallidum are thought to be ligands that mediate host cell surface attachment (2). Alderete and Baseman (2) inhibited attachment of T. pallidum cells to rabbit testes by the removal of surface proteins from T. pallidum by trypsin digestion. The non-pathogenic Reiter strain of T. phagedenis is incapable of host cell attachment.

Oral treponemes. Treponemes are common inhabitants of the human gingival crevice (95,133,137). The oral cavity offers favorable microenvironments for a large variety of microorganisms. Conditions in the gingival crevice favor growth of anaerobic and facultative microorganisms (108). The oral treponemes depend on the host and other microorganisms for growth factors, fermentable carbon and energy sources, and anaerobiosis (19). These treponemes are thought to derive nutrients from degraded host epithelial cells, saliva, ingested food, and gingival fluid (108). Saliva contains 18 free amino acids (108) as well as free and esterified long chain fatty acids (125). Gingival fluid also is rich in amino acids and contains serum proteins including albumin and the alpha globulin proteins (108,125).

Microbial associations are common in the oral cavity (49) and the oral treponemes are associated with fusospirochetal infections in the mouth (126,168). Oral treponemes also increase in numbers during periodontal disease (134). The oral treponemes require either serum, ascitic fluid, or short chain fatty acids for growth. Differential

characteristics of the described species of oral treponemes are listed in Table 2.

Treponema macrodentium is typical of an oral treponeme that depends upon its association with other oral organisms that supply required growth factors. Growth of T. macrodentium is obtained in a serum-free medium either cocultured with a fusobacterium and a diphtheroid or in medium supplemented with the fusobacterium-diphtheroid culture filtrate (136). The culture filtrate can be replaced by isobutyrate and thiamine pyrophosphate (TPP). The polyamines, spermine, spermidine, and putrescine can substitute for isobutyrate. Hardy and Munro (50) studied a different strain of T. macrodentium and reported that the diphtheroid-growth factors could be replaced by sodium isobutyrate and sodium bicarbonate. This strain, unlike the one used by Socransky et al. (136), did not require thiamine pyrophosphate and polyamines could not replace isobutyrate.

Treponema denticola, T. vincentii, T. scoliodontum, and most strains of T. macrodentium, require an exogenous supply of thiamine pyrophosphate (cocarboxylase) (Austin, M.S. thesis, 56, 104, 129, 149). Thiamine pyrophosphate is the pyrophosphoric ester of thiamine (vitamin B_1) and participates as a group transferring coenzyme in clostridial type pyruvate clastic reactions (157,158). Thiamine pyrophosphate also participates in α-keto decarboxylase and oxidase reactions as well as transketolase reactions (84). Growth of T. vincentii was stimulated in a medium containing the culture filtrate of an oral diphtheroid (105). The culture filtrate could be replaced by the addition of TPP to a medium containing ascitic fluid. Hampp and Nevin (48) replaced the

TABLE 2. Differentiating characteristics of the oral species of the genus *Treponema*[a]

Species	G + C (mol%)	Fermentation[b] end products	H_2S production	Indole production	Esculin hydrolyzed	Propionate from lactate
T. denticola	37-38	Apls	+	+	+	-
T. vincentii	ND[c]	ABls	w[d]	+	-	-
T. macrodentium	39	Als	+	-	+	-
T. oralis	37	Apls	+	+	+	+
T. scoliodontum	ND	Apbs	-	-	-	-

[a]Reference 57 for all but G+C mol%., references 103,127,131 for G+C mol%.

[b]Capital letters indicate major products, lower case letters indicate minor products. a = acetic acid; p = propionic acid; b = butyric acid; l = lactic acid; s = succinic acid.

[c]ND = no data available.

[d]w = weak reaction.

ascitic fluid requirement by adding TPP, coenzyme A, and ATP to their medium. However, growth often was poor in this medium.

Treponema denticola obtains energy by fermenting amino acids (56,126,129). Blakemore and Canale-Parola (17) demonstrated that T. denticola derives energy by the dissimilation of L-arginine via an arginine iminohydrolase pathway to yield citrulline, NH_3, CO_2, proline, and ornithine. Ornithine is either deaminated to form proline or decarboxylated to form putrescine (17,89). Glucose is not required by T. denticola, but glucose can be fermented via the Embden-Meyerhof-Parnas pathway as a second source of energy (56). Hespell and Canale-Parola (56) also demonstrated that T. denticola contains a coenzyme A dependent CO_2-pyruvate exchange system that is associated with a clostridial type clastic system. Treponema denticola also has phosphotransacetylase and acetate kinase activities.

Serum or ascitic fluid is required for growth of T. denticola and T. vincentii (129). Growth of T. vincentii is enhanced when oleic acid (0.5 µg/ml) is added to a medium containing ascitic fluid (104). Oleic acid alone can not substitute for ascitic fluid.

The alpha globulin fraction of whole serum can replace serum or ascitic fluid for growth of T. denticola and T. vincentii (Austin, M.S. thesis, 56, 135). Albumin, beta globulin, gamma globulin, hemoglobin, transferrin, fibrinogen, and ceruloplasmin fail to support growth. Steinman et al. could replace serum with albumin for growth of the oral treponeme, strain S-69 (149) and reported that the albumin detoxifies an essential lipid. Hespell and Canale-Parola (56) achieved better growth of T. denticola in a medium containing 3 to 5% serum and 0.05%

alpha 2 globulin than in a medium containing only 0.05% alpha 2 globulin. The factor(s) in alpha globulin was reported to be inactivated by heat (80°C for 30 min), not dialyzable, not volatile or steam-distillable, excluded by Sephadex G-75 but not by Sephadex G-100 or G-200, and precipitated by 30-50% ammonium sulfate (135).

Alpha globulin includes a group of proteins with similar electrophoretic mobilities that migrate directly after albumin during gel electrophoresis. The alpha globulins are found in Cohn fraction IV during serum fractionation. The major proteins of alpha globulin are either proteinase inhibitors or binding and transport proteins (144,163). They have molecular weights that range from 4×10^4 to $5\text{-}20 \times 10^6$. Other properties of the alpha globulin proteins are listed in Table 3.

The oral treponemes are thought to be incapable of de novo synthesis of long chain fatty acids. Their cellular fatty acid content resembles that of serum when grown in a medium that contains serum (126). Treponema vincentii, however, contains some branched-chain pentadecanoic acid (126) and T. denticola contains myristic acid and straight-chain pentadecanoic acid (129,166) which normally are not found in serum.

Treponema denticola has fibrinolytic activity (107) and both T. denticola and T. vincentii have proteolytic activity (47,107,109). Trevathan et al. (C. A. Trevathan, M.S. thesis, Virginia Polytechnic Institute and State University, Blacksburg, 1978, 155) demonstrated that these 2 oral treponemes also have phospholipase B activity and utilize lysophosphatidylcholine but not phosphatidylcholine. Trevathan also demonstrated acid phosphatase activity in T. denticola and in the

TABLE 3. Characteristics of the proteins of alpha globulin[a]

Protein	Function	Molecular weight (x 10^3)	Concentration in plasma (mg/100 ml)	Percent carbohydrate
α1-Acid glycoprotein	N.D.[b]	40	55-140	41.4
α1 T-glycoprotein	N.D.	60	5-12	N.D.
α1-Antitrypsin	Trypsin inhibitor	54	200-400	12.4
α1-Antichymotrypsin	Chymotrypsin inhibitor	68	30-60	N.D.
α1 B-glycoprotein	N.D.	50	15-30	N.D.
α1-Lipoprotein	Lipid transport	5-20,000	250-390	1.4
Inter α trypsin inhibitor	Protease inhibitor	160	20-70	9.1
Zn-α2-glycoprotein	N.D.	41	2-15	18.2
α2 HS-glycoprotein	N.D.	49	40-85	13.4
α2-Macroglobulin	Protease inhibitor	725	150-420	8.4
Gc globulin	N.D.	50.8	20-55	4.2
Haptoglobin (3 types)	Hemoglobin binding	100 or polymeric	100-300	19.3
Ceruloplasmin	Copper binding	151	15-60	8.0
Antithrombin III	Thrombin inhibitor	65	17-30	N.D.
Albumin	Binding and transport	65-69	3500-4000	0

[a]Reference 116

[b]N.D. = not determined

genital species T. phagedenis but not in T. vincentii. Lipase activity also was found in the oral treponemes (Trevathan, M.S. thesis, 155).

MATERIALS AND METHODS

Organisms. Treponema denticola strain T-32A was obtained from T. Rosebury, Washington University, St. Louis, Mo. Other treponemal strains used were: T. denticola strain ST-10; E. Canale-Parola, University of Massachusetts, Amherst, Mass.; and T. denticola strain N-39 and T. vincentii strain N-9, Center for Disease Control, Atlanta, Ga. All other strains were from the culture collection of this laboratory. All strains were isolated from the oral cavity of man. Stock cultures were maintained by weekly transfer in pre-reduced heart infusion-peptone-yeast extract-glucose broth (HPYG) prepared as outlined in the VPI Anaerobe Laboratory Manual (57) and supplemented with either 0.4% (wt/vol) human alpha globulin (Cohn fraction IV, Miles Laboratories, Elkhart, Ind.), 0.4% (wt/vol) bovine serum albumin (Cohn fraction V, Sigma Chemical Co., St. Louis, Mo.), or 10% vol/vol heat inactivated (60°C, 1 hr) rabbit serum. Cultures were stored in liquid nitrogen.

Basal medium. HPYG medium contained: Bacto-heart infusion broth, 5.0 g; polypeptone (Baltimore Biological Laboratories, Baltimore, Md.), 20.0 g; Bacto-yeast extract, 10.0 g; glucose, 10.0 g; Bacto-gelatin, 10.0 g; Bacto-agar, 1.6 g; $(NH_4)_2SO_4$, 0.5 g; $NaHCO_3$, 5.0 g; resazurin, 0.001 g; L-cysteine hydrochloride, 0.5 g; salt solution, 500 ml; and distilled water, 500 ml. The salt solution contained (g/liter): $CoCl_3 \cdot 6H_2O$, 0.04; $MnCl_2 \cdot 4H_2O$, 0.04 g; $MgSO_4$, 0.4 g; K_2HPO_4, 2.3 g; KH_2PO_4, 1.0 g; and NaCl, 2.0 g. The pH was adjusted to 7.2 to 7.4.

Preparation of supplements. The following stock solutions (wt/vol) were prepared in distilled water: 2 or 4% globulin fractions (alpha, beta, and gamma); 2, 4, 10 or 20% serum albumin; and 10% starch (Difco

Laboratories, Detroit, Mich.). The commercial sources of the various serum fractions are listed in Table 4. The pH was adjusted to 7.0 with 0.1 N NaOH. The serum fractions were filtered through non-sterile 5.0 μm, 1.2 μm, 0.8 μm, and 0.6 μm membrane filters (Gelman Sciences, Inc., Ann Arbor, Mich.) and sterilized by passage through a 0.45 μm filter. The protein content of all serum fractions was determined by a dye binding method (Bio-Rad Laboratories, Richmond, Ca.). The procedure is based on the binding of acidic Coomassie Brilliant Blue to protein which causes a shift in the absorption maximum. The exact procedure is described in the appendix. The starch solution was sterilized by autoclaving at 121°C for 15 min.

The stock solutions for supplementing HPYG broth contained filter-sterilized thiamine pyrophosphate (from a 5 mg/ml TPP stock solution) (Sigma Chemical Co.), 33 mg per liter and L-glutamine (Sigma Chemical Co.), 660 mg per liter.

Growth factor assay by serial transfer. Cultures were grown in duplicate in HPYG broth (7 mls per tube) supplemented with either whole serum, serum fraction, or starch as listed in Table 4. Prior to use the medium was incubated for several days. All assays used optically matched culture tubes. The gas phase was oxygen-free nitrogen. Test cultures were inoculated with 0.5 ml of a four-day culture of T. denticola or T. vincentii containing approximately 1 x 10^8 cells/ml. Optical densities at 540 nm were read and recorded immediately after inoculation with a Bausch and Lomb Spectronic 20 (Bausch and Lomb Co., Rochester, N.Y.) equipped with a Bausch and Lomb DR-37 digital readout. Optical densities at 540 nm were recorded after 3 and 8 days incubation at

TABLE 4. Serum fractions used as supplements for growth of Treponema denticola and Treponema vincentii

Serum fraction	Cohn fraction	Animal source	Commercial source	Final concentration used (%)[a]
Whole serum	-	Rabbit[b]	-	10.0
Albumin	V	Bovine	Sigma[c]	0.2, 0.4, 1.0-7.0
Albumin	V	Human	Miles	0.2, 0.4, 1.0-7.0
Albumin	V	Lapine	Miles	1.0
Alpha globulin	IV	Human	Miles	0.1-0.5
Alpha globulin	IV	Porcine	ICN	0.2
Alpha globulin	IV	Ovine	ICN	0.2
Beta globulin	III	Human	USB	0.2, 0.4
Beta globulin	III	Bovine	Miles	0.2
Gamma globulin	II	Human	USB	0.2
Gamma globulin	II	Bovine	Miles	0.2
Starch	-	-	Difco	0.5, 1.0

[a]Final concentrations as wt/vol in HPYG except for serum which represents vol/vol.

[b]Heat inactivated 60°C, 30 min.

[c]Sigma Chemical Co., St. Louis, Mo.
Miles Laboratories, Elkhart, Ind.
ICN Pharmaceuticals, Inc., Cleveland, Ohio.
United States Biochemical Corp., Cleveland, Ohio.
Difco Laboratories, Inc., Detroit, Mich.

37°C. Variation of the optical densities of the duplicate cultures generally ranged from ± 0.05. Cultures were transferred on day 3 to the same lot of HPYG medium containing the same supplement; at least five serial transfers were made for each experiment. All cultures were examined by dark-field microscopy at the time of transfer.

Dialysis and ultrafiltration. To determine molecular weight characteristics of the growth factor(s), the alpha globulin was dialyzed using Spectrapor membrane dialysis tubing (Spectrum Medical Industries, Inc., Los Angeles, Ca.) with the molecular weight (MW) cutoffs of 3500, 6-8000, and 12-14000. The dialysis tubing was prepared as described by the manufacturer and stored at 4°C in distilled water. Alpha globulin was dialyzed against distilled water at 4°C for 2 to 4 days with frequent changes of water. After dialysis, the pH was readjusted to 7.0 and the solution was filter sterilized.

Ultrafiltration was performed with a Model 8MC Amicon Micro-ultrafiltration system (Amicon Corp., Lexington, Mass.) and PM10 (> 10,000 MW exclusion), XM50 (> 50,000 MW exclusion), XM100A (> 100,000 MW exclusion), and XM 300 (> 300,000 MW exclusion) Amicon Diaflo membrane filters. Filters were prepared for use and ultrafiltration of alpha globulin performed as described by the manufacturer. Retentates were reconstituted with distilled water and recaptured on the same Diaflo membrane. The washed retentates were restored to the original volume with distilled water and both the retentates and the ultrafiltrates were adjusted to pH 7.0 and filter sterilized.

Enzymatic hydrolysis of alpha globulin. Alpha globulin was treated with the enzymes and conditions listed in Table 5. The enzyme-globulin

mixture was incubated for 24 h at 37°C. The enzymes were inactivated (Table 5) and the pH of the reaction mixture was adjusted to 7.0. Each mixture was filter sterilized and used as a growth supplement in HPYG broth. Each inactivated enzyme was added to HPYG broth to ensure that these proteins did not contribute to treponemal growth. Other control cultures contained alpha globulin that had been subjected only to the enzyme inactivation conditions listed in Table 5.

Delipification. Five g of each serum fraction, HPYG medium component, and starch was made lipid-poor by extraction with 100 ml of chloroform-methanol (2:1) at 4°C for one to two h. The insoluble protein was separated from the lipid-containing solvent by centrifugation at 500 x g for 15 min and the chloroform-methanol layer was removed. The procedure was repeated through four additional extractions. The chloroform-methanol layers were combined and evaporated to dryness at 50°C under a stream of nitrogen. The lipids were stored at -20°C.

The delipified material was dried at 50°C under a stream of nitrogen and placed in a desiccator jar under vacuum for 24 h to ensure dryness. Stock solutions of the delipified fractions and starch were prepared in distilled water, the pH adjusted to 7.0, and filter sterilized.

Lipids. TEM-4T (Witco Chemical Co., New York, N.Y.), a tartaric acid ester of tallow monoglycerides, was used as a source of fatty acids. Stock solutions of 2 mg/ml were prepared in distilled water, the pH adjusted to 7.0, and the solution was sterilized at 121°C for 15 min. Myristoleic, palmitoleic, oleic, elaidic, vaccenic, and linoleic acids were obtained from Supelco Inc., Bellefonte, Pa. Stearic acid

TABLE 5. Enzymes and conditions used to determine properties of the alpha globulin growth factor

Enzyme[a]	pH[b]	Enzyme inactivator	Commercial source[c]
Pepsin (EC 3.4.23.1)	2.5	70°C, 30 min	Sigma
Trypsin (EC 3.4.21.4)	8.2	Trypsin inhibitor (1 mg/ml trypsin)	Sigma
Papain (EC 3.4.22.2)	5.0	Iodoacetate (1 mg/ml)	BBL
Lipase (EC 3.1.1.3)	7.0	80°C, 30 min	Sigma
Phospholipase C (EC 3.1.4.3)	7.0	N.I.[d]	Sigma
Phospholipase D (EC 3.1.4.4)	5.6	N.I.	Sigma

[a]One mg of enzyme was added per ml of a 2% alpha globulin solution.

[b]Reference 79.

[c]Sigma Chemical Co., St. Louis, Mo. Baltimore Biological Laboratories, Baltimore, Md.

[d]N.I. - not subjected to inactivation.

was obtained from Fisher Scientific Co., Pittsburgh, Pa. Sodium salts of each acid were made by adding 1.0 ml of 1 N NaOH to 10 ml of 0.1 M fatty acid in chloroform. The mixture was stirred for 30 minutes to ensure completion of salt formation. The mixture was centrifuged at low speed (3500 x g) and the chloroform was removed. The sodium salts of the fatty acids were dried at room temperature under a stream of nitrogen. Sodium salts of myristic and palmitic acids were obtained from Pfaltz and Bauer, Stamford, Conn. Stock solutions of 0.5 mg/ml of the various fatty acids were prepared in distilled water, the pH was adjusted to 7.0, and the solution sterilized at 121°C for 15 min.

Tween 20, 40, 60, and 80 were obtained from Baker Chemical Co., Phillipsburg, N.J. Stock solutions of 2 mg/ml were prepared in distilled water, the pH was adjusted to 7.0, and the solutions were filter sterilized.

Charcoal treated Tween 80 was prepared by combining 100 ml of a ten percent Tween 80 solution with 20 g of Norit A charcoal (Fisher Scientific Co.) as described by Bey and Johnson (16). The mixture was stirred slowly for 18-24 h at room temperature before the charcoal was allowed to settle for 18-24 h at 4°C. The Tween 80 solution was then decanted from the charcoal. The Tween 80 solution was centrifuged (12,000 x g for 1 h), and filtered through an Amicon XM100A membrane filter (Amicon Corp.) with a molecular weight exclusion of 100,000. Tween 80 concentrations were determined by comparing the absorbance at 230 nm, read on a Gilford Model 250 recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio), to that of a Tween 80 standard curve. Stock solutions of 2 mg/ml were prepared in distilled

water, the pH was adjusted to 7.0, and the solution was filter sterilized.

The sterile lipid solutions were added in various concentrations to either sterile delipified serum fractions or starch stock solutions. These solutions were mixed for 30-60 seconds with a Vortex mixer. The supplements listed in Table 4 were added to HPYG broth. Sterile charcoal-treated Tween 80 solution was added directly to HPYG broth.

Volatile fatty acid solution. The volatile fatty acid (VFA) solution contained: acetic acid, 5 ml; N-butyric acid, 4 ml; N-valeric acid, 1 ml; isobutyric acid, 1 ml; isovaleric acid, 1 ml; and distilled water, 88 ml. The pH was adjusted to 7.2 and the solution was filter sterilized. The sterile fatty acid solution was added in various amounts (0.1 ml, 0.2 ml, and 0.3 ml of VFA solution) directly to HPYG broth containing 10 µg/ml TPP. The VFA solution was also added in various amounts (1.0, 2.0, and 3.0 ml) to 7 ml of 4% delipified albumin stock solutions. This supplement was added to basal HPYG broth to a final concentration of 0.4% delipified albumin.

Thin-layer chromatography (TLC). Lipids present in serum fractions and TEM-4T were detected by TLC. Precoated silica-gel G plates (Analtech, Inc., Newark, Del.) were inactivated by heat at 110°C for 1.5 h. Ten to 50 µl of lipid extract was applied to the plates. The plates were developed at room temperature in a sealed tank containing a petroleum ether-ethyl ether-acetic acid (90:10:2 vol/vol/vol) solvent system. The atmosphere of the tank was equilibrated with the solvent system by lining the tank with Whatman No. 1 filter paper (Whatman, Inc., Clifton, N.J.) prior to development of the plates. Standards

(Supelco, Inc.) contained phospholipid, mono-, di-, and triglycerides, and cholesterol and were chromatographed with the unknown samples. The developed plates were air dried, sprayed with 0.05% Rhodamine 6G (Allied Chemical Co., Morristown, N.J.) in ethanol and observed under ultraviolet light for red fluorescent spots. Phospray (Supelco, Inc.) was used to confirm the presence of phospholipids and observed for a blue spot which developed upon heating the TLC plates at 100°C.

Lipid extraction of whole cells. Treponema denticola cells were grown in either agar-free HPYG broth containing 10% serum or agar-free HPYG broth containing 0.4% delipified alpha globulin-0.03 mg/ml oleate. After 8 days of incubation at 37°C the cells were harvested by centrifugation at 15,000 x g for 15 min. The supernatant was decanted and saved for fatty acid analysis. The cells were washed by resuspension in 50 mM Tris-5 mM $MgCl_2$ buffer (pH 7.3) and recentrifugation. At least three washes were performed.

Cell lipids were extracted by the method of Bligh and Dyer (18). Five ml of chloroform-methanol-water (1:2:0.8 vol/vol/vol) was added to the whole cells in a 30 ml glass centrifuge tube. The mixture was incubated for 1 h at room temperature with intermittent gentle mixing. Ten ml of chloroform-methanol-water (1:2:1.8 vol/vol/vol) was added to form a biphasic mixture and this mixture was incubated for 1 h at room temperature with intermittent gentle mixing. The mixture was centrifuged (500 x g for 10 min), and the chloroform layer was removed. The aqueous layer was extracted twice more with 5 ml of chloroform. The three chloroform layers were combined and washed once with an equal volume of distilled water. The chloroform was evaporated to dryness at 50°C

under a stream of nitrogen and the dried extract was used for analysis of long chain fatty acids.

Fatty acid analysis. Extracts of HPYG medium components, whole cells, and various serum fractions were analyzed for short chain fatty acids. The solvents were extracted with ether and analyzed by gas liquid chromatography as outlined in the VPI Anaerobe Laboratory Manual (57).

Methyl-esters of long chain fatty acids were prepared by transmethylation. The lipid was dissolved in a mixture of 0.5 ml of chloroform, 0.5 ml of methanol:toluene (1:1), and 0.5 ml of a freshly prepared methanolic KOH (0.2 N solution). The solution was incubated at 40°C for 1.5 h and then neutralized with 1 M acetic acid. Methyl-esters of the long chain fatty acids were extracted with chloroform, and the chloroform was evaporated to dryness at 50°C under a stream of nitrogen. The methyl-esters were redissolved in 0.5 ml of hexane and 5 µl was injected into a Varian 1520 gas chromatograph (Varian Aerograph, Walnut Creek, Ca.) equipped with a Hewlett-Packard Model 3380A recorder/integrator (Hewlett-Packard, Avondale, Pa.). The chromatograph had a flame ionization detector. The oven temperature was 180-185°C; the detector oven temperature was 200°C, and the injector oven temperature was 200°C. The column, 6 ft x 1/4 inch aluminum column, was packed with SP-1000 (Supelco Inc.). The carrier gas was helium with a flow rate of 30 ml/min. The long chain fatty acid standard was obtained from Supelco Inc.

Alpha globulin fractionation. Alpha globulin (2% wt/vol in 0.02 M phosphate buffer) was fractionated by column chromatography using

100-200 mesh Affi-Gel Blue (Bio-Rad Laboratories) with an albumin binding capacity of 15 mg/ml. The column was equilibrated with 0.02 M phosphate buffer (pH 7.1). Buffer was eluted from the column (1.6 x 20 cm) at a flow rate of 15 ml/h. Alpha globulin was eluted with 0.02 M phosphate buffer and the adsorbed albumin was eluted with 1.4 M NaCl in 0.02 M phosphate buffer. Four ml fractions were collected. The column was regenerated with 8 M urea.

The eluted protein fractions were dialyzed, for 48 h against distilled water at 4°C with Spectropor 6-8000 MW cutoff dialysis tubing, to remove buffer and salts. The dialyzed protein fractions were lyophilized and stored at -20°C. Stock solutions of either 2 or 4% wt/vol of the protein fractions were made as previously described.

Agar gel double diffusion. Two dimensional immunodiffusion was performed by the method of Ouchterlony (110) to determine the presence of individual serum proteins in alpha globulin. Cleaned glass slides (2 x 3 inch) were overlayed with 6 ml of 1.0% agarose (Bio-Rad Laboratories) buffered with Tris-NaCl, pH 7.2. The agarose was allowed to harden and a circle of 4 mm diameter wells spaced 3-5 mm apart were made around a 4 mm center well. The center well was filled with an antiserum specific to an individual human serum protein. The antisera used and commercial suppliers are listed in Table 6. The outer wells were filled with the protein solution to be tested. The slides were incubated at room temperature in a moist chamber for 24 h. A precipitin line between the center well and an outer well indicated the presence of that specific human protein in the test solution.

TABLE 6. antisera used for immunodiffusion and rocket immunoelectrophoresis[a]

Antiserum (vs.)	Titer	Commercial supplier
Albumin	2.3 mg/ml	Miles[b]
Alpha 2-macroglobulin	2.0 mg/ml	Miles
Alpha 1-lipoprotein	3.0 mg/ml	Miles
Ceruloplasmin	2.0 mg/ml	Miles
Alpha 1-trypsin inhibitor	1:4 vs. human serum	Miles
Alpha 1-glycoprotein	0.8 mg/ml	Miles
Alpha 2-AP-glycoprotein	N.A.[c]	Behring[d]
Alpha 1-T glycoprotein	N.A.	Behring
Gc globulin	0.30	Behring
Inter alpha trypsin inhibitor	0.95	Behring
Alpha 1-antichymotrypsin	0.60	Behring
Alpha 1 B-glycoprotein	N.A.	Behring
Zn-alpha 2-glycoprotein	N.A.	Behring
Haptoglobin	1.50	Behring
Apolipoprotein	1.37	Behring
Lactoferrin	1.50	Behring

[a]Antisera are against human proteins.

[b]Miles Laboratories, Elkhart, Ind., antisera prepared in goats.

[c]N.A. = not available.

[d]Behring Diagnostics, Somerville, N.J., antisera prepared in rabbits.

Albumin determinations. Albumin content of commercial alpha globulin and of alpha globulin fractions was determined with a dye binding method and with rocket immunoelectrophoresis. The dye binding method, using methyl orange, is a modification of the method of Kachmar (77). The exact procedure is described in the appendix.

Rocket immunoelectrophoresis. For the rocket immunoelectrophoresis assay, antisera was incorporated into the agarose. Bio-Rad agarose immunoelectrophoresis tablets (Bio-Rad Laboratories) were dissolved by heating in distilled water. The molten agarose was allowed to cool to 55°C and 10 µl of antisera was added per ml of agarose solution. Six ml of the mixture was dispensed onto a clean, level, 2 by 3 inch glass slide and allowed to harden. The antisera used are listed in Table 6.

Six wells with diameters of 4 mm were made eight to ten mm apart in the agarose on the slide. Ten µl of alpha globulin sample was applied to each well and the slide was subjected to electrophoresis at 150V (40 milliamps) on a Zipzone electrophoresis chamber (Helena Laboratories, Beaumont, Tex.) that contained barbitol-sodium barbitol buffer (0.05 ionic strength, pH 8.6). After electrophoresis the wells were filled with distilled water and the gels covered with moistened Whatman No. 1 filter paper (Whatman, Inc.). A 2 cm layer of dry, folded Whatman No. 1 filter paper was placed on the gel and pressure was applied to the gel by a heavy glass plate for 30 min. The pressed gels were washed in saline for 24 hours then in distilled water for 24 hours and dried by warm air. The dried gels were stained using 0.2% Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories) in a water-methanol-acetic acid (4.5:4.5:1) mixture for 2-3 min. The gels were destained with the

same solvent mixture without stain. The stained gels were air dried and the peak heights measured and compared to an alpha globulin standard curve.

Thiamine binding. Thiamine pyrophosphate was added to 2% albumin, 5% starch, and 0.1% wt/vol agar to a final TPP concentration of 50 µg/ml and allowed to equilibrate for up to 24 hours at 4°C. Two ml of sample was added to washed Centriflo CF25 membrane filter cones (Amicon Corp.) with a molecular weight exclusion of 25,000. Samples were centrifuged at 500 x g for 30 min. Ultrafiltrates were collected and retentates restored to the original volume with 2 ml of distilled water. The restored CF25 retentates were recaptured on the same membrane cone and restored to the original volume with distilled water twice more.

Thiamine pyrophosphate concentrations (as thiamine) in both the restored retentates and the ultrafiltrates were determined by a thiochrome assay (151). The oxidizing solution was freshly prepared by adding 3 ml of an aqueous 1% wt/vol solution of potassium ferricyanide to 97 ml of cold 3.75 N NaOH. One ml of sample or distilled water (used as the blank) was added to 1 ml of freshly prepared oxidizing solution in a 1 cm quartz cuvette and mixed. The exact procedure for the assay is listed in the appendix.

The absorbance of thiochrome at 369 nm was read on a Gilford Model 250 recording spectrophotometer (Gilford Instrument Laboratories, Inc.). The absorbances of the retentates and ultrafiltrates were compared to TPP standards containing 1.5 through 50 µg/ml TPP.

Kinetic studies were performed to determine the rate of TPP binding and the effect of oleate on TPP binding. Oleate, when added, was at a

final concentration of 0.3 mg/ml and was allowed to bind to albumin for 24 h prior to adding TPP. Two ml samples were taken at various intervals and thiamine binding was determined as described above.

Proteolysis of serum fractions. Insoluble trypsin, 5 mg/ml (Sigma Chemical Co.), was added to 10 ml of a 2% (wt/vol) solution of either Affi-Gel Blue fractionated alpha globulin or human serum albumin at pH 8.2, and incubated at 37°C for 24 hours with constant shaking. The insoluble trypsin was removed by centrifugation at 12,000 x g for 10 min. The supernatant was filtered through Whatman No. 1 filter paper, the pH was adjusted to 7.0, and the enzyme-treated protein solution was filter sterilized.

Proteolytic activity of whole cells. Whole cells of T. denticola, grown, harvested, and washed as previously described for the lipid extraction of whole cells, were used to determine proteolytic activity of the cells. The spent culture supernatant also was assayed for proteolytic activity. Casein yellow (Calbiochem-Behring Corp., LaJolla, Ca.) or Bio-Rad Protease Detection Kit tablets (Bio-Rad Laboratories) were used as substrates. The procedure for using casein yellow is described in the appendix. The procedure used for the Bio-Rad Protease Detection Kit was described by the manufacturers. Casein substrate gel tablets were hydrated (1 tablet/5 ml distilled water) and heated in a boiling water bath until dissolved. The agar was cooled to 55°C and 6 ml pipetted onto a cleaned glass slide (2 x 3 inch) and allowed to harden. Four mm diameter wells were punched into the gel and 15 µl of sample was added per well. Trypsin solutions containing 0.12 through 2.4 BAEE (α-N-benzoyl-L-arginine methyl ester hydrochloride equivalents)

units was used as a standard. The plates were incubated for 24 h at room temperature then overlayed with 3% vol/vol acetic acid to stop hydrolysis and enhance the clarity of the hydrolyzed zones. The diameters, of the zones that were produced by proteolytic activity of whole cells and of culture supernatants, were compared to those of the trypsin standard.

Determination of serum proteins adsorbed to the cell surface of treponemes. Whole cells were grown in HPYG broth containing BSA-oleate for 5 days and harvested by centrifugation at 16,000 x g for 15 min. The cells were washed at least twice by resuspension in 50 mM Tris-5 mM $MgCl_2$ buffer (pH 7.3) and recentrifugation. To test these cells for proteolytic activity against the alpha globulin proteins, the T. denticola cells were incubated under nitrogen at 37°C for 4 days in a 0.4% alpha globulin solution. After incubation the cells were removed from the alpha globulin by sedimention at 16,000 x g for 10 min. The cells were washed twice in 50 mM Tris-5 mM $MgCl_2$ buffer, and stored in buffer at 4°C. The supernatant alpha globulin solution was filtered through a 0.45 µm filter to remove any remaining cells and stored at 4°C.

A coagglutination technique was used to determine if human alpha globulin proteins were tightly bound to the surface of T. denticola cells. The antisera against specific human proteins, listed in Table 5, were diluted 1:2 with saline. Twenty-five µl of diluted antisera was added to 25 µl of formalin-fixed staphylococci cells bearing protein-A (Bethesda Research Laboratories, Gaithersburg, Md.) and incubated at room temperature for 30 min. The antibody-coated staphylococci were washed once with one ml of saline and sedimented with an

Eppendorf 5412 (Brinkman Instruments, Westbury, N.Y.) centrifuge. The washed antibody (IgG)-coated cells were resuspended in 50 µl of saline. Twenty-five µl of the antibody coated cell suspension and 25 µl of T. denticola cells, previously suspended in human alpha globulin and harvested as described above, were added to a ring slide and mixed. The reaction mixture was rotated for 1 min at room temperature and observed for coagglutination. Treponema denticola cells incubated in 50 mM Tris-5 mM $MgCl_2$ buffer (pH 7.3), and non-antibody coated staphylococci were used as negative controls. Reactions were recorded as either negative or positive in comparison with the control.

Alpha globulin proteins that were tightly bound to the surface of T. denticola cells also were assayed by enzyme-linked immunosorbent assay (ELISA). Ten µl of the T. denticola cells that had been suspended in alpha globulin and harvested as described above was added to wells in a Costar Serocluster EIA plate (Costar Corp., Cambridge, Mass.) with ing 150 µl of coating buffer. The plates were incubated for 1 h at 37°C. Treponema denticola cells, incubated in 50 mM Tris-5 mM $MgCl_2$ wash buffer (pH 7.3), were used as a negative control. The wells were washed three times with 150 µl of buffer to remove unattached cells. The antisera against specific human alpha globulin proteins (Table 6) were diluted 1:20 in wash buffer and 150 µl was added to each of the wells. The plates were incubated for 2 h at 37°C. The wells were washed three times with 150 µl of buffer to remove unbound antibody. Alkaline phosphatase-conjugated anti-rabbit IgG serum (Miles Laboratories), diluted 1:400 in wash buffer and 150 µl was added to each well and the plates were incubated for 1 h at 37°C. The wells were washed

three times with 150 µl of buffer to remove unbound alkaline phosphatase. Phosphatase substrate (150 µl) (Sigma Chemical Co.) was added to the wells and the plates were incubated for 5 h at room temperature. Alkaline phosphatase-labeled antiserum and phosphatase substrate were added to an empty well as a positive control. Development of a yellow color indicated a positive reaction. The reagents and solutions used and the exact procedure are described in the appendix.

The cell-treated alpha globulin proteins were subjected to rocket immunoelectrophoresis and agar gel double diffusion as described previously, and to agarose gel electrophoresis to determine the effects that T. denticola cells may have on these proteins. Fifteen µl of sample was added to wells in an Isophore Gradipore Survey Gel (Isolab Inc., Akron, Ohio) containing 2.5 to 27% gradient polyacrylamide gel. The samples were electrophoresed for 24 hours at 125 V, 30 mamp in a 40 mM glycine-5 mM Tris buffer, pH 8.8. The gel was removed and stained for 1 h in a distilled water-methanol-acetic acid (4.5:4.5:1) solution containing 0.2% Coomassie Brilliant Blue G-250. The gels were destained with the same solvent mixture without stain.

RESULTS

Characterization of the Growth Factor(s) in Alpha Globulin

Comparison of the growth response of T. denticola and T. vincentii with alpha globulin. Treponema denticola and T. vincentii were grown in heart infusion peptone yeast extract glucose (HPYG) broth supplemented with 10% rabbit serum, 1% bovine serum albumin, or 0.2% alpha globulin to confirm the reports by Austin (M.S. thesis) and Socransky and Hubersak (135) that alpha globulin can support good growth of these two oral treponemes. Rabbit serum was used as the control for optimal growth and albumin as the minimal growth control. The results after 8 days incubation of the fifth transfer are shown in Table 7. Growth after 3 days incubation in this and subsequent tests generally was equivalent to or slightly less than growth after 8 days incubation. Growth after 8 days was recorded for all test results. Because T. denticola grew better in the alpha globulin supplemented medium than did T. vincentii, I decided to use T. denticola strain T-32A in subsequent tests.

Ten-fold serial dilutions of T. denticola in HPYG broth containing serum were made to determine approximate cell counts. Optical densities (O.D.) were found to correspond to the following approximate cell counts: 1.0 O.D. equivalent to 5×10^8 cells/ml,

0.5 O.D. equivalent to 1×10^8 cells/ml,

0.25 O.D. equivalent to 3×10^7 cells/ml, and

0.13 O.D. equivalent to 1×10^7 cells/ml.

TABLE 7. Comparison of the growth of Treponema denticola strain T-32A and Treponema vincentii strain N-9

Supplement	Final concentration (%)[a]	Optical density[b]	
		T. denticola	T. vincentii
Serum (lapine)	10.0	1.18	0.70
Alpha globulin (human)	0.2	0.50	0.24
Serum albumin (bovine)	1.0	0.12	0.02

[a]Final concentration of serum as vol/vol, other concentrations indicate protein content of supplements in HPYG broth.

[b]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 5th transfer.

To confirm the findings of Austin (M.S. thesis), and Socransky and Hubersak (135) that alpha globulin was the only serum fraction that supports growth of T. denticola, HPYG broth was supplemented with the following serum fractions: serum albumin (human, bovine, and lapine), alpha globulin (human, porcine, and ovine), beta globulin (human and bovine), and gamma globulin (human and bovine). Rabbit serum was used as the control for optimal growth. The alpha globulin fraction of serum from different animal sources was shown to support better growth through five serial transfers than did albumin, beta globulin, and gamma globulin fractions which supported minimal growth (Table 8). Minimal growth was usually observed after 2 to 3 transfers. Combinations of serum fractions gave no better growth than did alpha globulin alone (Table 9). Human alpha globulin was chosen for all subsequent tests because of commercial availability.

Effect of dialysis and ultrafiltration of alpha globulin. Alpha globulin was subjected to either dialysis or ultrafiltration to determine the approximate molecular weight of the active factor(s) in alpha globulin. There was a minor loss of growth of T. denticola when dialyzed alpha globulin was added to basal HPYG broth (Table 10).

Ultrafiltration of the alpha globulin also did not remove the growth factor(s) (Table 11). When these ultrafiltrates and retentates were recombined there appeared to be no decrease in O.D. (0.62) as compared to retentates alone (0.60 O.D.). No further characterization of any possible inhibitory substance was done.

The active factor(s) in alpha globulin was shown to be stable at pH 7.0 to heat at 121°C for 15 min. After autoclaving, alpha globulin

TABLE 8. Growth promoting activity of serum fractions for Treponema denticola strain T-32A

Supplement	Final concentration (%)[a]	Optical density[b]
Whole serum (lapine)	10.0	1.18
Albumin (bovine)	1.0	0.12
Albumin (human)	1.0	0.13
Albumin (lapine)	1.0	0.19
Alpha globulin (human)	0.2	0.42
Alpha globulin (porcine)	0.2	0.36
Alpha globulin (ovine)	0.2	0.47
Beta globulin (human)	0.2	0.13
Beta globulin (bovine)	0.2	0.24
Gamma globulin (human)	0.2	0.04
Gamma globulin (bovine)	0.2	0.16

[a]Final concentration of serum as vol/vol, other concentrations indicate protein content of supplements in HPYG broth.

[b]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 5th transfer.

TABLE 9. Effect of combinations of serum fractions on the growth of Treponema denticola strain T-32A

Supplements[a]	Final concentration (%)[b]	Optical density[c]
Whole serum	10	1.20
α-globulin	0.2	0.49
α-globulin plus β-globulin	0.2/0.2	0.42
α-globulin plus γ-globulin	0.2/0.2	0.39
α-globulin plus albumin	0.2/1.0	0.46
α-globulin plus β-globulin, and γ-globulin and albumin	0.2/0.2/0.2/1.0	0.43
Albumin	1.0	0.09
Albumin plus β-globulin	1.0/0.2	0.10
Albumin plus γ-globulin	1.0/0.2	0.08
β-globulin plus γ-globulin	0.2/0.2	0.04

[a]Abbreviations used α, alpha; β, beta; , gamma. Serum fractions were of human origin and the whole serum was from rabbits.

[b]Final concentration of serum as vol/vol, all other concentrations indicate protein content of supplements in HPYG broth.

[c]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 5th transfer.

TABLE 10. Effect of dialysis of human globulin on the growth of Treponema denticola strain T-32A

Supplement	Dialysis tubing M. W. cutoff[a]	Final concentration (%)[b]	Optical density[c]
Alpha globulin	-	0.2	0.45
Alpha globulin	3500	0.2	0.40
Alpha globulin	6-8000	0.2	0.41
Alpha globulin	12-14000	0.2	0.36
Albumin[d]	-	1.0	0.08
Albumin	6-8000	1.0	0.12

[a]Serum fractions dialyzed at 4°C for 24 h against distilled water. M. W. = molecular weight cutoff of Spectrapor dialysis tubing (Spectrum Medical Laboratories, Inc., Los Angeles, Ca.).

[b]Final concentrations indicate protein content of supplements in HPYG broth.

[c]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 5th transfer.

[d]Albumin (bovine) was added as a minimal growth control.

TABLE 11. Effect of ultrafiltration of human alpha globulin on the growth of Treponema denticola strain T-32[a]

Ultrafiltration fraction	Molecular weight exclusion	Final concentration (%)[b]	Optical density[c]
Whole alpha globulin	-	0.2	0.51
Retentate	10,000	0.2	0.44
Ultrafiltrate	10,000	10.0	0.02
Retentate	50,000	0.2	0.46
Ultrafiltrate	50,000	10.0	0.15
Retentate	100,000	0.2	0.60
Ultrafiltrate	100,000	10.0	0.15
Retentate	300,000	0.2	0.59
Ultrafiltrate	300,000	10.0	0.05

[a]Ultrafiltration of alpha globulin with an Amicon Micro-Ultrafiltration System (Amicon Corp., Lexington, Mass.) and retentates and ultrafiltrates collected.

[b]Final concentrations of retentates indicate protein content. The concentration of ultrafiltrates are vol/vol. Supplements were added to HPYG broth.

[c]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 5th transfer.

(0.2%) was added to HPYG broth. The optical density of the T. denticola culture containing autoclaved alpha globulin, was 0.45. Cultures with filter sterilized 0.2% alpha globulin had an O.D. of 0.41.

Characterization of the growth factor(s) by enzymatic hydrolysis. Alpha globulin was subjected to hydrolysis with various proteolytic enzymes and lipid-hydrolyzing enzymes to determine some biochemical properties of the growth factor(s). The proteolytic enzymes used were papain, pepsin, and trypsin. The lipid-hydrolyzing enzymes were lipase, and phospholipase C and D. The enzyme-treated alpha globulin was added to HPYG broth. The results are shown in Table 12. Trypsin and lipase treatment destroyed the growth factor(s) in alpha globulin. Cultures grown with papain-treated alpha globulin required up to 8 days to reach visible growth. The O.D. after 3 days of incubation was 0.02 and after 8 days the O.D. was 0.31. Controls that contained untreated alpha globulin, which was subjected to the appropriate enzyme inactivation conditions, had optical densities (0.40 to 0.48) similar to that of untreated 0.2% alpha globulin (0.51 O.D.). Enzymes that were not subjected to inactivation were added to growing cultures of T. denticola in alpha globulin supplemented HPYG broth to determine if the active enzymes would inhibit growth of T. denticola. No active enzyme inhibited growth when these cultures were serially transferred. These data indicate that the active growth factors in alpha globulin are a protein, inactivated by trypsin, and a lipid, inactivated by lipase. The lipid factor was investigated first.

Determination of the optimum final concentration of human alpha globulin. Growth of T. denticola in HPYG broth supplemented with

TABLE 12. Effect of enzyme treatment of hyman alpha globulin on the growth of Treponema denticola strain T-32A[a]

Enzyme[b]	Optical density[c]
Positive control[d]	0.51
Negative control[e]	0.09
Alpha globulin treated with	
Papain (EC 3.4.22.2)	0.31
Pepsin (EC 3.4.23.1)	0.25
Trypsin (EC 3.4.21.4)	0.02
Lipase (EC 3.1.1.3)	0.01
Phospholipase C (EC 3.1.4.3)	0.31
Phospholipase D (EC 3.1.4.4)	0.36

[a]Treated alpha globulin (2% stock solution) was added to a final concentration of 10% vol/vol to HPYG broth.

[b]The enzymes were incubated with alpha globulin for 24 h at 37°C at their pH optima.

[c]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 5th transfer.

[d]0.2% untreated alpha globulin in HPYG broth.

[e]1.0% bovine serum albumin in HPYG broth.

various concentrations of alpha globulin was measured to determine the optimum concentration of alpha globulin (Table 13). Alpha globulin at a final concentration of 0.4% in HPYG broth was chosen for subsequent tests. A stock solution of 4.0% (wt/vol) was used because solubility in water was poor at higher concentrations.

Characterization of the Lipid Growth Factor

Effect of delipification of alpha globulin on growth of T. denticola. Alpha globulin was delipified with chloroform-methanol to determine whether lipids are a required factor supplied by this serum fraction as indicated by the results obtained with lipase treatment. Although lipid extraction of alpha globulin greatly reduced the growth promoting activity of this serum fraction, the lipid extract alone did not support growth (Table 14). However, addition of both the crude lipid extract and the delipified protein portion to HPYG restored the growth of T. denticola.

Characterization of the lipids in human alpha globulin. The lipids extracted from alpha globulin were analyzed by thin layer chromatography and gas liquid chromatography. Austin (M.S. thesis) reported that human alpha globulin contained cholesterol, triglycerides, and phospholipids. The lipids of alpha and beta globulin were determined by thin layer chromatography of serum fraction lipid extracts with a petroleum ether-ethyl ether-acetic acid solvent system (Table 15).

Long chain fatty acids of the various human serum fractions were determined by gas liquid chromatography using transmethylated lipid extracts. The fatty acids of TEM-4T also were determined to see if this compound contained fatty acids similar to those of alpha globulin

TABLE 13. Effect of increased human alpha globulin concentrations on the growth of Treponema denticola strain T-32A

Supplement	Final concentration (%)[a]	Optical density[b]
Serum (lapine)	10.0	1.04
Albumin (bovine)	1.0	0.11
Alpha globulin	0.1	0.32
Alpha globulin	0.2	0.44
Alpha globulin	0.3	0.53
Alpha globulin	0.4	0.72
Alpha globulin	0.5	0.68
Alpha globulin	0.6	0.73

[a]Final concentration of serum as vol/vol. Other concentrations indicate protein content. Supplements were added to HPYG broth.

[b]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 4th transfer.

TABLE 14. Effect of lipid extraction of human alpha globulin on the growth of Treponema denticola strain T-32A

Supplement	Final concentration (%)[a]	Optical density[b]
Serum (lapine)	10.0	1.18
Alpha globulin	0.4	0.72
Delipified alpha globulin	0.4	0.15
Delipified alpha globulin plus lipid extract	0.4	0.52
Lipid extract only	0.4	0.07

[a]Final concentration of serum as vol/vol, serum fraction concentrations indicate protein content (wt/vol). Lipid extracts were added in amounts corresponding to the protein content. Supplements were added to HPYG broth.

[b]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 4th transfer.

TABLE 15. Lipids detected in various human serum fractions[a]

Lipid[b]	Alpha globulin	Beta globulin	Gamma globulin	Albumin	Tem-4T[c]
Phospholipids	+[d]	+	+	-	-
Cholesterol	+	+	-	-	-
Monoglycerides	+	+	-	+	+
Diglycerides	+	+	-	-	±
Triglycerides	+	+	-	-	-
Free fatty acids	+	+	-	-	+
Cholesterol esters	±	-	-	±	-

[a]Lipid extracts of the various serum fractions separated by Silica Gel G thin layer chromatography using a petroleum ether-ethyl ether-acetic acid (90:10:2) solvent system.

[b]Lipid controls included phospholipid, triglycerides, and cholesterol. Other spots were identified by comparing Rf values to standards shown in reference 115.

[c]TEM-4T, a tartaric acid ester of tallow monoglycerides was used as a monoglyceride control.

[d]Lipids were detected by spraying with Rhodamine 6G and observing fluorescence under ultraviolet light. + = strong fluorescent spot, ± = weakly fluorescent spot, - = no fluorescent spot detected.

and if it could be used as another source of fatty acids for growth of T. denticola. The fatty acid content of alpha and beta globulins and TEM-4T were similar qualitatively (Table 16). Quantitatively, human beta globulin and human serum albumin (HSA) contained only 40% and 14% of the total fatty acid content of human alpha globulin, whereas bovine serum albumin contained only trace amounts (less than 1%) of the total fatty acids detected in alpha globulin.

Characterization of the lipid(s) required for growth. TEM-4T was added to delipified alpha globulin to determine if this artificial lipid source could substitute for the lipids of alpha globulin. TEM-4T also was added to delipified beta globulin and albumin to determine if lack of growth observed in earlier tests was due to a fatty acid deficiency of these serum fractions. The results in Table 17 show that TEM-4T could substitute for the crude lipid extract of alpha globulin for growth of T. denticola. Higher concentrations of TEM-4T were inhibitory. Beta globulin (0.4%) and albumin (1.0% from a 20% stock solution containing 33 µg/ml of TPP) did not support good growth with added TEM-4T. This was further evidence that there is a non-lipid component found only in alpha globulin that is required for growth of T. denticola. TEM-4T alone did not support growth.

Since the fatty acid content of TEM-4T and alpha globulin were similar and TEM-4T could substitute for the lipid extract of alpha globulin, individual long chain fatty acids found in alpha globulin were tested for their growth promoting activity. Sodium salts of the long chain fatty acids found in alpha globulin and several isomers of these fatty acids were added alone or in various combinations to HPYG

TABLE 16. Fatty acid content of human serum fractions[a]

Fatty acid	Whole[b] serum	Alpha globulin	Beta globulin	Human albumin	Bovine albumin	TEM-4T
Myristic (14:0)	N.D.[c]	< 1.0	2	N.D.	N.D.	10
Myristoleic (14:1)	N.D.	< 1.0	< 1.0	97	N.D.	2
Palmitic (16:0)	23	30	30	3	20	18
Palmitoleic (16:1)	1	4	5	N.D.	N.D.	12
Stearic (18:0)	27	9	18	N.D.	31	14
Oleic (18:1)	15	26	24	N.D.	27	21
Linoleic (18:2)	30	28	16	N.D.	20	3
Eicosadienoic (20:2)	N.D.	N.D.	N.D.	N.D.	N.D.	20
Others[d]	4	2	5	N.D.	2	N.D.

[a]Fatty acids detected by a Varian 1520 gas chromatography with a flame ionization detector and a Hewlett-Packard recorder/integrator using transmethylated derivatives of lipid extracts. Values are % of the total fatty acid detected. Fatty acids were not detected in human gamma globulin.

[b]One ml of whole serum and one g of each of the serum fractions, albumin, and TEM-4T were extracted.

[c]N.D. = none detected.

[d]Includes trace amounts of pentadecanoic acid (15:0), septadecanoic acid (17:0), and unidentified peaks between linoleic and eicosadienoic acids.

TABLE 17. Growth of Treponema denticola strain T-32A using TEM-4T as an artificial lipid source with various serum fractions

Fraction[a]	TEM-4T (mg/ml)	Optical density[b]
Alpha globulin	-[c]	0.72
Delipified alpha globulin	-	0.15
Delipified alpha globulin	0.05	0.41
Delipified alpha globulin	0.10	0.82
Delipified beta globulin	-	0.13
Delipified beta globulin	0.05	0.03
Delipified beta globulin	0.10	0.02
Albumin	-	0.11
Albumin	0.05	0.10
Albumin	0.10	0.17

[a]All globulin fractions were of human origin at a final protein concentration of 0.4%, the albumin was of bovine origin at a final protein concentration of 1.0%.

[b]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 4th transfer. The medium was HPYG.

[c]- means no addition.

broth supplemented with 0.4% delipified alpha globulin. The fatty acids were added to the delipified alpha globulin stock solution to yield a final concentration of 0.03 mg of fatty acid per ml of HPYG broth.

Oleic acid (cis-18:1Δ9) and elaidic acid (trans-18:1Δ9) supported good growth of T. denticola strain T-32A (Table 18). Growth was poor with other unsaturated fatty acids, including vaccenic acid (cis-18:1Δ11) which differs from oleic acid only in the position of the double bond. Growth was better when oleate was used as the fatty acid source (0.81 O.D.) than with the same concentration of elaidate (0.51 O.D.). All saturated fatty acids tested supported poor growth, whether alone or in various combinations. All combinations of saturated and unsaturated fatty acids supported poor growth except in the presence of oleic or elaidic acids. The optical densities of fatty acid combinations containing either oleate or elaidate in delipified alpha globulin supplemented HPYG broth were equivalent to, but no higher than the optical densities achieved with either oleate or elaidate alone. A medium containing 0.03 mg/ml (final concentration) each of palmitic, oleic, and linoleic acids (the 3 most abundant fatty acids in alpha globulin) in HPYG broth supplemented with 0.4% delipified alpha globulin had an O.D. of 0.84 which was no higher than that with oleate alone.

Oleate concentration required for optimal growth. Various concentrations of oleate or elaidate were added to 0.4% delipified alpha globulin supplemented HPYG broth to determine the concentration that would give optimal growth of T. denticola strain T-32A. Higher optical densities were achieved with both of these fatty acids upon increasing their concentrations. The results in Table 19 show that a final concen-

TABLE 18. Growth of Treponema denticola strain T-32A with various long chain fatty acids[a]

Lipid	Concentration (mg/ml)	Optical density[b]
TEM-4T	0.10	0.80
Myristic	0.03	0.13
Myristoleic	0.03	0.31
Palmitic	0.03	0.21
Palmitoleic	0.03	0.34
Stearic	0.03	0.11
Oleic	0.03	0.81
Elaidic	0.03	0.51
Vaccenic	0.03	0.26
Linoleic	0.03	0.21
No lipid added	-	0.25

[a]Fatty acids were added as their sodium salts to HPYG broth supplemented with 0.4% delipified alpha globulin.

[b]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 3rd transfer.

TABLE 19. Growth response of *Treponema denticola* strain T-32A to various concentrations of oleate

Supplement[a]	Concentration (mg/ml)	Optical density[b]
Serum control (10%)	-	1.32
Oleate	0.00	0.25
Oleate	0.01	0.73
Oleate	0.02	0.92
Oleate	0.03	0.96
Oleate	0.04	1.05
Oleate	0.05	0.75
Oleate	0.06	0.00
Albumin (1%) plus oleate	0.03	0.26

[a]Sodium oleate was added to HPYG broth supplemented with 0.4% delipified human alpha globulin. No growth occurred in HPYG with only 0.03 mg/ml sodium oleate.

[b]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 3rd transfer.

tration of 0.04 mg/ml of sodium oleate gave optimal growth of T. denticola (1.05 O.D.). The weight ratio of delipified alpha globulin to sodium oleate that gave optimal growth was 100:1. Elaidic acid also gave optimal growth of T. denticola at a final concentration of 0.04 mg/ml (Table 20). Concentrations of oleate or elaidate above 0.04 mg/ml were inhibitory.

Bovine serum albumin (from 20% BSA stock solution containing 33 µg/ml of TPP) was used as a substitute for delipified alpha globulin to see if albumin could substitute for the delipified alpha globulin proteins when oleic acid was added as the lipid source. Basal HPYG broth was supplemented with 1% BSA (final concentration) and 0.03 mg/ml of oleate. Growth of T. denticola in the BSA-oleate medium was no better than growth in the delipified alpha globulin minimal growth control. Thus, BSA appeared not to be able to replace the requirement for the protein portion of alpha globulin.

Confirmation of the oleate requirement by using Tween 80. Tween compounds (polysorbitan mono-fatty acid) were used as the lipid source to confirm that oleate is the fatty acid required by T. denticola for growth. Tween 20 (lauric acid), Tween 40 (palmitic acid), Tween 60 (stearic acid), and Tween 80 (oleic acid) were used as the fatty acid sources. They were added in various concentrations to HPYG broth supplemented with 0.4% delipified alpha globulin. Tween 80 was the only Tween compound that could support good growth of T. denticola (Table 21). Higher concentrations of Tween 80 were inhibitory.

Bey and Johnson (16) reported that charcoal-treated Tween 80, could support growth of the leptospires in a protein-free medium. In order to

TABLE 20. Growth response of *Treponema denticola* strain T-32A to various concentrations of elaidate

Supplement[a]	Concentration (mg/ml)	Optical density[b]
Serum control (10%)	-	1.25
Elaidate	0.00	0.14
Elaidate	0.01	0.35
Elaidate	0.02	0.49
Elaidate	0.03	0.52
Elaidate	0.04	0.65
Elaidate	0.05	0.35
Elaidate	0.06	0.00
Oleate	0.04	1.05

[a]Sodium elaidate was added to HPYG broth supplemented with 0.4% delipified human alpha globulin. No growth occurred in HPYG broth with only 0.03 mg/ml sodium elaidate.

[b]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 3rd transfer.

TABLE 21. Growth response of Treponema denticola strain T-32A to various Tween compounds

Supplement[a]	Concentration (mg/ml)	Optical density[b]
Whole serum (10%)	-	1.18
Delipified alpha globulin (0.4%)	-	0.15
Tween 20	0.1	0.04
Tween 20	0.3	0.13
Tween 20	0.5	0.06
Tween 40	0.1	0.13
Tween 40	0.3	0.10
Tween 40	0.5	0.07
Tween 60	0.1	0.11
Tween 60	0.3	0.14
Tween 60	0.5	0.08
Tween 80	0.1	0.72
Tween 80	0.3	0.95
Tween 80	0.5	0.47

[a]Tween compounds were added to HPYG broth supplemented with 0.4% delipified human alpha globulin. No growth occurred in HPYG broth with only 0.3 mg/ml Tween 80.

[b]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 3rd transfer.

determine if T. denticola could grow in a protein-free medium, Tween 80 was treated with charcoal as described by Bey and Johnson. Treated Tween 80, containing 33 μg/ml of TPP, was added directly to HPYG broth in various concentrations (0.1 to 0.5 mg/ml). No increase in growth was observed when compared with cultures grown in HPYG broth with delipified alpha globulin. The O.D. of T. denticola grown in HPYG broth containing charcoal detoxified Tween 80 (0.3 mg/ml final concentration) was 0.20. Cultures grown in 0.4% delipified alpha globulin had an O.D. of 0.19.

Growth of other strains of oral treponemes in an oleate medium. Five strains of T. denticola and three strains of T. vincentii were tested to determine if oleate was a general requirement for these two oral species. All strains of T. denticola and T. vincentii were able to grow in HPYG broth supplemented with 0.4% delipified alpha globulin and 0.03 mg/ml of sodium oleate. A slightly suboptimal concentration of oleate was used to ensure that there was no fatty acid inhibition. The results are shown in Table 22.

Utilization of oleate by T. denticola. Cellular fatty acids of T. denticola were examined by gas liquid chromatography of lipid extracts of whole cells to determine if this organism incorporated fatty acids directly from the medium as reported for other treponemes (67,71). Treponema denticola strain T-32A was grown in HPYG broth containing either 10% rabbit serum or 0.4% delipified alpha globulin and 0.03 mg/ml of oleate. After 5 to 6 days incubation, 75% of the oleate had been removed from the medium by T. denticola. The remaining 25% of the oleate was recovered in the spent culture medium. These percentages were based on the amount of oleate detected in an uninoculated control

TABLE 22. Growth of Treponema denticola strains and of Treponema vincentii strains using oleic acid as the lipid source[a]

Organism	Strain	Optical density[b]	
		Serum	Oleate
T. denticola	T32-A	1.18	1.05
	ST-10	1.14	1.00
	N-39	1.04	0.94
	D3A-5	0.76	0.67
	D3A-9	0.78	0.34
T. vincentii	N-9	0.70	0.63
	D7B-1	0.43	0.39
	D3A-1	0.79	0.37

[a]Sodium oleate (0.03 mg/ml) added to HPYG broth supplemented with 0.4% delipified human alpha globulin. Whole serum (final concentration 10% vol/vol) added to HPYG broth.

[b]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 3rd transfer.

medium containing oleate and compared with the amount of oleate that remained in the culture supernatant after growth of the treponeme cells. The cellular fatty acids of T. denticola grown in serum and oleate are shown in Table 23.

Effect of Treponemal Cells on Alpha Globulin Proteins

Proteolytic activity of T. denticola cells. Treponema denticola cells were grown for five days in a medium containing alpha globulin and sedimented by centrifugation. Both the spent culture supernatant and the 4% alpha globulin solution, which had been subjected to 4 days incubation with the harvested cells, were analyzed by electrophoresis in gradient polyacrylamide gels. Several protein bands present in the alpha globulin control were missing from the polyacrylamide gels of the spent medium and whole cell treated alpha globulin. These results indicated that the T. denticola cells may have proteolytic activity toward some alpha globulin proteins.

Culture supernatants and whole cells of T. denticola were assayed for proteolytic activity with casein substrates (casein yellow or Bio-Rad agar containing casein). Various concentrations of trypsin were used as positive controls for casein degradation. The proteolytic activity of whole cells and culture supernatant were calculated as BAEE unit equivalents of trypsin by comparison of the proteolytic activity of the cells and of the supernatant to the controls. Whole cells of T. denticola had proteolytic activity toward casein equivalent to 0.3 BAEE units of trypsin, but the culture supernatants had the equivalent of only 1×10^{-3} BAEE units of trypsin activity.

TABLE 23. Cellular fatty acids of *Treponema denticola* strain T-32A grown with whole serum or sodium oleate[a]

Fatty acid	Lipid source		Retention time (min)
	serum	oleate	
Myristic	17[b]	26	7
Pentadecanoic	5	23	10
Palmitic	27	26	13
Stearic	8	N.D.[c]	25
Oleic	14	6	28
Linoleic	16	N.D.	33
Eicosadienoic (20:2)	N.D.	11	62
Unknown	N.D.	6	88

[a]Sodium oleate (0.03 mg/ml) added to HPYG broth supplemented with 0.4% delipified human alpha globulin. Whole serum (final concentration 10% vol/vol) added to HPYG broth.

[b]Values are as % of total fatty acid detected.

[c]N.D. = none detected.

Effect of whole cells on alpha globulin. The activity of T. denticola cells on human alpha globulin was investigated. The total protein content of a 4% alpha globulin solution decreased to approximately 3% after four days incubation with the T. denticola cells as detected by the Bio-Rad dye binding assay. These results indicated that the proteolytic activity detected in whole cells may have been directed against alpha globulin proteins. The individual proteins of alpha globulin were examined qualitatively by agar-gel immunodiffusion using commercial antisera, prepared in either rabbits or goats, against individual human alpha globulin proteins. Whole alpha globulin served as the control. The commercial alpha globulin (Miles Laboratories, Fraction IV, Catalog No. 82-306-4) was found to contain the following proteins: albumin, alpha 1-trypsin inhibitor, alpha 1 B-glycoprotein, alpha 1-antichymotrypsin, inter alpha trypsin inhibitor, alpha 2-macroglobulin, alpha 2 Zn-glycoprotein, ceruloplasmin, Gc globulin, and haptoglobin. Inter alpha trypsin inhibitor and alpha 1 B-glycoprotein were not detected in the T. denticola cell treated alpha globulin.

A 4% cell treated alpha globulin solution, either dialyzed or ultrafiltered, was used to determine if the proteolytic activity of these cells released a peptide that is required for the growth of T. denticola. If a required peptide was released from alpha globulin by the proteolytic activity of whole cells then the peptide should be removed from the treated alpha globulin either by dialysis (12-14,000 molecular weight cutoff) or by ultrafiltration (300,000 molecular weight exclusion). There was no decrease in growth (0.78 O.D. for dialyzed and 0.76 O.D. for ultrafiltered) of T. denticola when the treated alpha

globulin was added to HPYG broth and compared to growth in the untreated alpha globulin control (0.79 O.D.). Ultrafiltrates of the treated alpha globulin added to HPYG broth did not support growth.

Delipified alpha globulin (4% stock solution) was exposed to whole cells of T. denticola to determine the effects of proteolytic activity on the ability of alpha globulin to bind fatty acid. The protein content of the whole cell treated-delipified alpha globulin stock solution was determined and this solution was added to HPYG broth to a final protein concentration of 0.4% wt/vol. Various concentrations of sodium oleate were added to these HPYG broths (Table 24). The protein to lipid weight ratio that supported optimal growth with treated delipified alpha globulin was 150:1. Untreated delipified alpha globulin (0.4% final concentration) gave optimal growth with 0.04 mg/ml of oleate (Table 19) which is a protein to lipid weight ratio of 100:1. It appeared that some fatty acid binding protein had been effected by the proteolytic activity of T. denticola cells.

Identification of the Protein Requirement

Determination of the albumin content of alpha globulin. Commercial bovine serum albumin (Sigma Chemical Co., Fraction V, Catalog No. A-4503) is contamined with one to four percent serum globulins as described by the manufacturer. Increased concentrations of commercial HSA or BSA were tried as a source of globulin for growth of T. denticola. Optimal growth was obtained with a final concentration of 7% BSA and 5% HSA (Table 25). Seven percent serum albumin was calculated to contain a maximum contaminating alpha globulin concentration of 0.28%. However, this concentration of alpha globulin did not support optimal growth (0.42 O.D.).

TABLE 24. Effect of Treponema denticola strain T-32A on the oleic acid binding capacity of delipified alpha globulin[a]

Supplement[b]	Oleate concentration (mg/ml)	Optical density[c]
Alpha globulin control	-	0.86
Treated delipified alpha globulin	-	0.11
Treated delipified alpha globulin	0.01	0.41
Treated delipified alpha globulin	0.02	0.56
Treated delipified alpha globulin	0.03	0.98
Treated delipified alpha globulin	0.04	0.02

[a]Treponema denticola cells added to 4% delipified alpha globulin stock solution and incubated for 4 days at 37 C. The cells were sedimented by centrifugation and the solution filter sterilized.

[b]Added to HPYG to a final protein concentration of 0.4%.

[c]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 4th transfer.

TABLE 25. Growth of Treponema denticola strain T-32A using increased concentrations of albumin

Supplement	Final concentration (%)[a]	Optical density[b]
Whole serum	10	1.18
Alpha globulin	0.2	0.42
Alpha globulin	0.4	0.95
Bovine serum albumin	1.0	0.36
Bovine serum albumin	3.0	0.39
Bovine serum albumin	5.0	0.75
Bovine serum albumin	7.0	1.08
Human serum albumin	1.0	0.35
Human serum albumin	3.0	0.97
Human serum albumin	5.0	1.23
Human serum albumin	7.0	1.18

[a]Final concentration of serum as vol/vol, serum fraction concentrations indicate protein content. The serum fractions were added to HPYG broth.

[b]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 3rd transfer.

Higher albumin concentrations did not yield better growth. These results indicated that albumin may play a role in supporting growth of T. denticola.

The albumin content in commercial alpha globulin was determined by a methyl orange dye binding assay and by rocket immunoelectrophoresis. Forty-five to fifty percent of the total protein content of the commercial human alpha globulin used in this investigation was found to be albumin.

Separation of albumin from human alpha globulin. The albumin in commercial alpha globulin was selectively removed by affinity column chromatography using Affi-Gel Blue (Bio-Rad Laboratories) to determine the roles of albumin and the alpha globulin proteins for growth of T. denticola. The alpha globulin proteins eluted with 0.02 M phosphate buffer as two overlapping peaks. These two globulin fractions were designated fraction 1, which eluted first, and fraction 2 which eluted immediately after fraction 1. Albumin was eluted from the column with 1.4 M NaCl in 0.02 M phosphate buffer and designated fraction 3. The albumin content of each fraction was determined by the methyl orange dye binding assay and by rocket immunoelectrophoresis. Rocket immunoelectrophoresis with antisera against specific human proteins also was used to determine the presence of individual alpha globulin proteins in each fraction.

Fraction 3 contained mainly albumin (65% of the total protein). Fraction 2 contained less than 10% albumin and fraction 1 had no detectable albumin. The individual serum proteins detected in each fraction

are listed in Table 26. No attempt to quantify the individual proteins was performed.

The fractions separated by Affi-Gel Blue were added to HPYG broth individually and in combinations to determine which fraction(s) was required for T. denticola growth. The final concentration of each fraction was 0.2% wt/vol in HPYG broth because 0.4% alpha globulin contained 0.2% albumin. A combination of fraction 1 and fraction 3 supported optimal growth of T. denticola (Table 27).

Determination of the protein growth factor. Since fraction 3 contained mostly albumin, HSA and BSA were used as substitutes for fraction 3. Serum albumin and either fraction 1 or fraction 2 were added to HPYG broth to yield a final concentration of 0.2% of each protein fraction and 0.2% albumin. These final concentrations were based on the fact that 0.4% alpha globulin contained 0.2% albumin. Both HSA and BSA could substitute for fraction 3 which contained albumin (Table 28). Both serum albumin and fraction 1 were required for optimal growth of T. denticola (1.03 O.D.).

Fraction 1 and HSA were hydrolyzed with insoluble trypsin to determine if intact proteins are required for growth of T. denticola. Intact albumin (Table 29) was required for optimal growth. Intact proteins were not required in fraction 1, which supplied another growth factor, presumably the lipid.

Lipids were extracted from both fraction 1 and HSA to determine if fraction 1 supplied the lipid growth factor. The delipified supplements were added to HPYG broth. Fraction 1, as shown in Table 29, supplied the lipid required for T. denticola growth. The fatty acid content of

TABLE 26. Proteins detected in human alpha globulin fractions separated by Affi-Gel Blue affinity chromatography[a]

Proteins[b]	Fraction 1	Fraction 2	Fraction 3
Alpha 1 B-glycoprotein	+[c]	-	-
Alpha 1-antichymotrypsin	+	+	+
Alpha 1-trypsin inhibitor	+	+	+
Inter alpha trypsin inhibitor	-	+	-
Alpha 2-macroglobulin	+	+	+
Alpha 2 Zn-glycoprotein	+	-	-
Gc globulin	-	+	+
Haptoglobin	+	-	-
Ceruloplasmin	+	-	-
Albumin	-	+	+

[a]Fraction 1 and 2 were eluted with 0.02 M phosphate buffer. Fraction 3 was eluted with 1.4 M NaCl in phosphate buffer.

[b]Detected by rocket immunoelectrophoresis using specific antisera.

[c]+ means protein present in that fraction, - means not detected.

TABLE 27. Growth promoting activity of human alpha globulin proteins separated by Affi-Gel Blue affinity chromatography for Treponema denticola strain T-32A

Supplement[a]	Final concentration (%)[b]	Optical density[c]
Alpha globulin	0.4	0.91
Fraction 1	0.2	0.06
Fraction 2	0.2	0.07
Fraction 3	0.2	0.47
Fraction 1 + fraction 2	0.2/0.2	0.09
Fraction 1 + fraction 3	0.2/0.2	1.02
Fraction 2 + fraction 3	0.2/0.2	0.49

[a]Fraction 1 and fraction 2 were eluted with 0.02 M phosphate buffer. Fraction 3 was eluted with 1.4 M NaCl in phosphate buffer.

[b]Final concentrations indicate protein content (wt/vol) and are based on alpha globulin containing 50% albumin. The medium was HPYG.

[c]Average optical density of duplicate cultures read at 540 nm after 8 days of the 3rd transfer.

TABLE 28. Substitution of Affi-Gel Blue fraction 3 with albumin for growth of Treponema denticola strain T-32A

Supplement[a]	Albumin source	Final concentration (%)[b]	Optical density[c]
Alpha globulin	-	0.4	0.89
-	Bovine	0.2	0.23
Fraction 1	Bovine	0.2/0.2	1.03
Fraction 2	Bovine	0.2/0.2	0.30
-	Human	0.2	0.32
Fraction 1	Human	0.2/0.2	0.97
Fraction 2	Human	0.2/0.2	0.40

[a]Fraction 1 and fraction 2 were eluted with 0.02 M phosphate buffer. These fractions were added to albumin and the supplement added to HPYG broth.

[b]Final concentrations indicate protein content (wt/vol) and are based on alpha globulin containing 50% albumin.

[c]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 3rd transfer.

TABLE 29. Effect of lipid extraction and trypsinization of Affi-Gel Blue fraction 1 and albumin on the growth of Treponema denticola strain T-32A

Supplement[a]	Final concentration (%)[b]	Optical density[c]
HSA[d]	0.2	0.32
HSA plus fraction 1	0.2/0.2	0.97
Delipified HSA plus fraction 1	0.2/0.2	0.96
HSA plus delipified fraction 1	0.2/0.2	0.37
Trypsinized HSA plus fraction 1	0.2/0.2	0.00
HSA plus trypsinized fraction 1	0.2/0.2	0.91
Trypsinized HSA	0.2	0.30
Alpha globulin	0.4	0.89

[a]Fraction 1 was eluted from Affi-Gel Blue with 0.02 M phosphate buffer. The supplements were mixed and added to HPYG broth.

[b]Final concentrations indicate protein content (wt/vol) and are based on alpha globulin containing 50% albumin.

[c]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 3rd transfer.

[d]HSA = human serum albumin.

fraction 1, fraction 2, and fraction 3 was determined to explain why fraction 1 added to albumin supported growth and fraction 2 did not. The fatty acids detected in fraction 1 were those found in whole alpha globulin, 30% palmitic acid, 35% oleic acid, 14% linoleic acid, 8% palmitoleic acid, and 10% stearic acid. Minor amounts of myristic (2%) and myristoleic (1%) acids were also detected. The values are listed as the percent of total fatty acid detected in all three fractions. Fraction 2 contained only trace amounts (< 1%) of palmitic and palmitoleic acids. No fatty acids were detected in fraction 3.

Optimal concentrations of albumin and oleate. Albumin-oleate supplemented HPYG broth was previously shown (Table 19) to support only minimal growth of T. denticola (0.26 O.D.). The roles of TPP, albumin, and oleic acid in the growth of T. denticola were investigated. Thiamine pyrophosphate was reported to be required for growth of T. denticola (Austin, M.S. Thesis, 57, 119). Albumin and oleic acid have been determined during this investigation to be required for growth of T. denticola.

The roles of albumin and oleate were investigated by adding TPP to stock solutions of albumin to yield a final concentration of 25 µg/ml TPP in HPYG broth. Sodium oleate was added in various concentrations to a constant amount of delipified albumin to determine the oleate concentration that would support maximal growth of T. denticola. A final concentration of 0.4% delipified albumin was chosen because 10% vol/vol serum (amount added to HPYG) contains 0.4% albumin (serum = 80 mg/ml protein, of which approximately 50% is albumin). The results in Table 30 show that a final oleate concentration of 0.08 mg/ml (0.02 mg

TABLE 30. Growth response of Treponema denticola strain T-32A to various concentrations of oleic acid

Supplement	Final concentration (%)[a]	Oleate concentration (mg/ml)[b]	Optical density[c]
Serum	10	-	1.50
Delipified BSA[d]	0.4	-	0.19
Delipified BSA	0.4	0.04	0.90
Delipified BSA	0.4	0.08	0.98
Delipified BSA	0.4	0.20	0.01
Delipified HSA	0.4	-	0.19
Delipified HSA	0.4	0.04	0.93
Delipified HSA	0.4	0.08	1.00
Delipified HSA	0.4	0.20	0.17

[a]Final concentration of serum as vol/vol, albumin concentrations indicate protein content. The supplements were added to HPYG broth.

[b]Oleate was added to albumin stock solutions 24 hours prior to the test. Amounts represent final concentrations in HPYG broth containing 25 µg/ml of TPP.

[c]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 3rd transfer.

[d]BSA = bovine serum albumin; HSA = human serum albumin.

oleate per mg of albumin) in HPYG broth gave optimal growth with either HSA or BSA (1.00 O.D. and 0.98 O.D. respectively). Higher concentrations of oleate were inhibitory. The optimum protein to lipid weight ratio was calculated to be 50:1. Neither increased (1%) nor decreased (0.2%) final concentrations of delipified albumin plus 0.02 mg of oleate per mg of albumin gave any better growth than did 0.4% delipified albumin with added oleate. No growth differences were observed in cultures grown in HPYG broth supplemented with either human or bovine serum albumin.

Starch was used as a supplement with various oleate concentrations to determine if another compound could replace the albumin requirement (Table 31). Higher concentrations of starch (2%) with oleate gave no better growth of T. denticola than did the 1% starch-oleate cultures.

Thiamine pyrophosphate binding. The concentration of TPP added to albumin stock solutions was critical for optimal growth of T. denticola in HPYG broth containing albumin-oleate. Thiamine pyrophosphate was added to 2, 10, and 20% wt/vol albumin stock solutions, containing 0.02 mg of oleate per mg of albumin, to yield 33 µg/ml final concentration of TPP in the stock solutions. These stock solutions were each added to basal HPYG broth to a final concentration of 0.2% albumin- 0.03 mg/ml oleate. HPYG broth, containing albumin and oleate, supplemented from the 2% stock solution supported good growth (0.89 O.D.), whereas cultures supplemented from the 10 and 20% albumin-oleate stock solutions did not grow as well (Table 32). These results indicated that albumin may bind TPP making it unavailable to T. denticola for growth.

Two percent solutions of albumin that contained known amounts of TPP (50 µg/ml) were subjected to ultrafiltration by centrifugation

TABLE 31. Growth of Treponema denticola strain T-32 using starch as a substitute for the albumin requirement.

Supplement	Final concentration (%)[a]	Oleate concentration (mg/ml)[b]	Optical density[c]
Albumin (bovine)	0.4	-	0.19
Albumin (bovine)	0.4	0.06	0.93
Starch	0.5	-	0.21
Starch	0.5	0.03	0.42
Starch	0.5	0.06	0.45
Starch	0.5	0.15	0.00
Starch	1.0	-	0.25
Starch	1.0	0.03	0.50
Starch	1.0	0.06	0.56
Starch	1.0	0.09	0.01

[a]Albumin concentrations indicate protein content and starch concentrations are wt/vol. The supplements were added to HPYG broth.

[b]Oleate was added to stock solutions 24 hours prior to the test. Amounts represent final concentration in HPYG broth.

[c]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 3rd transfer.

TABLE 32. Effect of various concentrations of thimaine pyrophosphate in albumin solutions on the growth of Treponema denticola strain T-32A

Albumin stock solution (%)[a]	Thiamine pyrophosphate (µg/ml)[b]	Optical density[c]
2.0	33	0.89
2.0	250	0.91
10.0	33	0.55
10.0	250	0.82
20.0	33	0.44
20.0	250	0.92

[a]Bovine albumin stock solutions were added to HPYG broth to a final concentration of 0.2% and contained sodium oleate sufficient to yield a final concentration of 0.03 mg/ml.

[b]Final concentration in albumin stock solutions.

[c]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 3rd transfer.

through Amicon Centriflo CF 25 (25,000 molecular weight exclusion) membrane filter cones and tested for TPP content by a thiochrome assay to determine how much TPP binds to albumin. Both human and bovine serum albumin bound TPP (Table 33), but starch and agar did not. One mg of BSA bound 0.5 µg of TPP and one mg of HSA bound 0.6 µg of TPP. The binding assays were repeated using 2% albumin that contained 0.3 mg/ml of oleate to determine if added oleate effected the TPP binding. No appreciable change in the amount of TPP bound to albumin was detected when oleate was added. The rate of TPP binding was investigated in albumin and albumin-oleate solutions. Thiamine pyrophosphate was found to reach maximum binding four-times faster in the albumin-oleate solution than in albumin alone.

The tightness of TPP binding was determined by washing the Amicon Centriflo CF 25 membrane filter cone that contained the albumin-TPP retentate. The retentates were restored to the original volume in the CF25 cones with distilled water, allowed to equilibrate for up to 24 h, and the solution recentrifuged with the same membrane filter cones. This wash was repeated twice and TPP concentrations were determined in all of the ultrafiltrates and in the final retentate. Binding of TPP to albumin (or to albumin-oleate) appeared to be very tight because TPP was not detected at the µg level in any ultrafiltrates and there was no detectable loss of TPP in the final washed retentates. The limits of detection of thiamine pyrophosphate were one to two µg/ml with the thiochrome assay.

The fatty acids of beta globulin were shown (Table 16) to be qualitatively similar to those of alpha globulin, but the albumin content

TABLE 33. Binding of thiamine pyrophosphate (TPP) by various compounds[a]

Compound	Concentration (mg/ml)	Oleate (0.3 mg/ml)[b]	TPP bound (μg/ml)
Bovine albumin	20	-	11.0
Bovine albumin	20	+	10.0
Human albumin	20	-	13.0
Human albumin	20	+	12.0
Starch	50	-	0.0
Agar	1.0	-	< 0.5

[a]Binding was tested, after 24 hours incubation of the compound and TPP, by ultrafiltration with Centriflo CF25 (25,000 M.W. cutoff) membrane filters. TPP was detected in ultrafiltrates and retentates by a thiochrome assay.

[b]Oleate, when added, was allowed to bind to albumin 24 hours before adding TPP.

of beta globulin was less than 10% as detected by the methyl orange dye binding assay. Because beta globulin plus albumin (from a 20% stock solution containing 33 µg/ml of TPP) was shown previously (Table 9) to support only limited growth (0.10 O.D.), excess TPP (125 µg/ml in a 10% albumin stock solution) was added to 0.2% beta globulin-1.0% albumin supplemented HPYG broth to determine if the limited growth was due to a lack of unbound TPP. The beta globulin-albumin medium, containing excess TPP, supported good growth of T. denticola (0.78 O.D.). Growth was nearly as good as in the 0.4% alpha globulin control (0.85 O.D.). Limited growth of T. denticola was observed in HPYG broth containing excess TPP and either 0.2% beta globulin (0.15 O.D.) or 1.0% albumin (0.25 O.D.). Media containing excess TPP and supplemented with 0.2% gamma globulin and 1.0% albumin supported only limited growth (0.22 O.D.) because no long chain fatty acids were detected in gamma globulin.

Minimal growth of T. denticola. Because minimal growth (0.15 to 0.25 O.D.) of T. denticola was always observed in control HPYG broth, it was necessary to determine the reason for this slight growth. Minimal growth was observed in HPYG broth supplemented with either 0.4% delipified alpha globulin or 0.4% delipified albumin (Table 34) and in HPYG broth containing only 10 µg/ml of TPP.

The lipids present in HPYG broth were investigated to determine if they play a role in supporting minimal growth of T. denticola. Individual HPYG components were delipified then the medium was prepared and tested for growth supporting ability. Delipified HPYG broth, that contained 10 µg/ml of TPP, did not support growth (Table 34). Gas liquid chromatography of the extracts of the HPYG components showed that the

TABLE 34. Minimal growth requirements of Treponema denticola strain T-32A.

Medium[a]	Supplement[b]				Optical density[c]
	TPP	VFA	Delip-albumin	Oleate	
HPYG	-[d]	-	-	-	0.00
HPYG	+	-	-	-	0.20
Delip-HPYG	-	-	-	-	0.00
Delip-HPYG	+	-	-	-	0.00
HPYG	+	+	-	-	0.40
Delip-HPYG	+	+	-	-	0.37
HPYG	+	+	+	-	0.46
HPYG	+	-	+	+	0.92
Delip-HPYG	+	-	+	+	1.00

[a]HPYG = heart infusion peptone yeast extract glucose broth; delip-HPYG = lipids extracted by chloroform-methanol.

[b]Supplements added to medium; TPP = thiamine pyrophosphate added to a final concentration of 10 µg/ml; VFA = volatile fatty acid solution containing acetic, n-butyric, n-valeric, isobutyric, and isovaleric acids was added (0.2 ml); delip-albumin = delipified albumin added to a final concentration of 0.4% wt/vol; oleate added to a final concentration of 0.06 mg/ml.

[c]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 5th transfer.

[d]+ means compound added; - means no addition.

medium contained acetic, n-butyric, and lactic acids. Trace amounts of propionic, isobutyric, isovaleric, n-valeric, isocaproic, n-caproic, and succinic acids were detected. Long chain fatty acids were not detected. A short chain volatile fatty acid solution containing acetic, n-butyric, n-valeric, isobutyric, and isovaleric acids was added (0.2 ml) to either HPYG broth or delipified HPYG broth to determine if short chain fatty acids could enhance the growth of T. denticola above minimal levels. Thiamine pyrophosphate was added to each medium to a final concentration of 10 µg/ml. Cultures of T. denticola grew better (0.40 O.D.) in the short chain fatty acid-TPP supplemented medium (either HPYG or delipified HPYG) than in HPYG broth with only TPP (0.20 O.D.). However, abnormal treponeme cell morphology was observed (many straight forms and coccoid bodies) in these cultures. The fatty acid solution (0.2 ml) also was added to HPYG broth supplemented with 0.4% delipified albumin to determine if short chain fatty acids could substitute for the oleate requirement. Growth of T. denticola in HPYG broth with short chain fatty acid and delipified albumin was not nearly as good as growth in the albumin-oleate control. Neither higher nor lower concentrations of the short chain fatty acid solution gave any better growth of T. denticola whether albumin was present or not.

Growth of other strains of oral treponemes in an albumin-oleate medium. Five strains of T. denticola and three strains of T. vincentii were used to determine if albumin and oleate were general requirements for these two oral species. All strains of T. denticola and T. vincentii were able to grow in HPYG broth containing 0.4% bovine serum albumin, 0.06 mg/ml sodium oleate, and 12.5 µg/ml thiamine pyrophosphate

(Table 35). A slightly suboptimal oleate concentration was used to ensure that there was no fatty acid inhibition.

Treponema denticola activity on alpha globulin proteins. Whole cells of T. denticola, grown in a medium containing BSA-oleate, were harvested by centrifugation then added to a 0.4% human alpha globulin solution and incubated at 37°C for four days. Disappearance of alpha globulin proteins was determined by sedimention of the cells by centrifugation and filteration of the cell treated alpha globulin. The treated alpha globulin was assayed for decreases in individual protein content by rocket immunoelectrophoresis. The sedimented whole cells were washed in Tris-$MgCl_2$ buffer and assayed for alpha globulin proteins attached to the treponeme cell surface by coagglutination with staphylococci bearing protein-A and by ELISA.

The peak heights of each sample were compared to the peak heights of control gels with various concentrations of untreated alpha globulin. The concentrations of several alpha globulin proteins decreased by treatment with whole cells (Table 36). The limit of detection of the rocket immunoelectrophoresis technique was approximately ±5%. The same results were obtained by growing T. denticola in a medium containing 0.4% alpha globulin and testing the spent culture medium by rocket immunoelectrophoresis for the amount of each individual protein that remained. Uninoculated HPYG broth containing 0.4% alpha globulin was used as the control.

The method of Alderete and Baseman (1) was used to determine if the missing alpha globulin proteins were bound to T. denticola cells. They reported that host serum proteins were adsorbed to the cell surface

TABLE 35. Growth of *Treponema denticola* strains and *Treponema vincentii* strains using an albumin-oleate medium[a]

Organism	Strain	Optical density[b]	
		Serum	Albumin-oleate
T. denticola	T-32A	1.50	1.00
	ST-10	1.47	1.00
	N-39	1.18	1.05
	D3A-5	0.76	0.51
	D3A-9	0.78	0.39
T. vincentii	N-9	1.08	0.97
	D7B-1	0.48	0.33
	D3A-1	1.18	0.72

[a]Sodium oleate (0.6 mg/ml) added to 4% wt/vol delipified albumin stock solution 24 hours prior to adding 1:10 to HPYG broth. Whole serum (final concentration 10% vol/vol) added to basal HPYG broth. Thiamine pyrophosphate (125 µg/ml) was also added to the stock solution.

[b]Average optical density of duplicate cultures read at 540 nm after 8 days incubation of the 3rd transfer.

TABLE 36. Activity of Treponema denticola strain T-32A cells toward human alpha globulin proteins[a]

Protein	Protein detected by rocket immunoelectrophoresis (%)[b]	Proteins adsorbed to treponeme cells[c]	
		Coagglutination	ELISA
Albumin	90	+	N.D.[d]
Alpha 1-trypsin inhibitor	100	-	N.D.
Alpha 1 B-glycoprotein	80	+	N.D.
Alpha 1-antichymotrypsin	< 10	+	+
Inter alpha trypsin inhibitor	0	+	+
Apha 2 Zn-glycoprotein	100	-	N.D.
Alpha 2-macroglobulin	100	-	N.D.
Haptoglobin	20	-	-
Ceruloplasmin	100	+	N.D.
Gc globulin	55	+	+

[a]Treponema denticola cells grown in bovine serum albumin-oleate medium, sedimented by centrifugation, washed with Tris-$MgCl_2$ buffer, added to 0.4% alpha globulin and incubated four days. Cells were harvested by centrifugation and washed with Tris-$MgCl_2$ buffer.

[b]Values are percent of alpha globulin protein remaining after incubation with whole cells when compared to an untreated alpha globulin control.

[c]Alpha globulin proteins detected on T. denticola cells by coagglutination using protein A bearing staphylococci and by enzyme linked immunosorbent assay using alkaline phosphatase. + indicates protein detected, - indicates no protein detected.

[d]N.D. = not done.

of Treponema pallidum. Washed cells of T. denticola, harvested from a 0.4% alpha globulin solution after four days of incubation, were assayed for cell surface-associated alpha globulin proteins. Several alpha globulin proteins were found to be avidly bound to T. denticola cells (Table 36) as detected by coagglutination with staphylococci cells bearing protein-A cells that contained IgG antibody against the specific alpha globulin proteins. Whole cells incubated in Tris-$MgCl_2$ buffer were used as a negative control. The proteins that gave a positive coagglutination reaction also gave a positive reaction with the ELISA test. Albumin, ceruloplasmin, alpha 1 B-glycoprotein, alpha 1-antichymotrypsin, inter alpha trypsin inhibitor, and Gc globulin were found to be avidly bound to the surface of T. denticola cells. Haptoglobin was the only protein that was lost from the alpha globulin and was not bound to the cells.

DISCUSSION

Alpha globulin requirement. Alpha globulin has been reported to replace serum or ascitic fluid for the growth of Treponema denticola and Treponema vincentii (Austin, M.S. thesis, 56,135). Socransky and Hubersak (135) and Austin reported that serum albumin and other serum globulin fractions were incapable of supporting growth of T. denticola. Austin reported similar results with T. vincentii. Serum albumin replaced whole serum for the growth of Treponema phagedenis (Austin, M.S. thesis, 33,91,111,126,146), while the serum globulins did not support growth (Austin, M.S. Thesis, 91). Albumin also replaced serum for the growth of Treponema strain S-69, an oral isolate (149). Results from the present investigation confirmed earlier reports that T. denticola and T. vincentii grew in a medium supplemented with alpha globulin (Miles Laboratories, Cohn fraction IV). These two oral species did not grow in a medium containing either serum albumin (Cohn fraction V), beta globulin (Cohn fraction III), or gamma globulin (Cohn fraction II).

Some characteristics of the active growth factor in alpha globulin were reported by Socransky and Hubersak (135). The active factor was non-dialyzable and not excluded by Sephadex G-100 and Sephadex G-200. The active factors in the present study were non-dialyzable through Spectrapor dialysis tubing with a molecular weight cutoff of 12-14,000 and did not pass through an Amicon ultrafiltration membrane filter with a molecular weight exclusion of 300,000. These results were due to the lipids being part of the lipoprotein component (5-20 x 10^6 molecular weight) of alpha globulin. Minimal growth in medium supplemented with

the ultrafiltrates was due to the passage of excess TPP, which was not bound to albumin, through the Amicon filters. In contrast to the report by Socransky and Hubersak that the factor was destroyed by heat at 80°C for 30 min, the factors in alpha globulin were found in this study to be stable at pH 7.0 to autoclaving at 121°C for 15 min. Alpha globulin can be sterilized by autoclaving. The growth promoting activity of alpha globulin was destroyed by either trypsin or lipase treatment which suggested that a protein and a lipid are required for growth of T. denticola and T. vincentii. Treponema phagedenis (67,111,126) and the leptospires (34,35,68,70,73,126,162) have been reported to require both albumin and either a lipid or lipids for growth.

This study was to determine the growth factors present in alpha globulin that are required for the growth of T. denticola and T. vincentii. The results show that the commercial alpha globulin used in this study provides both serum albumin and oleic acid that were required for the growth of these two oral treponemes. Thiamine pyrophosphate was shown to be required for growth; however, TPP was also shown to be tightly bound to albumin and thus not available as a growth factor for the oral treponemes. Therefore, sufficient concentrations of TPP are required in the medium to saturate the binding sites on albumin and also to provide additional TPP for growth of these treponemes.

Lipid requirement. Extraction of lipids from serum or serum fractions, before use as supplements in basal media, has been reported to reduce the growth-promoting activity for T. phagedenis (67,111) and for the leptospires (35,68,162). When the lipids were added to the extracted material, growth of these organisms was restored. Lipid extraction of

alpha globulin greatly reduced its growth-promoting activity for T. denticola in this study. Good growth of T. denticola was restored by the addition of the extracted lipids to the delipified alpha globulin which suggested that both the protein and lipid were required for growth. These results also indicated that the delipification procedure was gentle enough not to destroy the growth factors in alpha globulin. The fatty acids in alpha globulin (palmitic, palmitoleic, stearic, oleic, and linoleic acids) detected in this study reflect those found in serum (43,129).

To examine the lipid requirement, TEM-4T was chosen as a source of fatty acids to replace the lipids extracted from alpha globulin that were required for growth of T. denticola. This compound was chosen because it has been reported to stimulate the growth of the Reiter strain of T. phagedenis (114) and has been used as a routine supplement for growth of T. vincentii in media containing either serum or alpha globulin (Austin, M.S. Thesis). In this study, TEM-4T replaced the lipids of alpha globulin that were required for growth of T. denticola when the medium also was supplemented with delipified alpha globulin. The fatty acids in TEM-4T were found in this study to be similar to those found in alpha globulin. Long chain fatty acids are required for growth of T. phagedenis (67,111,126), the leptospires (35,68,70,73,124, 141), and the borreliae (81,113). Motility of Treponema pallidum is prolonged by the addition of long chain fatty acids to a medium containing alblumin (97). Oleic acid (cis-18:1Δ9), when added to a medium containing ascitic fluid, enhanced the growth of T. vincentii, but oleic acid alone did not substitute for ascitic fluid

(104). In the present study either sodium oleate or sodium elaidate (trans-18:1Δ9)) added to either delipified alpha globulin or delipified albumin supplemented media, supported growth of T. denticola and T. vincentii. Growth was not as good in the medium containing elaidate as it was in the oleate medium. Growth of these two oral species in the medium containing oleate was nearly as good as growth in a medium containing serum. Other saturated and unsaturated fatty acids, including vaccenic acid (cis-18:1Δ11) which differs from oleate only in the double bond position, did not support growth. These results indicate that T. denticola and T. vincentii have a strict growth requirement for a mono-unsaturated fatty acid of 18 carbons with the double bond in the Δ9 position. There is also a steric preference because the cis form of the C18:1Δ9 acid is much more active than the trans form. Treponema denticola and T. vincentii have a simpler fatty acid nutritional requirement than does the Reiter strain of T. phagedenis which requires a pair of fatty acids, one saturated of at least 14 carbons and one unsaturated of at least 15 carbons with one, two, or three double bonds (67). Elaidic acid substituted for this pair of fatty acids. A similar observation was made for Mycoplasma strain Y (117) and for two parasitic leptospires (73). Oleic acid has been reported to enhance the growth of some Lactobacillus species (5,62,83,167,169), Corynebacterium diphtheriae (25), Mycobacterium tuberculosis (30,31,32, 53), Clostridium tetani (36), and Erysipelothrix rhusiopathiae (63).

Tween 80 (polysorbitan monooleate) was used to confirm that the lipid requirement for growth of T. denticola was oleic acid. Tween 80 has been reported to be an excellent oleic acid substitute for growth

of Mycobacterium tuberculosis (28,31,32) and for growth of the leptospires in a medium containing albumin (34,35,70,71,164). Recently the leptospires have been cultivated in protein-free media containing Tween 80 detoxified either by anion exchange chromatography (142) or by charcoal treatment (16). Long chain fatty acids are often toxic to bacteria (78). Tween 80 was found, in the present study, to be the only Tween compound capable of replacing the oleate requirement for optimal growth of T. denticola in a medium containing delipified alpha globulin. Charcoal detoxified Tween 80 (16), in protein free HPYG, did not support growth of T. denticola in this study. Delipified alpha globulin was still required to detoxify the fatty acid. Thus, T. denticola appears to be more sensitive to the toxicity of long chain fatty acids than are the leptospires.

Short chain fatty acids were reported to be required for growth of Treponema succinifaciens (27), Treponema bryantii (143), Treponema strain PR-7 (132), and the oral isolates Treponema macrodentium (50,136), Treponema strain HO-19, and Treponema strain HO-27 (94). In the present study, minimal growth of T. denticola was observed in HPYG broth that contained either delipified alpha globulin, delipified albumin, or TPP. HPYG broth was found to contain short chain fatty acids. Treponema denticola failed to grow in delipified HPYG broth that was supplemented with only TPP indicating that the short chain fatty acids are required for minimal growth. Short chain fatty acids were found to support only limited growth of T. denticola when added to either unsupplemented HPYG broth containing TPP or delipified albumin supplemented HPYG broth containing TPP. Many straight cells and coccoid bodies were observed

in these cultures which suggested suboptimal growth conditions. Short chain fatty acids were not required to initiate growth since maximal growth of T. denticola was achieved in delipified HPYG broth supplemented with delipified albumin, oleate, and TPP.

Protein requirement. The results suggested that alpha globulin may also be required for some other purpose such as the need for a specific protein. Treponema denticola and T. vincentii ferment amino acids (17,56,89). Treponema denticola was shown in this study to have surface-associated proteolytic activity toward casein. Dialysis of alpha globulin that had been exposed to whole cells of T. denticola, in this study, failed to show that either a peptide or an amino acid required for growth was released from the alpha globulin by the proteolytic activity of T. denticola cells. These results indicated that a protein in alpha globulin was responsible only for detoxifying oleic acid and not for supplying a peptide nutrient. Both the protein and lipid portions of alpha globulin were required for optimal growth of T. denticola.

The protein requirement for growth of T. denticola was investigated by determining the proteins present in the commercial alpha globulin used. Albumin was found to be 50% of the total protein content of the commercial alpha globulin. Albumin was reported to be selectively removed from the other serum proteins by affinity column chromatography using a Sepharose-blue dextran conjugate which allowed 94% of the alpha 1-globulins to pass through (154,163). Albumin was removed from the commercial alpha globulin in the present study with Bio-Rad Affi-Gel Blue affinity chromatography. The alpha globulin proteins eluted from

the column as two fractions. Albumin was desorbed from the column, as fraction 3, with 1.4 M NaCl. Fraction 3, which contained albumin, and fraction 1, which contained alpha globulin proteins, were both required as supplements in HPYG broth for the growth of T. denticola. Fraction 1 was required because it was the only fraction found to contain oleic acid. Fraction 1 alone did not support growth and trypsinization of the proteins of fraction 1 did not cause a decrease in growth when intact albumin was present in the culture medium. No function for the proteins in fraction 1, other than a supply of oleic acid, was determined.

Serum albumin (human or bovine) replaced the fraction 3 requirement for growth of T. denticola in HPYG broth containing fraction 1. Intact albumin was required because trypsin hydrolysis of HSA destroyed the growth-promoting activity. Intact albumin has been reported to be required for growth of the leptospires (140), M. tuberculosis (28,31), and the Reiter strain of T. phagedenis (145).

Serum albumin has been shown to bind fatty acids with oleic acid reported to bind the tightest (4,45,138,139). Human serum albumin has the greatest affinity for binding oleate, of the fatty acids tested (119). Albumin binds to the carboxyl group of long chain fatty acids (139) and the carboxyl group has been suggested as necessary for the antibacterial activity of long chain fatty acids (78). A molar ratio of serum albumin to oleate of 1:4 was reported to be of critical importance to detoxify oleate for growth of the Reiter strain of T. phagedenis (111). Higher concentrations of oleate were inhibitory to T. phagedenis. A molar ratio of albumin to fatty acid of 1:6 (68) and a weight ratio

of albumin to oleate of 100:1 (35) were found to support maximal growth of the leptospires. The weight ratio of protein to oleic acid was determined in this investigation with either delipified alpha globulin or delipified serum albumin and various concentrations of sodium oleate. The commercial serum albumin was delipified because it has been reported that serum albumins contain small amounts of fatty acids, including oleate (24,119,138). Weight ratios of delipfied alpha globulin to sodium oleate of 100:1 and delipified albumin to sodium oleate of 50:1 gave maximal growth of T. denticola. The weight ratios obtained are equivalent because albumin is 50% of the total protein content of the commercial alpha globulin. The albumin to oleate weight ratio obtained in this investigation was lower than that for the leptospires because T. denticola required only a single fatty acid and the leptospires are reported to require a pair of fatty acids (73).

Starch also has been reported to adsorb long chain fatty acids (90,123). In this study, starch plus oleate supported only limited growth of T. denticola because starch may not bind oleate as tightly as does albumin. Optimal growth was obtained only in media containing oleate and albumin.

Commercial alpha globulin was found to contain two required growth factors for T. denticola and T. vincentii. The first was oleic acid which was ester-linked and bound to alpha globulin lipoprotein. The treponemes, which have lipase and phospholipase B activity (Trevathan, M.S. thesis), may release the oleic acid for use by the cells. The second, albumin present in commercial alpha globulin preparations, functions to bind the released oleic acid and other fatty acids, thus

detoxifying the medium. Slow dissociation of oleate from the albumin makes the oleate available to the treponemes for growth.

Fatty acid composition of whole cells. The cellular fatty acid composition of Treponema minutum, T. refringens, T. phagedenis, T. denticola, and T. vincentii grown in media containing serum has been reported to reflect the fatty acids found in serum (101,159,166). The cellular fatty acids of T. denticola have been shown also to contain myristic acid (129,166) and pentadecanoic acid (129). The cellular fatty acids of Borrelia hermsi, grown in Kelly's serum medium (80), also reflect those of serum (92). Livermore and Johnson (93) used a lipid free albumin supplemented medium containing added oleate and palmitate to show that the cellular fatty acids of Treponema scoliodontum, T. phagedenis, T. denticola, and T. vincentii were oleate and palmitate. Treponema denticola and T. vincentii contained small amounts of myristic acid and T. denticola also contained pentadecanoic acid (93). Media containing either the required pair of fatty acids or elaidic acid were used to show that the cellular fatty acids of the Kazan 5 (67,71) and Reiter strains (67) of T. phagedenis were those that were added to the culture medium. The treponemes studied were concluded to be incapable of de novo synthesis, chain length modification, saturation, and unsaturation of fatty acids (71,93). The two oral treponemes, strain HO-19 and strain HO-27, that required short chain fatty acids for growth, were reported to be capable of synthesizing normal and branched chain saturated fatty acids of 14 to 16 carbon chain length (94). The leptospires are capable of beta-oxidation and unsaturation of long chain fatty acids (11,55,72), but incapable of de

novo synthesis (150) and chain elongation (72,150) of fatty acids. Borrelia hermsii (92) and T. pallidum (122) directly incorporated serum fatty acids unaltered into their cellular lipids. Treponema pallidum cells isolated from rabbit testes contained cellular fatty acids that reflected those of the rabbit tissue (98).

The results from this study indicated for the first time that T. denticola, in contrast to previous reports (71,93), may be capable of chain modification of oleic acid. Treponema denticola cells grown in an oleate supplemented medium contained myristic, pentadecanoic, and palmitic acids as the main cellular fatty acids. Smaller amounts of oleic acid, eicasadienoic acid (20:2), and an unidentified fatty acid (retention time 88 min.) also were detected. Thus, T. denticola appears capable of saturation, unsaturation, chain shortening, and chain elongation of oleic acid. Limited growth of T. denticola with short chain fatty acids suggested that this organism may have some de novo synthetic ability of fatty acids to allow the cells to survive until a long chain fatty acid source is available. The true nature of the fatty acid modification and synthetic capabilities of T. denticola would best be identified in future investigations by using radioactively labeled fatty acids.

Thiamine pyrophosphate requirement. Thiamine pyrophosphate (TPP) was required for growth of the oral species T. vincentii, T. denticola, T. scoliodontum, T. macrodentium, and Treponema strain S-69 (Austin, M.S. thesis, 48,104,105,129,135,136,149). Thiamine is required for growth of the Reiter strain of T. phagedenis (91,148) and for the leptospires (141,164). Albumin has been reported to bind a wide variety

of compounds (37, 160), including those that contain phosphate (161). The results of this study indicated that the concentrations of TPP in albumin stock solutions were of critical importance for achieving optimal growth of T. denticola. Both BSA and HSA tightly bound TPP. When oleate was added to the albumin stock solutions there was no increase in the amount of TPP bound to albumin but the rate of TPP binding was enhanced. This agrees with a report by Wosilait and Ryan (170) that oleate enhanced the binding of drugs to HSA.

Poor growth in albumin-oleate supplemented media in early experiments, using a 20% albumin stock solution that contained 33 µg/ml of TPP, was due to the unavailabilty of free TPP in the medium because it was all bound to albumin. The 10 and 20% stock solutions, containing 33 µg/ml of TPP, were calculated to be capable of binding 50 and 100 µg/ml of TPP respectively. Minimal growth of T. denticola may have been due to the slow release of small amounts of TPP which were below the limits of detectability of the thiochrome assay. Treponema vincentii has been reported to require only nanogram quantities of TPP for growth (Austin, M.S. thesis).

The requirement for free TPP in this study also was indicated by determining that beta globulin plus albumin supported growth only when excess TPP was added. The fatty acids of beta globulin were shown to be qualitatively similar to those of alpha globulin (both contained oleate), but beta globulin alone did not support good growth of T. denticola because less than 10% of its total protein content was found to be albumin. Therefore, beta globulin plus added albumin should have supported growth of T. denticola, but when albumin from a 20% stock

solution containing 33 µg/ml of TPP was used, growth was poor. All of the TPP was bound to the albumin in the stock solution and unavailable to T. denticola for growth. Good growth was obtained when excess TPP was added to the albumin stock solution before adding it to beta globulin supplemented HPYG broth. Gamma globulin plus albumin did not support good growth of T. denticola in HPYG broth regardless of the concentration of TPP because no fatty acids were detected in gamma globulin. Thus, excess non-albumin bound TPP is required for optimal growth of T. denticola.

Proteolytic activity of whole cells. The Reiter strain of T. phagedenis was reported to have peptidase activity (152) and Hampp et al. (147) reported that the oral treponemes studied had proteolytic activity. Omata and Hampp (109) noted a decrease in the protein content of the culture media used for growth of either T. vincentii or a small unidentified oral treponeme. Omata and Hampp also suggested that T. vincentii has peptidases. An extracellular protease, with fibrinolytic activity that degraded gelatin, has been isolated from T. denticola culture supernatants (107). Treponema denticola strain T-32A cells were shown in this study to have proteolytic activity toward casein. Very little proteolytic activity was demonstrated in the T. denticola culture supernatants. The proteolytic activity of T. denticola cells as measured by polyacrylamide gradient gel electrophoresis, double immunodiffusion, and rocket immunoelectrophoresis may have caused the decreases observed in the amounts of several alpha globulin proteins after incubation of alpha globulin with T. denticola cells. These decreases could have been due either to cellular proteolytic activity

or to the proteins being tightly bound to the surface of the cells. Coagglutination, with staphylococci bearing protein-A, and ELISA of T. denticola cells that had been incubated in an alpha globulin solution, indicated in this investigation that albumin, alpha 1-antichymotrypsin, alpha 1 B-glycoprotein, inter alpha trypsin inhibitor, Gc globulin, and ceruloplasmin were tightly bound to the T. denticola cells. Haptoglobin did not bind to the cells; however, rocket immunoelectrophoresis results indicated a loss of this protein. Thus, this indicated that whole cells of T. denticola have proteolytic activity toward haptoglobin. Ceruloplasmin was found to adhere to T. denticola cells, but there was no detectable decrease in its content in alpha globulin presumably becuase only small amounts were bound at the limits of detection of the rocket immunoelectrophoresis. Albumin, alpha 1-antichymotrypsin, inter alpha trypsin inhibitor, Gc globulin, and alpha 1 B-glycoprotein were found to decrease in quantity and also were found tightly bound to T. denticola cells. The protease inhibitors (alpha 1-antichymotrypsin and inter alpha trypsin inhibitor) may have been bound as inhibitors to the outer membrane proteolytic enzymes of T. denticola.

A further examination of the role of tightly bound serum proteins might be beneficial to determine the role of T. denticola and other oral treponemes in periodontal disease. Alderete and Baseman (1) observed that albumin, alpha 2-macroglobulin, ceruloplasmin, transferrin, immunoglobulin G (IgG), immunoglobulin M (IgM), and C3 were host serum proteins that were strongly associated with the outer envelope of T. pallidum. No other proteins were tested. The presence of transferrin, IgG, IgM, and C3 on T. denticola cells was not determined in this study

because these were not found in commercial alpha globulin. The consequences of this protein attachment to oral treponeme cells may be either to inhibit phagocytosis of the treponeme cells or to act as an altered antigen to produce autoantibodies against the attached host proteins. These autoantibodies may act to destroy the proteins of the complement system that may be present and active against treponemes in the oral microenvironments that these treponemes inhabit.

SUMMARY

Alpha globulin replaced whole serum for the growth of Treponema denticola and Treponema vincentii. The growth factors in alpha globulin were oleic acid (cis-18:1Δ9) and albumin. Either bovine serum albumin and sodium oleate or human serum albumin and sodium oleate could replace alpha globulin for growth of T. denticola and T. vincentii. The two oral treponemes had a specific growth requirement for oleate, an unsaturated fatty acid of 18 carbon chain length with a single double bond in the Δ9 position. There also was a steric preference for the cis form of the fatty acid. Albumin, either in the alpha globulin or alone, acted to detoxify the oleic acid and render the medium suitable for growth of these two oral treponemes. Albumin also tightly bound thiamine pyrophosphate (TPP) making the TPP unavailable to the treponemes for growth. Sufficient concentrations of TPP were required to saturate all of the albumin binding sites and to have free TPP available for growth of the treponemes. Minimal growth was obtained in a medium containing short chain fatty acids and TPP. Treponema denticola was capable of chain shortening, chain elongation, saturation, and unsaturation of oleic acid.

Proteolytic activity was detected on the surface of T. denticola cells, but not in spent culture media. Several alpha globulin proteins were found to be lost or decreased in concentration from stock alpha globulin solutions that had been incubated with whole treponemal cells. Albumin, alpha 1 B-glycoprotein, alpha 1-antichymotrypsin, inter alpha trypsin inhibitor, ceruloplasmin, and Gc globulin were found to be avidly bound to the surface of T. denticola cells. Haptoglobin was not

bound to the cells but had decreased in concentration from the treated alpha globulin solution due to the proteolytic activity of T. denticola cells.

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APPENDIX

I. Protein determination by a dye binding method (Bio-Rad Laboratories, Richmond, Ca.).

Reagent	Protein[a] Standard	Blank	Test
Working dye reagent[b]	5.0 ml	5.0 ml	5.0 ml
Bovine gamma globulin	0.1	-	-
Protein sample	-	-	0.1
Distilled water	-	0.1	-

[a]Lyophilized Bio-Rad bovine gamma globulin standard was rehydrated with 20 ml of distilled water to yield 1.4 mg/ml of protein. The standard was serially diluted until the OD_{595} was 0.1 to 1.0.

[b]One part Bio-Rad dye concentrate added to 4 parts distilled water and filtered through Whatman No. 1 filter paper (Whatman, Inc., Clifton, N.J.).

The reaction mixture was incubated at room temperature for 5-60 min. Following incubation the O.D. at 595 nm was measured using a Gilford Model 250 recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The protein content of unknown solutions (mg/ml) was calculated from the standard curve.

II. Albumin determination. Dye binding method of Kachmar (77).

Reagents	Albumin[a] standard	Blank	Test
Methyl orange[b]	10.0 ml	10.0 ml	10.0 ml
Human albumin	0.2	-	-
Test sample	-	-	0.2
Distilled water	-	0.2	-

[a]A human albumin standard containing 1.0 to 6.0 mg/100 ml of distilled water.

[b]Prepared by adding an aqueous 0.1% (wt/vol) methyl orange solution to 0.055 M citrate buffer, pH 3.5, until the O.D. at 540 nm was 0.78 to 0.85.

The reaction mixture was incubated at room temperature for 20 min. Following incubation, the O.D. at 540 nm was measured using a Bausch and Lomb spectronic 20 spectrophotometer (Bausch and Lomb, Rochester, N.Y.). The albumin concentration (mg/ml) of the samples was calculated from the standard curve.

III. Determination of thiamine pyrophosphate (TPP). Thiochrome assay of Strohecker and Henning (151).

Reagent	Thiamine[a] Standard	Blank	Test
Working reagent[b]	1.0 ml	1.0 ml	1.0 ml
TPP	1.0	-	-
Test sample[c]	-	-	1.0
Distilled water	-	1.0	-

[a]Thiamine pyrophosphate in distilled water (1.5 to 50 µg/ml).

[b]Freshly prepared by adding 3 ml of an aqueous 1% potassium ferricyanide to 97 ml of 3.75 N NaOH.

[c]Retentates and ultrafiltrates of albumin, starch, or agar containing TPP.

The reaction mixture was incubated at room temperature for 5 min. Following incubation the O.D. at 369 nm was read in a Gilford Model 250 recording spectrophotometer. The thiamine concentration of retentates and ultrafiltrates was calculated from the standard curve. Albumin, starch, and agar not containing TPP were used as negative controls.

IV. Proteolytic assay of whole cells using casein yellow (Calbiochem-Behring Corp., LaJolla, Ca.).

Reagent[a]	Trypsin[b] Standard	Blank	Whole cells	Test culture supernatant
Casein yellow[c]	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Buffer	-	0.5	-	-
Trypsin	0.5	-	-	-
Whole cells	-	-	0.5	-
Culture supernatant	-	-	-	0.5
Color developer[d]	0.2	0.2	0.2	0.2

[a]All reagents were prepared in 50 mM Tris-5 mM $MgCl_2$ buffer (pH 7.3) except the culture supernatant.

[b]Trypsin standard contained 0.12 to 2.4 BAEE units in Tris-$MgCl_2$ buffer.

[c]Prepared by adding 1.0 g of casein yellow to 100 ml of buffer.

[d]A solution of 0.1 N NaOH in saline added after incubation.

The reaction mixture was incubated at 37°C for 1 h. Following incubation one ml of 5% (wt/vol) trichloroacetic acid was added to stop the reaction and precipitate intact proteins. The precipitate was removed by low speed (5000 rpm) centrifugation. The O.D. at 280 nm was measured followed by the addition of the color developing reagent and the O.D. at 423 nm read on a Gilford Model 250 recording spectrophotometer. Proteolytic activity in trypsin BAEE units was calculated from the standard curve. Uninoculated HPYG was used as a negative control.

V. Enzyme linked immunosorbent assay using alkaline phosphatase conjugated anti-rabbit IgG serum (Miles Laboratories, Elkhart, Ind.).

Reagent	Blank	Antiserum control	Negative control	Postive control	Test
Whole cells[a]		10 µl	10 µl		10 µl
Coating buffer[b]	150 µl	150	150		150
Specific antiserum[c]		150			150
Labeled antiserum[d]			150	150 µl	150
Enzyme substrate[e]	150	150	150	50	150

[a] *Treponema denticola* cells grown in HPYG containing BSA-oleate were suspended in either 0.4% human alpha globulin or 50 mM Tris-5 mM $MgCl_2$ buffer (negative control cells), harvested, and washed with Tris-$MgCl_2$ buffer.

[b] Prepared by adding 1.6 g Na_2CO_3, 2.93 g $NaHCO_3$, and 0.2 g NaN_3 to 1000 ml distilled water, pH 9.6.

[c] Antisera to individual human proteins (Table 6) was diluted 1:20 in wash buffer, prepared by adding 8.0 g NaCl, 0.2 g KH_2PO_4, 2.2 g $Na_2HPO_4 \cdot 7H_2O$, 0.2 g KCl, 0.2 g NaN_3, and 0.5 ml Tween 20 to 1000 ml distilled water, pH 7.4.

[d] Prepared by diluting 1:400 in wash buffer.

[e] Prepared by adding 6 mM phosphatase substrate (Sigma Chemical Co., St. Louis, Mo.) to a 0.1 M glycine-1.0 mM $MgCl_2$ buffer, pH 10.0.

Test cells and control cells (10 µl in Tris-$MgCl_2$ buffer) were added to two separate wells in a Costar serocluster EIA plate (Costar Corp., Cambridge, Mass.) containing the coating buffer, and mixed. The plates were incubated for 1 h at 37°C. After incubation, the wells were washed three times each with 150 µl of wash buffer. The specific antisera were added to each well, mixed, and the plates incubated for 2 h at 37°C then the wells washed three times each with 150 µl of wash buffer. Labeled antisera were then added to each well, mixed, and the plates incubated for 1 h at 37°C then the wells washed three times each with 150 µl of wash buffer. Enzyme substrate was added to the wells and incubated for 5 h at room temperature. Labeled antiserum and enzyme substrate were added to an empty well to serve as a positive control. Development of a yellow color indicated a positive reaction.

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Fatty acid requirement of *Treponema denticola* and *Treponema vincentii*

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Accepted December 9, 1981

VAN HORN, K. G., and R. M. SMIBERT. 1982. Fatty acid requirement of *Treponema denticola* and *Treponema vincentii*. Can. J. Microbiol. **28**: 344–350.

Treponema denticola and *Treponema vincentii* were cultured in a medium supplemented with either 0.2 or 0.4% (w/v) alpha globulin in place of serum. The active factor(s) in alpha globulin was stable at pH 7.0 to autoclaving and was nondialyzable. Extraction of lipids from alpha globulin showed that both protein and lipid, supplied by the alpha globulin, were required for maximal growth of these two oral treponemes. The lipid component was investigated by adding sodium salts of long-chain fatty acids to the basal medium supplemented with 0.4% delipified alpha globulin. The lipid component of alpha globulin was replaced by either oleic acid (*cis*-18:1 (9)) or by elaidic acid (*trans*-18:1 (9)). No other saturated or unsaturated fatty acid tested could support good growth. Tween 80 (polysorbitan monooleate) was the only Tween compound able to support maximal growth of *T. denticola*. The cellular lipids of *T. denticola*, grown with oleate in broth supplemented with 0.4% delipified alpha globulin, were extracted and analyzed by gas chromatography. The principle fatty acids were myristic, pentadecanoic, and palmitic acids. Lesser amounts of oleic acid, eicosadienoic acid, and an unidentified fatty acid (retention time, 88 min) were also detected. *Treponema denticola* appears to be capable of limited synthesis of cellular fatty acids such as myristic, pentadecanoic, and palmitic acids from oleic acid.

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Nous avons cultivé *Treponema denticola* et *Treponema vincentii* dans un milieu enrichi de 0,2 ou 0,4% (m/v) d'alpha-globuline au lieu de sérum. Le(s) composé(s) actif(s) de l'alpha-globuline résistai(en)t à l'autoclavage à pH 7,0 et n'étai(en)t pas dialysable(s). L'extraction des lipides de l'alpha-globuline a démontré que la protéine et les lipides fournis par cette dernière étaient tous deux requis pour la croissance maximale des deux tréponèmes oraux. Nous avons étudié le composant lipidique en ajoutant des sels de sodium d'acides gras à longues chaînes au milieu de base additionné de 0,4% d'alpha-globuline délipidée. Le composant lipidique de l'alpha-globuline était remplacé soit par l'acide oléique (*cis*-18:1 (9)) ou l'acide élaïdique (*trans*-18:1 (9)). Aucun autre acide gras saturé ou insaturé mis à l'essai ne pouvait supporter une bonne croissance. Le Tween 80 (polysorbitan monooléate) était le seul Tween capable de soutenir une croissance maximale de *T. denticola*. Nous avons extrait et analysé par chromatographie en phase gazeuse les lipides cellulaires de *T. denticola* après que ce dernier eut été cultivé en présence d'oléate dans un bouillon additionné de 0,4% d'alpha-globuline délipidée. Les principaux acides gras étaient les acides myristique, pentadécanoique et palmitique. Nous avons aussi détecté des quantités moindres d'acide oléique, d'acide eicosadiènoique et d'un acide gras non identifié (temps de rétention de 88 min). *Treponema denticola* semble capable d'une synthèse limitée d'acides gras cellulaires tels que les acides myristique, pentadécanoique et palmitique à partir d'acide oléique.
[Traduit par le journal]

Introduction

Some cultivable treponemes have been reported to require serum or ascitic fluid for growth (Hampp and Nevin 1959; Rose and Morton 1952; Smibert 1973, 1976). These include three oral species (*Treponema denticola*, *Treponema vincentii*, and *Treponema scoliodontum*) and three genital species (*Treponema phagedenis*, *Treponema refringens*, and *Treponema minutum*). Albumin was the only serum fraction reported to replace serum for the growth of *T. phagedenis* (Little and Subbarow 1945). Oyama *et al.* (1953) reported that the function of albumin for the growth of *T. phagedenis* was to detoxify lipids. When grown in a fatty acid free albumin containing medium, *T. phagedenis* was reported to require a pair of fatty acids, one saturated of at least 14 carbons and one unsaturated of at least 15 carbons with one, two, or three double bonds (Johnson and Eggebraten 1971). Elaidic acid (*trans*-18:1 (9)) could substitute for this pair. The leptospires have also been reported to require a pair of fatty acids (one saturated and one unsaturated) of at least 15 carbons and fatty acid free albumin for growth (Johnson and Walby 1972).

The alpha globulin fraction of serum was reported by Socransky and Hubersak (1967), Hespell and Canale-Parola (1971), and F. E. Austin (1978. M.S. Thesis, Virginia Polytechnic Institute and State University, Blacksburg, VA) to replace serum for the growth of *T. denticola*. Austin also reported that alpha globulin could replace serum for the growth of *T. vincentii*. Other

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0008-4166/82/030344-07$01.00/0

serum fractions were found to be incapable of replacing serum for good growth of these two oral treponemes.

The purpose of this investigation was to determine the lipid requirement supplied by alpha globulin for the growth of *T. denticola* and *T. vincentii*.

Materials and methods

Organism and medium

Treponema denticola strain T-32A was obtained from T. Rosebury, Washington University, St. Louis, MO. Other treponemal strains used were obtained from the following: *T. denticola* strain ST-10, E. Canale-Parola, University of Massachusetts, Amherst, MA, and *T. denticola* strain N-39 and *T. vincentii* strain N-9, Center for Disease Control, Atlanta, GA. All other strains were obtained from the culture collection of this laboratory. All strains were isolated from the oral cavity of man. Stock cultures were maintained in prereduced heart infusion peptone yeast extract glucose broth (HPYG), prepared as outlined in the *VPI anaerobe laboratory manual* (Holdeman *et al.* 1977), supplemented with either 0.4% (w/v) alpha globulin or 10% (v/v) heat-inactivated (60°C, 1 h) rabbit serum. Cultures were stored in liquid nitrogen. HPYG medium contained Bacto-heart infusion broth, 5.0 g; polypeptone (BBL), 20.0 g; Bacto-yeast extract, 10.0 g; glucose, 10.0 g; Bacto-gelatin, 10.0 g; Bacto-agar, 1.6 g; $(NH_4)_2SO_4$, 0.5 g; $NaHCO_3$, 5.0 g; resazurin, 0.001 g; L-cysteine hydrochloride, 0.5 g; salt solution, 500 mL; and distilled water, 500 mL. The salt solution contained (grams per litre) $CoCl_3 \cdot 6H_2O$, 0.04; $MnCl_2 \cdot 4H_2O$, 0.04 g; $MgSO_4$, 0.4 g; K_2HPO_4, 2.3 g; KH_2PO_4, 1.0 g; and NaCl, 2.0 g. The pH was adjusted to 7.2–7.4.

Test cultures were grown in 7 mL of HPYG broth supplemented with 1.0% (w/v) serum albumin (Cohn fraction V; bovine, human, or lapine), 0.2 or 0.4% (w/v) alpha globulin (Cohn fraction IV; human, porcine, or ovine), 0.2% (w/v) beta globulin (Cohn fraction III; human or bovine), 0.2% (w/v) gamma globulin (Cohn fraction II: human or bovine), or 10% (v/v) serum (lapine). Stock solutions of the above supplements contained filter-sterilized thiamine pyrophosphate (TPP), 33 mg/L, and L-glutamine, 660 mg/L. The gas phase was oxygen-free nitrogen.

All media were inoculated with 0.5 mL of a 4-day culture of *T. denticola* strain T-32A containing approximately 1×10^8 cells/mL. They were incubated at 37°C and optical densities at 540 nm were recorded after 3 and 8 days. Cultures were transferred on day 3, and at least five serial transfers were made for each experiment. At the time of transfer all cultures were examined by dark-field microscopy.

Preparation of serum fractions

Stock solutions of 2 or 4% (w/v) of the various globulin fractions (Miles Laboratories, Elkhart, IN) and 20% (w/v) albumin (Sigma Chemical Co., St. Louis, MO) were prepared in distilled water and the pH adjusted to 7.0 with 0.1 *N* NaOH. These were filtered through nonsterile 5.0-, 1.2-, 0.8-, and 0.6-μm membrane filters and finally sterilized with a 0.45-μm filter. Protein content was determined by the dye-binding method (Bio-Rad Laboratories, Richmond, CA).

Lipid-poor serum fractions

Five grams of the serum fractions was extracted with 100 mL of cold chloroform–methanol (2:1) for 1 h at 4°C. The chloroform–methanol was removed and the procedure repeated through four additional extractions. The delipified serum fraction was dried at 50°C under a stream of nitrogen and placed in a desiccator jar under vacuum for 24 h. Delipified globulin fractions (2 or 4%, w/v) or bovine serum albumin (20%, w/v) were dissolved in distilled water, the pH adjusted to 7.0, and filter sterilized. Protein content was determined by the dye-binding method.

Lipids

TEM-4T (Witco Chemical Co., New York, NY), a tartaric acid ester of tallow monoglycerides, was used as a source of fatty acids. Stock solutions of 2 mg/mL were prepared in distilled water, the pH adjusted to 7.0, and the solution autoclaved. Myristoleic, palmitoleic, oleic, elaidic, vaccenic, and linoleic acids were obtained from Supelco Inc., Bellefonte, PA. Stearic acid was obtained from Fisher Scientific Co. Sodium salts of each acid were made by adding 1.0 mL of 1 *N* NaOH to 10 mL of 0.1 *M* fatty acid in chloroform. The mixture was stirred for 30 min to ensure completion of salt formation. The mixture was centrifuged at low speed (5000 rpm) and the chloroform removed. The sodium salts were dried at room temperature under a stream of nitrogen. Sodium salts of myristic and palmitic acids were obtained from Pfaltz and Bauer, Stamford, CT. Stock solutions of 0.5 mg/mL of the various fatty acids were prepared in distilled water, the pH adjusted to 7.0, and autoclaved.

Tween 20, 40, 60, and 80 were obtained from Baker Chemical Co., Phillipsburg, NJ. Stock solutions of 2 mg/mL were prepared in distilled water, the pH adjusted to 7.0, and filter sterilized.

The sterile lipid solutions were added in varying concentrations to sterile bovine serum albumin, delipified alpha globulin, or delipified beta globulin solutions. These solutions were mixed for 30–60 s with a vortex mixer. These supplements were added to basal HPYG broth to a final concentration (w/v) of 0.2 or 0.4% alpha globulin, 0.2% beta globulin, or 1.0% bovine serum albumin.

Lipid extraction

The lipid fraction of alpha globulin was obtained from the solvent portion of the previously described delipification procedure. The chloroform–methanol was evaporated to dryness at 50°C under a stream of nitrogen. Lipid from whole cells was extracted by the method of Bligh and Dyer (1959).

Methyl esters of fatty acids were prepared by transmethylation. The lipid was dissolved in 0.5 mL of chloroform, 0.5 mL of methanol–toluene (1:1), and 0.5 mL of a freshly prepared methanolic KOH (0.2 *N*) solution. The solution was incubated at 40°C for 1.5 h and then neutralized with 1 *M* acetic acid. Methyl esters were extracted into chloroform, and the chloroform was evaporated to dryness at 50°C under a stream of nitrogen. The methyl esters were redissolved in 0.5 mL of hexane and 5 μL were injected into a Varian 1520 gas chromatograph equipped with a flame ionization detector. The oven temperature was 180–185°C, the detector oven temperature was 200°C, and the injector oven temperature was 200°C.

The column, a 72 in. long × 0.25 in. diameter (1 in. = 25.4 mm) aluminum column, was packed with SP-1000 (Supelco Inc.). The carrier gas was helium with a flow rate of 30 mL/min. The long-chain fatty acid standard was obtained from Supelco Inc.

Results

The alpha globulin fraction of serum from different animal sources is shown (Table 1) to support better growth of *T. denticola* strain T-32A through five transfers than did the albumin, beta globulin, and gamma globulin fractions which supported minimal growth. The optical density of cultures with the alpha globulin fractions was 0.36–0.47, while optical densities with the other serum fractions were 0.04–0.24. Similar results using *T. vincentii* strain N-9 were obtained during this investigation and were also reported by F. E. Austin (1978. M.S. Thesis, Virginia Polytechnic Institute and State University, Blacksburg, VA).

The active growth factor(s) in alpha globulin was nondialyzable against distilled water for 24 h at 4°C through Spectropor membrane tubing (12 000 molecular weight cutoff). It also did not pass through an Amicon membrane (XM 300) with a 300 000 molecular weight cutoff and was stable at pH 7.0 to autoclaving. Treatment of alpha globulin with trypsin, papain, or lipase inactivated the growth-promoting activity of alpha globulin.

To determine whether lipids are a required factor supplied by the alpha globulin fraction, growth was compared in cultures grown in HPYG broth supplemented with whole serum, alpha globulin, delipified alpha globulin, delipified alpha globulin plus lipid extract, and the lipid extract alone. The results in Table 2 show that chloroform–methanol extraction of alpha globulin greatly reduced the growth-promoting activity of this serum fraction and that readdition of the crude lipid extract to the protein portion restored good growth. The lipid extract alone did not support growth. A final concentration of 0.4% alpha globulin in HPYG broth yielded better growth (0.72 OD) of *T. denticola* than did 0.2% (0.44 OD) alpha globulin. Higher concentrations of alpha globulin had growth yields no higher than those with 0.4% alpha globulin. The same increase in concentration of delipified alpha globulin did not increase growth (0.13–0.15 OD).

TABLE 1. Growth-promoting activity of serum fractions for *T. denticola* strain T-32A

Fraction	Final concentration,[a] %	Optical density[b]
Whole serum (lapine)	10.0	1.18
Albumin (bovine)	1.0	0.12
Albumin (human)	1.0	0.13
Albumin (lapine)	1.0	0.19
Alpha globulin (human)	0.2	0.42
Alpha globulin (porcine)	0.2	0.36
Alpha globulin (ovine)	0.2	0.47
Beta globulin (human)	0.2	0.13
Beta globulin (bovine)	0.2	0.24
Gamma globulin (human)	0.2	0.04
Gamma globulin (bovine)	0.2	0.16
No supplement	—	0.00

[a]Final concentration of serum as v/v, serum fraction concentrations indicate protein content (w/v). The serum fractions were added to basal HPYG medium.

[b]Optical density of cultures read at 540 nm after 8 days of incubation of the fifth transfer. Optical densities correspond to the following approximate cell counts: 1.0 OD equivalent to 5×10^8 cells/mL, 0.5 OD equivalent to 1×10^8 cells/mL, 0.25 OD equivalent to 3×10^7 cells/mL, and 0.13 OD equivalent to 1×10^7 cells/mL.

TABLE 2. Effect of lipid extract from alpha globulin on the growth of *T. denticola* strain T-32A

Supplement[a]	Final concentration,[b] %	Optical density[c]
Serum	10.0	1.18
Alpha globulin	0.2	0.44
Alpha globulin	0.4	0.72
Delipified alpha globulin	0.2	0.13
Delipified alpha globulin	0.4	0.15
Delipified alpha globulin plus lipid extract	0.2	0.35
Delipified alpha globulin plus lipid extract	0.4	0.52
Lipid extract only	0.2	0.07

[a]All globulin fractions were of human origin and the serum was from rabbits.

[b]Final concentrations of serum as v/v, serum fraction concentrations indicate protein content (w/v). Lipid extracts added in amounts corresponding to the protein content (w/v). The basal medium was HPYG.

[c]Optical density of cultures read at 540 nm after 8 days of incubation of the fourth transfer. Optical densities correspond to the following approximate cell counts: 1.0 OD equivalent to 5×10^8 cells/mL, 0.5 OD equivalent to 1×10^8 cells/mL, and 0.13 OD equivalent to 1×10^7 cells/mL.

To see if an artificial lipid source could be used, TEM-4T was substituted for the lipid extract of alpha globulin. Results in Table 3 show that TEM-4T could substitute for the crude lipid extract of alpha globulin. However, even with TEM-4T, beta globulin and albumin did not support good growth. Increased concentrations of TEM-4T did not result in increased growth with beta globulin and albumin. Maximum growth of *T. denticola* occurred with 0.1 mg/mL of TEM-4T and 0.4% delipified alpha globulin. Concentrations of TEM-4T greater than 0.1 mg/mL were inhibitory.

Alpha globulin was found to contain as major fatty acids 30% palmitic acid, 26% oleic acid, 28% linoleic acid, 4% palmitoleic acid, and 9% stearic acid, while TEM-4T contained 18% palmitic acid, 21% oleic acid, 3% linoleic acid, 12% palmitoleic acid, 14% stearic acid, 10% myristic acid, and 20% eicosadienoic acid (20:2).

Sodium salts of fatty acids were added alone or in various combinations to the basal medium supplemen-

TABLE 3. Growth of *T. denticola* strain T-32A using TEM-4T as an artificial lipid source with various serum fractions

Fraction[a]	TEM-4T, mg/mL	Optical density[b]
Alpha globulin	—[c]	0.72
Delipified alpha globulin	—	0.15
Delipified alpha globulin	0.05	0.41
Delipified alpha globulin	0.10	0.82
Delipified alpha globulin	0.20	0.03
Delipified beta globulin	—	0.13
Delipified beta globulin	0.05	0.03
Delipified beta globulin	0.10	0.02
Albumin	—	0.11
Albumin	0.05	0.10
Albumin	0.10	0.17
No protein added	0.10	0.00

[a]All globulin fractions were of human origin at a final protein concentration of 0.4% (w/v); the albumin was of bovine origin at a final protein concentration of 1.0% (w/v). The basal medium was HPYG.

[b]Optical density of cultures read at 540 nm after 8 days of incubation of the fourth transfer. Optical densities correspond to the following approximate cell counts: 1.0 OD equivalent to 5×10^8 cells/mL, 0.5 OD equivalent to 1×10^8 cells/mL, and 0.13 OD equivalent to 1×10^7 cells/mL.

[c]No addition.

ted with 0.4% delipified alpha globulin. Only oleic acid (*cis*-18:1 (9)) or elaidic acid (*trans*-18:1 (9)) supported good growth of *T. denticola* (Table 4). Growth was poor with other unsaturated and all saturated long-chain fatty acids tested alone or in various combinations. Growth was better when oleic acid was used as the lipid source than with the same concentration of elaidic acid. Bovine serum albumin (1%) could not replace delipified alpha globulin when oleic acid was used as the lipid source.

TABLE 4. Growth of *T. denticola* strain T-32A with various long-chain fatty acids[a]

Lipid[b]	Concentration, mg/mL	Optical density[c]
TEM-4T	0.10	0.80
Oleic acid	0.03	0.81
Elaidic acid	0.03	0.51
Myristoleic acid	0.03	0.31
Palmitoleic acid	0.03	0.34
Vaccenic acid	0.03	0.26
Linoleic acid	0.03	0.21
No lipid added	—	0.25
Albumin plus oleic acid	0.03	0.26

[a]Fatty acids were added as their sodium salts to basal HYPG broth supplemented with 0.4% delipified alpha globulin (w/v) or 1% bovine serum albumin (w/v).

[b]There was poor growth with myristic, palmitic, or stearic acids (0.11–0.17 OD).

[c]Optical density of cultures read at 540 nm after 8 days of incubation of the third transfer. Optical densities correspond to the following approximate cell counts: 1.0 OD equivalent to 5×10^8 cells/mL, 0.5 OD equivalent to 1×10^8 cells/mL, and 0.25 OD equivalent to 3×10^7 cells/mL.

Higher growth yields were achieved with both of these fatty acids upon increasing their concentrations. The results are shown in Table 5 of a typical experiment using increasing concentrations of oleic acid. Maximum growth was achieved with a final concentration of 0.04 mg/mL of sodium oleate. The optimal weight ratio of delipified alpha globulin to sodium oleate was 100:1. The turbidity of cultures with 0.04 mg/mL of oleate was nearly as good as that of cultures with serum in the medium. *Treponema denticola* grown on elaidic acid achieved a maximum optical density of 0.65 at a final elaidate concentration of 0.04 mg/mL. Higher concentrations of oleic and elaidic acids were inhibitory.

Results using Tween compounds as the lipid source showed that only Tween 80 (polysorbitan monooleate) could support maximal growth of *T. denticola* strain T-32A. When added to the HPYG medium containing 0.4% delipified alpha globulin, a concentration of 0.3 mg/mL of Tween 80 yielded maximum growth with an optical density of 0.95. Increasing amounts of Tween 80 were inhibitory. Tween 20, 40, or 60 supported only poor growth with optical densities ranging from 0.04 to 0.14.

Utilization of oleic acid by *T. denticola* was also investigated. After 5–6 days of incubation, 75% of the oleic acid in the medium had been incorporated into the cells. The remaining 25% of oleic acid was recoverable in the spent medium. Methyl esters of the long-chain fatty acids extracted from whole cells grown in HPYG broth containing 10% whole serum, and from cells grown in HPYG broth containing 0.4% delipified alpha globulin and 0.03 mg/mL sodium oleate, are shown in Table 6. The major cellular fatty acids in oleate-grown cells were myristic, pentadecanoic, and palmitic acids. Oleic acid represented only a minor (6%) component of the cellular lipids of these cells. A similar cellular fatty

TABLE 5. Growth response of *T. denticola* strain T-32A to various concentrations of oleic acid

Supplement[a]	Concentration, mg/mL	Optical density[b]
Serum control (10%, w/v)	—	1.32
Oleic acid	0.00	0.25
Oleic acid	0.01	0.73
Oleic acid	0.02	0.92
Oleic acid	0.03	0.96
Oleic acid	0.04	1.05
Oleic acid	0.05	0.75
Oleic acid	0.06	0.00

[a]Sodium oleate added to basal HPYG broth supplemented with 0.4% delipified alpha globulin (w/v).

[b]Optical density of cultures read at 540 nm after 8 days of incubation of the third transfer. Optical densities correspond to the following approximate cell counts: 1.0 OD equivalent to 5×10^8 cells/mL, 0.5 OD equivalent to 1×10^8 cells/mL, and 0.25 OD equivalent to 3×10^7 cells/mL.

TABLE 6. Cellular fatty acids of *T. denticola* strain T-32A grown with whole serum or sodium oleate[a]

Fatty acid	Lipid source[b]	
	Serum	Oleate
Myristic	17	26
Pentadecanoic	5	23
Palmitic	27	26
Stearic	8	ND
Oleic	14	6
Linoleic	16	ND
Eicosadienoic (20:2)	ND[c]	11
Unknown[d]	ND	6

[a]Sodium oleate (0.03 mg/mL) added to HPYG broth supplemented with 0.4% delipified alpha globulin (w/v). Whole serum (final concentration 10%, v/v) added to basal HPYG broth.
[b]Values are as percentage of total fatty acid detected.
[c]ND, none detected.
[d]Retention time of unknown, 88 min.

acid pattern was found in serum-grown cells. Eicosadienoic acid (20:2), 11%, and an unidentified fatty acid (retention time 88 min), 6%, were only found in the cellular lipids of oleate-grown cells.

Five strains of *T. denticola* and three strains of *T. vincentii* were able to grow in HPYG broth supplemented with 0.4% delipified alpha globulin plus 0.03 mg/mL sodium oleate. These results are shown in Table 7. All strains except D3A-9 and D3A-1 grew nearly as well in the oleate medium as they did in the serum medium.

TABLE 7. Growth of *T. denticola* strains and of *T. vincentii* strains using oleic acid as the lipid source[a]

Organism	Strain	Optical density[b]	
		Serum	Oleate
T. denticola	T32-A	1.18	1.05
	ST-10	1.14	1.00
	N-39	1.04	0.94
	D3A-5	0.76	0.67
	D3A-9	0.78	0.34
T. vincentii	N-9	0.70	0.63
	D7B-1	0.43	0.39
	D3A-1	0.79	0.37

[a]Sodium oleate (0.03 mg/mL) added to HPYG broth supplemented with 0.4% delipified alpha globulin (w/v). Whole serum (final concentration 10%, v/v) added to basal HPYG broth.
[b]Optical density of cultures read at 540 nm after 8 days of incubation of the third transfer. Optical densities correspond to the following approximate cell counts: 1.0 OD equivalent to 5×10^8 cells/mL, 0.5 OD equivalent to 1×10^8 cells/mL, and 0.25 OD equivalent to 3×10^7 cells/mL.

Discussion

These results indicate that alpha globulin (Cohn fraction IV) is the only serum fraction that can support good growth of *T. denticola* and *T. vincentii*. These results support the findings of Socransky and Hubersak (1967), Hespell and Canale-Parola (1971), and F. E. Austin (1978. M.S. Thesis, Virginia Polytechnic Institute and State University, Blacksburg, VA). Austin reported that neither albumin nor any other globulin fraction could support good growth of these two oral treponemes, while Socransky and Hubersak (1967) reported the same results using *T. denticola*. They also reported that *T. denticola* would not grow when either ceruloplasmin or transferrin was added to a complex medium. Austin reported that *T. vincentii* and *T. denticola* could grow with alpha globulin, while *T. refringens* and *T. phagedenis* could not grow with alpha globulin. *Treponema phagedenis* was found to require albumin for growth. Little and Subbarow (1945), Eagle and Steinman (1948), and Oyama *et al.* (1953) have also reported that albumin is required for growth of *T. phagedenis* and for motility retention of *T. pallidum* (Matthews *et al.* 1978).

Some characteristics of the active growth factor(s) of alpha globulin have been reported by Socransky and Hubersak (1967). In contrast with their finding that it was labile to heating at 80°C for 30 min, we were able to show that it was stable at pH 7.0 to autoclaving.

We have demonstrated that the alpha globulin supplies both a nondialyzable fraction, presumably protein, and a lipid for maximal growth of *T. denticola*. The leptospires and *T. phagedenis* have been reported to require serum albumin which provides both protein and lipid growth factors (Ellinghausen and McCullough 1965; Johnson and Eggebraten 1971; Johnson and Gary 1963; Johnson *et al.* 1969; Johnson and Walby 1972; Oyama *et al.* 1953; Smibert 1973). Oyama *et al.* (1953) reported that the molar ratio of albumin to oleate (1:4) was of critical importance for the growth of the Reiter strain of *T. phagedenis*. It has been reported that a weight ratio of albumin to sodium oleate of 100:1 (Ellinghausen and McCullough 1965) yielded maximum growth of *Leptospira interrogans* serovar *pomona*. Our results indicate that a weight ratio of delipified alpha globulin to sodium oleate of 100:1 yields maximal growth of *T. denticola*.

Long-chain fatty acids are required for growth of the leptospires (Bey and Johnson 1978; Ellinghausen and McCullough 1965; Johnson and Gary 1963; Johnson *et al.* 1969; Johnson and Walby 1972; Smibert 1973; Staneck *et al.* 1973; Vaneseltine and Staples 1961) and for growth of *T. phagedenis* (Johnson and Eggebraten 1971; Oyama *et al.* 1953; Smibert 1973). Johnson and Eggebraten (1971) reported that the Reiter strain of *T. phagedenis* required a pair of fatty acids, one saturated

of at least 14 carbons and one unsaturated of at least 15 carbons with one, two, or three double bonds. Elaidic acid (*trans*-18:1(9)) could substitute for this pair of fatty acids. A similar observation has been made for *Mycoplasma* strain Y (Rodwell 1968) and for the leptospires (Johnson and Walby 1972). In our investigations, oleic acid alone was able to support maximal growth of *T. denticola* and *T. vincentii* when delipified alpha globulin was added to the basal medium. Growth of *T. denticola* and *T. vincentii* using the oleate-supplemented medium approached the growth of these two species in a serum-containing medium. *Treponema denticola* and *T. vincentii* have a simpler nutritional requirement for fatty acids than does *T. phagedenis* which required a pair of fatty acids (Johnson and Eggebraten 1971).

Tween 80 has been demonstrated as an excellent substitute for oleic acid (Davis and Dubos 1947; Dubos 1947; Dubos and Middlebrook 1948; Ellinghausen and McCullough 1965; Oyama *et al.* 1953; Razin 1973; Vaneseltine and Staples 1961). By using various Tween compounds we were able to show that Tween 80 was the only Tween compound, in a medium containing delipified alpha globulin, that could support maximal growth of *T. denticola*.

The cellular fatty acid composition of *T. phagedenis*, *T. refringens*, *T. minutum*, *T. vincentii*, and *T. denticola* grown in a serum-containing medium has been reported (C. B. Walker and R. M. Smibert. 1975. Abstr. Annu. Meet. Am. Soc. Microbiol. 1107. p. 134). The principle fatty acids of these five species were palmitic, stearic, oleic, and linoleic acids. Walker and Smibert also reported that the cellular lipids of *T. denticola* contained myristic acid. Using a lipid-free albumin medium containing oleate and palmitate, Livermore and Johnson (1974) reported that the cellular fatty acids of *T. scoliodontum*, *T. vincentii*, *T. denticola*, and *T. phagedenis* contained only oleate and palmitate. Johnson *et al.* (1970) used a medium containing lipid-free albumin and a pair of fatty acids (one saturated of at least 14 carbons and one unsaturated of at least 15 carbons with one, two, or three double bonds) to investigate the cellular fatty acids of the Kazan 5 strain of *T. phagedenis*. They reported that the cellular fatty acids of *T. phagedenis* Kazan 5 consisted of the pair of fatty acids that were added to the culture medium. Livermore and Johnson (1974) and Johnson *et al.* (1970) thus concluded that the treponemes studied were incapable of *de novo* synthesis, chain length modification, saturation, and desaturation of fatty acids. *Treponema pallidum* when suspended in a serum-free medium containing only oleate and palmitate has been reported by Schiller and Cox (1977) to incorporate the oleate and palmitate unaltered into their cellular fatty acids. Our investigation with *T. denticola* has indicated that this organism, in contrast with the report of Livermore and Johnson (1974), may be capable of chain modification of oleic acid. When grown in oleic acid – delipified alpha globulin supplemented medium the cellular fatty acids of *T. denticola* contain myristic, pentadecanoic, and palmitic acids as the major cellular fatty acids. Smaller amounts of oleic acid, eicosadienoic acid (20:2), and an unidentified fatty acid of retention time 88 min were also detected as cellular fatty acids. Thus, *T. denticola* appears capable of saturation, desaturation, chain shortening, and chain elongation of oleic acid.

Treponema denticola and *T. vincentii* require alpha globulin for maximal growth, while *T. phagedenis* requires albumin. The two oral treponemes have a lipid requirement that can be satisfied by either oleic acid or elaidic acid. Oleic acid and albumin do not support good growth of *T. denticola* and *T. vincentii*. Alpha globulin, in addition to supplying and detoxifying fatty acids for these two oral treponemes, may also be required for some other purpose such as the need for a specific protein subfraction. The protein requirement supplied by alpha globulin will be the subject of future investigations.

Acknowledgements

This work was supported in part by Public Health Service grant AI 12726 from the National Institute of Allergy and Infectious Diseases, grant DE-05139 from the National Institute of Dental Research, and by the Commonwealth of Virginia.

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APPENDIX VII. Submitted to the Canadian Journal of Microbiology, June 1982

Albumin requirement of *Treponema denticola* and *Treponema vincentii*

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Abstract

Treponema denticola and Treponema vincentii were found to require albumin, oleic acid, and thiamine pyrophosphate (TPP) for growth. Previous studies indicated that commercial human alpha globulin, which is 50% albumin, was the only serum fraction that supported growth of these two oral treponemes. The protein requirement of alpha globulin was investigated by separating the alpha globulin proteins from the contaminating albumin with Affi-Gel Blue affinity chromatography. The fraction containing albumin and a fraction containing alpha globulin protein were both required for growth of T. denticola. Serum albumin (bovine or human) could substitute for the alpha globulin fraction that contained albumin. Oleic acid was supplied by the fraction that contained alpha globulin protein. Optimal growth of T. denticola and T. vincentii was obtained in a medium supplemented with 0.4% (w/v) delipified albumin, 0.08 mg/ml of sodium oleate, and 25 µg/ml of TPP. Starch did not replace albumin for optimal growth.

Serum albumin solutions tightly bound TPP (0.5 µg of TPP per mg of albumin). Optimal growth of T. denticola was only achieved in an albumin-oleate supplemented medium with sufficient TPP to saturate the albumin binding sites and to provide excess free TPP. Albumin acted to detoxify long chain fatty acids. Charcoal treated Tween 80 (polysorbitan monooleate) did not support good growth of T. denticola in a protein-free medium that contained TPP. Short chain fatty acids supported limited growth of T. denticola when added to a medium with TPP or to a medium that contained 0.4% delipified albumin and TPP.

Short chain fatty acids were not required to initiate T. denticola growth in a delipified medium supplemented with 0.4% delipified albumin, 0.06 mg/ml of sodium oleate, and 25 µg/ml of TPP.

Introduction

The oral treponemes (Treponema denticola, T. vincentii, and T. scoliodontum) and genital treponemes (T. phagedenis, T. refringens, and T. minutum) have been reported to require serum and ascitic fluid for growth (Hampp and Nevin 1959; Rose and Morton 1952; Smibert 1973, 1976). Little and Subbarow (1945) reported that bovine serum albumin could replace serum for the growth of the Reiter strain of T. phagedenis. Serum albumin reversibly binds free fatty acids (Goodman 1958; Spector 1975) and the function of albumin for the growth of T. phagedenis was to detoxify the medium from the effects of the fatty acids (Oyama et al. 1953). Albumin is also needed for the growth of leptospires (Johnson and Gary 1963; Johnson and Walby 1972) unless the required fatty acids are treated by other methods (Bey and Johnson 1978; Staneck et al. 1973).

A commercial alpha globulin fraction of serum was able to replace whole serum for the growth of T. denticola (F. E. Austin, M.S. Thesis, Virginia Polytechnic Institute and State University, Blacksburg, 1978; Hespell and Canale-Parola 1971; Socransky and Hubersak 1967; Van Horn and Smibert 1982). Austin, and Van Horn and Smibert also reported that T. vincentii could grow when whole serum was replaced by alpha globulin. Other serum fractions did not support growth of these two oral treponemes. The alpha globulin fraction supplied oleic acid (C18:1) and a protein(s) that are both required for growth of these two treponemes (Van Horn and Smibert 1982).

The purpose of this investigation was to determine the protein requirement supplied by alpha globulin for the growth of T. denticola and T. vincentii.

Materials and Methods

Organism and medium. Treponema denticola strain T-32A was obtained from T. Rosebury, Washington University, St. Louis, MO. Other treponemal strains used were obtained from the following: T. denticola strain ST-10; E. Canale-Parola, University of Massachusetts, Amherst, MA, and T. denticola strain N-39 and T. vincentii strain N-9; Center for Disease Control, Atlanta, GA. All other strains were obtained from the culture collection of this laboratory. All strains were isolated from the oral cavity of man. Stock cultures were maintained in pre-reduced heart infusion peptone yeast extract glucose broth (HPYG), prepared as outlined in the VPI Anaerobe Laboratory Manual (Holdeman et al. 1977), supplemented with either 0.4% (w/v) human alpha globulin or 10% (v/v) heat inactivated (60°C, 1 hr) rabbit serum. Cultures were stored in liquid nitrogen. HPYG medium contained: Bacto-heart infusion broth, 5.0 g; polypeptone (BBL), 20.0 g; Bacto-yeast extract, 10.0 g; glucose, 10.0 g; Bacto-gelatin, 10.0 g; Bacto-agar, 1.6 g; $(NH_4)_2SO_4$, 0.5 g; $NaHCO_3$, 5.0 g; resazurin, 0.001 g; L-cysteine hydrochloride, 0.5 g; salt solution, 500 ml; and distilled water, 500 ml. The salt solution contained (g/liter): $CoCl_3 \cdot 6H_2O$, 0.04; $MnCl_2 \cdot 4H_2O$, 0.04 g; $MgSO_4$, 0.4 g; K_2HPO_4, 2.3 g; KH_2PO_4, 1.0 g; and NaCl, 2.0 g. The pH was adjusted to 7.2 to 7.4.

Test cultures were grown in 7 ml HPYG broth supplemented with 0.2, 0.4 or 1.0% (w/v) serum albumin (Cohn fraction V; bovine or human), 0.2 or 0.4% (w/v) alpha globulin (Cohn fraction IV; human), 0.5 or 1.0% (w/v) starch (Difco), or 10%

(v/v) inactivated rabbit serum. Stock solutions of these supplements contained filter sterilized thiamine pyrophosphate (TPP), 33 mg per liter and L-glutamine, 660 mg per liter. The gas phase was oxygen-free nitrogen. Culture tubes were inoculated with 0.5 ml of a four day culture of T. denticola strain T-32A containing approximately 1 x 10^8 cells/ml. They were incubated at 37°C and optical densities of duplicate cultures at 540 nm were recorded after 3 and 8 days. Cultures were transferred on day 3 and at least 5 serial transfers were made for each experiment. At the time of transfer all cultures were examined by dark field microscopy.

Preparation of supplements. Stock solutions of 2 or 4% (w/v) alpha globulin (Miles Laboratories, Elkhart, IN), 2, 4, 10 or 20% (w/v) albumin (Sigma Chemical Co., St. Louis, MO), and 10% (w/v) starch (Difco Laboratories, Detroit, MI) were prepared in distilled water and the pH adjusted to 7.0 with 0.1 N NaOH. The serum fractions were filtered through non-sterile 5.0 µm, 1.2 µm, 0.8 µm, and 0.6 µm membrane filters and sterilized by passage through a 0.45 µm filter. The starch solution was sterilized by autoclaving. The protein content of all serum fractions was determined by a dye binding method (Bio-Rad Laboratories, Richmond, CA).

Lipid-poor fractions. Supplements (alpha globulin, albumin, and starch) and HPYG medium components were delipified with chloroform:methanol (2:1) as previously described (Van Horn and Smibert 1982). Delipified serum fractions and starch were dissolved in distilled water, the solutions were adjusted to pH 7.0, and the solutions were sterilized as mentioned above.

Lipids. Oleic acid was obtained from Supelco Inc., Bellefonte, PA. Sodium oleate was made by adding 1.0 ml of 1 N NaOH to 10 ml of 0.1 M oleic acid in chloroform. The mixture was stirred for 30 minutes to ensure completion of salt formation. The mixture was centrifuged at low speed (5,000 rpm) and the chloroform removed. The sodium oleate was dried at room temperature under a stream of nitrogen. A stock solution of 0.5 mg/ml of sodium oleate was prepared in distilled water, the pH adjusted to 7.0, and the solution sterilized by autoclaving.

Charcoal treated Tween 80 (Baker Chemical Co., Phillipsburg, NJ) was prepared by combining 100 ml of a ten percent Tween 80 solution with 20 g of Norit A charcoal as described by Bey and Johnson (1978). Tween 80 concentrations were determined by comparing the absorbances at 230 nm to a Tween 80 standard curve. Stock solutions of 2 mg/ml were prepared in distilled water, the pH was adjusted to 7.0, and the solution was sterilized by filtration.

The sterile lipid solutions were added in various amounts to sterile serum fractions or starch. These solutions were mixed for 30-60 seconds with a Vortex mixer. The supplements were added to HPYG broth to a final concentration (w/v) of 0.2 or 0.4% alpha globulin, 0.5 or 1.0% starch, and 0.2, 0.4 or 1.0% serum albumin. Sterile charcoal treated Tween 80 was added directly to HPYG broth.

The volatile fatty acid (VFA) solution contained: acetic acid, 5 ml; N-butyric acid, 4 ml; N-valeric acid, 1 ml; isobutyric acid, 1 ml; isovaleric acid, 1 ml; and distilled

water, 88 ml. The pH was adjusted to 7.2 and the solution sterilized by filtration. The sterile fatty acid solution was added in various amounts (0.1 ml, 0.2 ml, and 0.3 ml of the VFA solution) directly to HPYG broth that contained 10 µg/ml of TPP. The VFA solution was also added in various amounts (1.0, 2.0, and 3.0 ml) to 7 mls of a 4% delipified albumin stock solution. This supplement was added to HPYG broth to a final concentration of 0.4% w/v delipified albumin.

Lipid extraction. Lipids from HPYG medium components and various serum fractions were obtained from the solvent portion of the previously described delipification procedure. Short chain fatty acids were extracted and analyzed by gas liquid chromatography as outlined in the VPI Anaerobe Laboratory Manual (Holdeman et al. 1977). Chloroform:methanol extracts that contained long chain fatty acids were evaporated to dryness at 50°C under a stream of nitrogen. Methyl-esters of long chain fatty acids were prepared by transmethylation and chromatographed (Van Horn and Smibert 1982).

Albumin determinations. The albumin content of commercial alpha globulin was determined by both a dye binding method and rocket immunoelectrophoresis. The dye binding method is a modification of the method of Kachmar (1970). The reagent consisted of 0.055 M citrate buffer, pH 3.5, to which an aqueous 0.1% w/v methyl orange solution had been added until the absorbance at 540 nm was 0.78 to 0.85. The albumin standards contained 1.0 through 6.0 g of human serum albumin per 100 ml of distilled water. Standard, test sample or saline (0.2 ml) was added to 10 ml of the methyl orange reagent,

mixed, and the absorbance at 540 nm read after 20 min. Albumin concentrations of the test sample were obtained by comparison with the standard curve.

Rocket immunoelectrophoresis was performed by making a 1% agarose solution, pH 8.6, with Bio-Rad agarose immunoelectrophoresis tablets (Bio-Rad Laboratories). The molten agarose was cooled to 55°C and 10 µl of antisera was added per ml of agarose. Six ml of the agarose that contained antisera was dispensed onto a clean, level 2 by 3 inch glass slide and allowed to harden. Antisera against human albumin, alpha 2-macroglobulin, alpha 1-lipoprotein, ceruloplasmin, alpha 1-trypsin inhibitor, and alpha 1-glycoprotein were obtained from Miles Laboratoreis (Elkhart, IN) and antisera against alpha 2 AP-glycoprotein, alpha 1 T-glycoprotein, Gc globulin, inter alpha trypsin inhibitor, alpha 1-antichymotrypsin, alpha 1 B-glycoprotein, Zn-alpha 2-glycoprotein, and haptoglobin were obtained from Behring Diagnostics (Somerville, NJ).

Six 4 mm wells, eight to ten mm apart, were made on the slide. Ten µl of sample was applied to each well and the slide was subjected to electrophoresis at 150 V (40 milliamps) on a Zipzone electrophoresis chamber (Helena Laboratories, Beaumont, TX) that contained barbitol-sodium barbitol buffer (0.05 ionic strength, pH 8.6). After electrophoresis the wells were filled with distilled water and the gels were covered with moistened Whatman No. 1 filter paper. A 2 cm layer of folded, dry, filter paper was placed on the gel and pressure was applied with a glass plate. The pressed gels were washed in saline for 24 h then in distilled water for 24

h and dried with warm air. They were stained using 0.2% Coomassie blue in a water:methanol:acetic acid (200:200:44) mixture for 2-3 min. The slides were destained with the same mixture that did not contain stain. The slides were air dried and peak heights were measured and compared to a standard curve.

Alpha globulin fractionation. Alpha globulin (2% w/v in buffer) was fractionated with Affi-Gel Blue (Bio-Rad Laboratories) column chromatography that had an albumin binding capacity of 15 mg/ml. Alpha globulin was eluted with 0.02 M phosphate buffer (pH 7.1) and albumin was eluted with 1.4 M NaCl in 0.02 M phosphate buffer. The column was regenerated with 8 M urea.

Protein fractions were dialyzed, with two changes of water, at 4°C with Spectrapor 6-8,000 MW cutoff dialysis tubing. The protein fractions were lyophilized and stored at 4°C.

Thiamine binding. Thiamine pyrophosphate (5 mg/ml stock solution) was added to 2% w/v albumin, 5% w/v starch, and 0.1% w/v agar and allowed to equilibrate for 24 h. Controls contained 50 µg/ml of TPP in distilled water. Oleate, when added, was at a final concentration of 0.3 mg/ml and was allowed to bind to albumin for 24 hours prior to the addition of TPP.

Two ml of sample was added to washed Centriflo CF25 membrane cones (Amicon Corp., Lexington, MA) with a molecular weight cutoff of 25,000. The samples were centrifuged at 5,000 x g for 30 min. Ultrafiltrates were collected and the

retentates were restored to the original volume with 2 ml of distilled water.

Thiamine pyrophosphate concentrations (as thiamine) in both the retentates and the ultrafiltrates were determined by a thiochrome assay (Strohecker and Henning 1966). The oxidizing solution was freshly prepared by adding 3 ml of an aqueous 1% w/v solution of potassium ferricyanide to 97 ml of cold 3.75 N NaOH. One ml of sample was added to 1 ml of the fresh oxidizing solution and mixed. The absorbance of thiochrome at 369 nm was read with a Gilford spectrophotometer. Absorbances of samples were compared to those of a standard that contained 1.5 through 50 µg/ml of TPP.

Proteolysis of serum fractions. Insoluble trypsin, 5 mg/ml, (Sigma Chemical Co., St. Louis, MO) was added to 10 ml of a 2% (w/v) solution of Affi-Gel Blue fractionated alpha globulin or human serum albumin at pH 8.2 and incubated at 37°C for 24 hours with constant shaking. The insoluble trypsin was removed by centrifugation at 12,000 x g for 10 min. The supernatant was filtered through Whatman No. 1 filter paper, the pH was adjusted to 7.0, and the enzyme treated solution was sterilized by filtration.

Results

Commercial serum albumin (bovine, BSA or human, HSA) supported growth of Treponema denticola strain T-32A when the final concentration of albumin in HPYG broth was increased from 1 to 7% (Table 1). Higher albumin concentrations did not give better growth.

Forty-five to fifty percent of the total protein content of the commercial alpha globulin preparations that were used in this and our previous investigation (Van Horn and Smibert 1982) was found to be albumin. The albumin in the commercial alpha globulin was selectively removed by affinity column chromatography with Affi-Gel Blue (Bio-Rad). The alpha globulin proteins were eluted with 0.02 M phosphate buffer as 2 overlapping peaks (fractions 1 and 2). Albumin (fraction 3) was eluted with 1.4 M sodium chloride in 0.02 M phosphate buffer. It was shown by a methyl orange dye binding assay and rocket immunoelectrophoresis that fraction 3 contained mostly albumin (65% w/v) and that fraction 2 contained less than 10% (w/v) albumin. Fraction 2 also contained alpha 1-antichymotrypsin, inter alpha trypsin inhibitor, Gc globulin, alpha 2-macroglobulin, and alpha 1-trypsin inhibitor. Albumin was not detected in fraction 1 which contained alpha 1-antichymotrypsin, alpha 2-macroglobulin, haptoglobin, ceruloplasmin, and alpha 1-trypsin inhibitor. Fractions 1 and 3 are shown in Table 2 to both be necessary for optimal growth of T. denticola strain T-32A.

Serum albumin (bovine and human) could replace the fraction 3 of alpha globulin when added to HPYG broth that contained

TABLE 1. Growth of *Treponema denticola* strain T-32A using increasing concentrations of albumin

Supplement	Final concentration (%)[a]	Optical density[b]
Whole serum	10	1.18[c]
Alpha globulin	0.4	0.95
Bovine serum albumin	1.0	0.36
Bovine serum albumin	3.0	0.39
Bovine serum albumin	5.0	0.75
Bovine serum albumin	7.0	1.08
Human serum albumin	1.0	0.35
Human serum albumin	3.0	0.97
Human serum albumin	5.0	1.23
Human serum albumin	7.0	1.18

[a]Final concentration of serum as v/v, serum fraction concentrations indicate protein content (w/v). The serum fractions were added to HPYG medium.

[b]Optical density of cultures read at 540 nm after 8 days incubation of the 3rd transfer.

[c]Optical densities correspond to the following approximate cell counts: 1.0 O.D. equivalents to 5×10^8 cells/ml, 0.5 O.D. equivalent to 1×10^8 cells/ml, and 0.25 O.D. equivalent to 3×10^7 cells/ml.

TABLE 2. Growth promoting activity of alpha globulin proteins separated by Affi-Gel blue affinity chromatography for *Treponema denticola* strain T-32A

Supplement[a]	Final concentration (%)[b]	Optical density[c]
Alpha globulin	0.4	0.91[d]
Fraction 1	0.2	0.06
Fraction 2	0.2	0.07
Fraction 3	0.2	0.47
Fraction 1 + fraction 2	0.2/0.2	0.09
Fraction 1 + fraction 3	0.2/0.2	1.02
Fraction 2 + fraction 3	0.2/0.2	0.49

[a]Fraction 1 and fraction 2 eluted with 0.02M phosphate buffer, Fraction 3 eluted with 1.4 M NaCl in phosphate buffer.

[b]Final concentrations indicate protein content (w/v) and are based on alpha globulin containing 50% albumin. The medium was HPYG.

[c]Optical density of cultures read at 540 nm after 8 days of the 3rd transfer.

[d]Optical densities correspond to the following approximate cell counts: 1.0 O.D. equivalent to 5×10^8 cells/ml, 0.5 O.D. equivalent to 1×10^8 cells/ml, and 0.13 O.D. equivalent to 1×10^7 cells/ml.

fraction 1 (Table 3). Concentrations of albumin, used as HPYG supplements, were based on the commercial alpha globulin containing 50% albumin (0.2% albumin in a 0.4% alpha globulin solution).

Results with HPYG broth supplemented with delipified fraction 1 and HSA (Table 4) indicate that fraction 1 supplied the lipid required for growth of T. denticola. The fatty acid content of fraction 1 was 30% palmitic, 8% palmitoleic, 10% stearic, 35% oleic, and 14% linoleic acids. Minor amounts of myristic (2%) and myristoleic (1%) acids were also found. Fraction 2 had only trace amounts of palmitic and palmitoleic acids.

Fraction 1 and HSA were hydrolyzed with insoluble trypsin. The results in Table 4 show that an intact protein (albumin) was required for optimal growth of T. denticola strain T-32A.

Oleic acid was the only fatty acid required for growth of T. denticola and T. vincentii when HPYG broth was supplemented with delipified commercial alpha globulin (Van Horn and Smibert 1982). Because a medium supplemented with albumin and oleate supported only minimal growth of these 2 organisms (Van Horn and Smibert 1982), we investigated the roles of TPP, albumin, and oleic acid for the growth of T. denticola. For all subsequent tests, stock supplements contained sufficient TPP to yield a final concentration of 25 µg/ml of TPP in HPYG broth unless otherwise noted.

Sodium oleate was added in various amounts to delipified albumin to determine the concentration of oleate that would support optimal growth of T. denticola. The results in Table

TABLE 3. Substitution of Affi-Gel Blue fraction 3 with albumin for growth of *Treponema denticola* strain T-32A

Supplement[a]	Albumin source	Final concentration (%)[b]	Optical density[c]
Alpha globulin	–	0.4	0.89[d]
–	Bovine	0.2	0.23
Fraction 1	Bovine	0.2/0.2	1.03
Fraction 2	Bovine	0.2/0.2	0.30
–	Human	0.2	0.32
Fraction 1	Human	0.2/0.2	0.97
Fraction 2	Human	0.2/0.2	0.40

[a]Fraction 1 and fraction 2 were eluted with 0.02 M phosphate buffer. These fractions were added to albumin and the supplement added to HPYG medium.

[b]Final concentrations indicate protein content (w/v) and are based on alpha globulin containing 50% albumin.

[c]Optical density of cultures read at 540 nm after 8 days of the 3rd transfer.

[d]Optical densities correspond to the following approximate cell counts: 1.0 O.D. equivalent to 5×10^8 cells/ml, 0.5 O.D. equivalent to 1×10^8 cells/ml, and 0.25 O.D. equivalent to 3×10^7 cells/ml.

TABLE 4. Effect of lipid extraction and trypsinization of Affi-Gel Blue Fraction 1 and albumin on the growth of *Treponema denticola* strain T-32A

Supplement[a]	Final concentration (%)[b]	Optical density[c]
HSA[d]	0.2	0.32[e]
HSA plus fraction 1	0.2/0.2	0.97
Delipified HSA plus fraction 1	0.2/0.2	0.96
HSA plus delipified fraction 1	0.2/0.2	0.37
Trypsinized HSA plus fraction 1	0.2/0.2	0.00
HSA plus trypsinized fraction 1	0.2/0.2	0.91
Trypsinized HSA	0.2	0.30
Alpha globulin	0.4	0.89

[a]Fraction 1 was eluted from Affi-Gel Blue with 0.02 M phosphate buffer. The supplements were mixed and added to HPYG medium.

[b]Final concentrations indicate protein content (w/v) and are based on alpha globulin containing 50% albumin.

[c]Optical density of cultures read at 540 nm after 8 days of the 3rd transfer.

[d]HSA, human serum albumin.

[e]Optical densities correspond to the following approximate cell counts: 1.0 O.D. equivalent to 5×10^8 cells/ml, 0.5 O.D. equivalent to 1×10^8 cells/ml, and 0.25 O.D. equivalent to 3×10^7 cells/ml.

5 show that HPYG broth that contained a final concentration of 0.08 mg/ml of oleate allowed optimal growth of T. denticola strain T-32A. Increased concentrations of albumin (1% w/v final concentration), with 0.02 mg of oleate/mg of albumin added, gave no better growth than did 0.4% albumin. Trace amounts of palmitic, stearic, oleic, and linoleic acids were detected in BSA and only a trace amount of myristoleic acid was detected in HSA.

Starch was used as a supplement, with various oleate concentrations, to see if another compound could replace the albumin requirement for growth of T. denticola. A final concentration of 1% w/v starch (Table 6) in HPYG broth supported better growth (0.56 O.D.) than did albumin alone (0.19 O.D.); however, growth was not as good as with albumin and oleate (0.93 O.D.). Higher concentrations of starch (2% w/v final concentration) and oleate in HPYG broth gave no better growth of T. denticola strain T-32A than did a medium that contained 1% starch and oleate.

Charcoal treated Tween 80 (0.25 mg/ml), (Bey and Johnson 1978), added to HPYG broth that contained TPP (25 µg/ml), supported poor growth (0.21 O.D.) of T. denticola. The optical density of cultures supplemented with 0.4% albumin and 0.25 mg/ml of Tween 80 was 0.90.

Thiamine pyrophosphate requirements were investigated to determine the amounts of TPP needed in albumin stock solutions to satisfy the TPP requirement of T. denticola strain T-32A. The concentration of TPP that was added to albumin stock solutions was critical for good growth of T. denticola in an

TABLE 5. Growth response of *Treponema denticola* strain T-32A to various concentrations of oleic acid

Supplement	Final concentration (%)[a]	Oleate concentration (mg/ml)[b]	Optical density[c]
Serum	10%	–	1.50[e]
Delipified BSA[d]	0.4	–	0.19
Delipified BSA	0.4	0.04	0.90
Delipified BSA	0.4	0.08	0.98
Delipified BSA	0.4	0.20	0.01
Delipified HSA	0.4	–	0.19
Delipified HSA	0.4	0.04	0.93
Delipified HSA	0.4	0.08	1.00
Delipified HSA	0.4	0.20	0.17

[a]Final concentration of serum as v/v, albumin concentrations indicate protein content (w/v). The supplements were added to HPYG medium.

[b]Oleate was added to albumin stock solutions 24 hours prior to test. Amounts represent final concentration in HPYG medium containing 25 μg/ml of TPP.

[c]Optical density of cultures read at 540 nm after 8 days incubation of the 3rd transfer.

[d]BSA, bovine serum albumin; HSA, human serum albumin.

[e]Optical densities correspond to the following approximate cell counts: 1.0 O.D. equivalent to 5×10^8 cells/ml, 0.25 O.D. equivalent to 1×10^7 cells/ml, and 0.13 O.D. equivalent to 1×10^7 cells/ml.

TABLE 6. Growth of *Treponema denticola* strain T-32A using starch as a substitute for the albumin requirement

Supplement	Final concentration (%)[a]	Oleate concentration (mg/ml)[b]	Optical density[c]
Albumin (bovine)	0.4	–	0.19[d]
Albumin	0.4	0.06	0.93
Starch	0.5	–	0.21
Starch	0.5	0.03	0.42
Starch	0.5	0.06	0.45
Starch	0.5	0.15	0.00
Starch	1.0	–	0.25
Starch	1.0	0.03	0.50
Starch	1.0	0.06	0.56
Starch	1.0	0.09	0.01

[a]Albumin concentrations indicate protein content (w/v) and starch concentrations are w/v. The supplements were added to HPYG medium.

[b]Oleate was added to stock solutions 24 hours prior to test. Amounts represent final concentration in HPYG medium.

[c]Optical density of cultures read at 540 nm after 8 days incubation of the 3rd transfer.

[d]Optical densities correspond to the following approximate cell counts: 1.0 O.D. equivalent to 5×10^8 cells/ml, 0.5 O.D. equivalent to 1×10^8 cells/ml, and 0.25 O.D. equivalent to 3×10^7 cells/ml.

albumin-oleate supplemented HPYG medium. Thiamine pyrophosphate (33 µg/ml) was added to 2, 10 or 20% w/v albumin stock solutions that contained 0.02 mg of oleate/mg of albumin. These stock solutions were added to HPYG broth to a final concentration of 0.2% albumin and 0.03 mg/ml of oleate. HPYG broth supplemented from the 2% albumin stock solution supported good growth (0.89 O.D.), whereas the optical densities of cultures supplemented from the 10 or 20% albumin stocks were only 0.55 and 0.44 respectively. However, the medium supplemented from 10 and 20% albumin-oleate stock solutions that contained higher concentrations of TPP (250 µg/ml) gave good growth (0.82 O.D. and 0.92 O.D., respectively). Thiamine pyrophosphate was shown to be tightly bound to albumin (Table 7) but not to starch or agar. One mg of BSA bound 0.5 µg of TPP and one mg of HSA bound 0.6 µg of TPP. When oleate was added to albumin there was no change in the amount of TPP bound; however, the rate of binding of TPP to the albumin-oleate complex was increased four times over that of albumin alone. In order to determine the tightness of the binding of TPP to albumin, the albumin retentates from the original binding studies were restored to the original volume with 2 ml of distilled water and recaptured on the same Amicon Centriflo (CF25) membrane filter cone (25,000 molecular-weight cutoff). This procedure was repeated twice more. The thiochrome assay was performed on all ultrafiltrates and on the final retentate. Binding of TPP to albumin appeared to be very tight because TPP was not detected in any of the ultrafiltrates and there was no loss of

TABLE 7. Binding of thiamine pyrophosphate by various compounds[a]

Compound	Concentration (mg/ml)	Oleate (0.3 mg/ml)[b]	TPP bound (μg/ml)
Bovine albumin	20	–	11.0
Bovine albumin	20	+	10.0
Human albumin	20	–	13.0
Human albumin	20	+	12.0
Starch	50	–	0.0
Agar	1.0	–	⟨0.5

[a]Binding tested after 24 hours incubation of compound and TPP by ultrafiltration using Centriflo CF25 (25,000 M.W. cutoff) membrane filters. TPP was detected in ultrafiltrates and retentates by a thiochrome assay.

[b]Oleate, when added, was allowed to bind to albumin 24 hours before adding TPP.

TPP detected in the final retentate. The limit of detection of the thiochrome assay was one to two µg/ml of TPP.

Next we investigated why T. denticola would grow slightly (0.15 to 0.25 O.D.) in HPYG broth that contained either delipified albumin and TPP or delipified alpha globulin and TPP. Minimal growth (0.20 O.D.) was observed in cultures grown in HPYG broth supplemented only with 10 µg/ml of TPP. There was no growth of T. denticola in HPYG broth that lacked TPP. Delipified HPYG broth that contained 10 µg/ml of TPP failed to support growth. The HPYG broth components contained acetic, n-butyric, and lactic acids. Trace amounts of propionic, isobutyric, isovaleric, n-valeric, isocaproic, n-caproic, and succinic acids were also detected. Long chain fatty acids were not found in HPYG broth. A short chain fatty acid solution (FA) that contained acetic, n-butyric, n-valeric, isobutyric, and isovaleric acids was added to HPYG broth supplemented with 10 µg/ml of TPP. There was better growth (0.38 O.D.) of T. denticola in HPYG-FA-TPP broth than in HPYG-TPP broth without the fatty acids (0.20 O.D.). The fatty acid solution was added to HPYG broth that contained 0.4% delipified albumin to see if the short chain fatty acids could substitute for the oleate requirement. Growth of T. denticola in HPYG broth supplemented with delipified albumin, oleate and TPP had an optical density of 0.92, whereas, growth in HPYG broth that contained 0.4% delipified albumin, short chain fatty acids, and TPP had an optical density of only 0.46. Higher concentrations of the short chain fatty acid solution gave no better growth. Many coccoid bodies and straightened treponemes were

observed in these cultures. Delipified HPYG broth that contained delipified albumin, oleate, and TPP supported good growth (1.00 O.D.) of T. denticola.

Five strains of T. denticola and 3 strains of T. vincentii were able to grow in HPYG broth supplemented with 0.4% bovine serum albumin, 0.06 ml of oleate, and 12.5 µg/ml of TPP (Table 8). Strains D3A-5, D3A-9, D7B-1 and D3A-1 were recent isolates.

TABLE 8. Growth of *Treponema denticola* strains and *Treponema vincentii* strains using an albumin-oleate medium[a]

Organism	Strain	Optical density[b]	
		Serum	Albumin-oleate
T. denticola	T-32A	1.50[c]	1.00
	ST-10	1.47	1.00
	N-39	1.18	1.05
	D3A-5	0.76	0.51
	D3A-9	0.78	0.39
T. vincentii	N-9	1.08	0.97
	D7B-1	0.48	0.33
	D3A-1	1.18	0.72

[a]Sodium oleate (0.6 mg/ml) added to 4% w/v delipified albumin stock solution 24 hours prior to adding (1:10) to HPYG medium. Whole serum (final concentration 10% v/v) was added to HPYG medium. TPP (125 μg/ml) was also added to the stock solution.

[b]Optical density of cultures read at 540 nm after 8 days incubation of the 3rd transfer.

[c]Optical densities correspond to the following approximate cell counts: 1.0 O.D. equivalent to 5×10^8 cells/ml, 0.5 O.D. equivalent to 1×10^8 cells/ml, and 0.25 O.D. equivalent to 3×10^7 cells/ml.

Discussion

In the present study, albumin and oleic acid, in combination with sufficient concentrations of TPP, supported optimal growth of Treponema denticola and Treponema vincentii. Previous reports indicated that alpha globulin was the only serum fraction that could support optimal growth of T. denticola and T. vincentii (F. E. Austin, 1978, M.S. thesis, Virginia Polytechnic Institute and State University, Blacksburg, VA; Van Horn and Smibert 1982). The present results show that 50% of the total protein content of commercial human alpha globulin (Cohn fraction IV) was albumin. The albumin contaminant was found to be a necessary component for the growth supporting capability of alpha globulin.

Albumin was removed from the commercial alpha globulin by Bio-Rad Affi-Gel Blue affinity chromatography. Alpha globulin proteins were eluted from the column as two overlapping peaks (fraction 1 and fraction 2) while the albumin was desorbed from the column as fraction 3. These results are similar to those reported by Virca et al. (1978), who used Cibacron Blue Sepharose column chromatography to selectively remove albumin from plasma. Fraction 3, which contained albumin, and fraction 1, which contained alpha globulin proteins, were both required for optimal growth of T. denticola. Either bovine or human serum albumin (Cohn fraction V) replaced the fraction 3 requirement for the growth of T. denticola in a medium that contained fraction 1, which functions to supply long chain fatty acids to the treponemes. Fraction 1 was the only alpha globulin fraction that contained oleic acid, which was reported to be

required by T. denticola (Van Horn and Smibert 1982). Treponema denticola was reported to have lipase activity (C. A. Trevathan, 1978, M. S. thesis, Virginia Polytechnic Institute and State University, Blacksburg, VA), thus, fatty acids, including oleic acid, would be released from fraction 1 and become available in the free form to the treponemes. Albumin has been reported to bind long chain fatty acids (Goodman 1958; Spector 1975) and the function of the albumin in this study was to bind the released fatty acids, thus render the medium non-toxic to the treponemes. Albumin and oleic acid were found to replace alpha globulin for optimal growth of T. denticola and T. vincentii. Albumin has been reported to bind oleic acid, at a molar ratio of albumin to oleate of 1:4, for the growth of T. phagedenis (Oyama et al. 1953). A weight ratio of albumin to oleate of 100:1 (Ellinghausen and McCullough 1965) supported maximal growth of Leptospira interrogans serovar pomona. A weight ratio, in this study, of delipified albumin to sodium oleate of 50:1 gave optimal growth of T. denticola which is equivalent to the 100:1 weight ratio previously reported (Van Horn and Smibert 1982) because albumin was 50% of the total protein content of commercial alpha globulin.

Starch has been reported to adsorb long chain fatty acids (Ley and Mueller 1946; Schoch and Williams 1944). In this study, starch could not replace albumin for optimal growth of T. denticola. Starch plus oleate supported only limited growth because starch may not bind oleate as tightly as albumin.

Bey and Johnson (1978) grew the leptospires in a protein-free medium that contained charcoal detoxified Tween 80. Tween 80 has been reported to be an excellent substitute for oleic acid (Davis and Dubos 1947; Dubos 1947; Dubos and Middlebrook 1948; Ellinghausen and McCullough 1965; Oyama et al. 1953; Van Eseltine and Staples 1961). We previously reported that Tween 80 supported maximal growth of T. denticola in a medium that contained delipified alpha globulin (Van Horn and Smibert 1982). Charcoal treated Tween 80, in this study, did not support good growth of T. denticola when added to protein-free HPYG broth with TPP, but does support good growth of T. denticola in HPYG broth that contained albumin and TPP. Thus, T. denticola appears more sensitive to the toxicity of long chain fatty acids than are the leptospires.

Thiamine pyrophosphate was reported to be required for growth of the following oral treponemes: T. denticola, T. vincentii, T. scoliodontum, T. macrodentium, and Treponema strain S-69 (Austin, M.S. thesis; Hampp and Nevin 1959; Nevin and Hampp 1959; Nevin et al. 1960; Smibert 1976; Socransky and Hubersak 1967; Socransky et al. 1964; Steinman et al. 1954). Austin reported that T. vincentii required only nanogram quantities of TPP. The concentration of TPP added to albumin stock solutions was found to be of critical importance for achieving optimal growth of T. denticola. Vandegrift and Evans (1981) reported that albumin binds compounds that contain phosphate. We demonstrated that serum albumin (bovine and human) tightly bound TPP making it unavailable to the treponemes. Growth of T. denticola was poor when either ten or twenty percent albumin

stock solutions that contained oleate and 33 µg/ml of TPP were used to supplement HPYG broth. Serum that contained 33 µg/ml of TPP supported optimal growth of T. denticola when added to a culture medium to give a final serum concentration of 10% (v/v). The ten and twenty percent albumin stock solutions were found in this study to bind 50 and 100 µg/ml of TPP respectively. Thus, the poor growth of T. denticola in an albumin-oleate supplemented medium in our previous study (Van Horn and Smibert 1982) was due to the unavailability of free TPP in the medium.

Minimal growth (O.D. = 0.15 to 0.25) of T. denticola was observed in control cultures in this and our previous study (Van Horn and Smibert 1982). The HPYG broth contained short chain fatty acids that were required with added TPP to produce minimal growth of T. denticola. A short chain volatile fatty acid solution supported limited growth when added to basal HPYG broth supplemented with either TPP or delipified albumin and TPP. Many coccoid bodies and poorly motile straight treponeme cells were observed in this medium, whereas the treponeme cells grown in an albumin-oleate-TPP supplemented medium were highly motile helical forms. Oleate was the best source of fatty acid, however T. denticola seems capable of limited synthetic ability with short chain fatty acids. Short chain fatty acids were not required to initiate growth because optimal growth (O.D. = 1.00) was achieved in delipified HPYG broth supplemented with delipified albumin, oleate, and TPP.

Acknowledgements

This work was supported in part by Public Health Service grant AI 12726 from the National Institute of Allergy and Infectious Diseases, grant DE-05139 from the National Institute of Dental Research, grant AI-12726 from the National Institute of Allergy and Infectious Diseases and by the Commonwealth of Virginia.

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NUTRITIONAL REQUIREMENTS OF *TREPONEMA DENTICOLA* AND *TREPONEMA VINCENTII*

by

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(ABSTRACT)

Treponema denticola and *Treponema vincentii* were grown in a medium supplemented with 0.4% (wt/vol) alpha globulin in place of whole serum. Other serum fractions did not support growth. The growth factors in alpha globulin were destroyed by trypsin and by lipase. Lipid extraction of alpha globulin showed that both a protein and a lipid fraction were required for growth. Sodium salts of either oleic acid (*cis*-18:1) or elaidic acid (*trans*-18:1), added to 0.4% delipified alpha globulin supplemented media at a final concentration of 0.04 mg/ml, replaced the alpha globulin lipids required for optimal growth of these two oral treponemes. Tween 80 (polysorbitan monooleate) also supported growth in a medium containing protein. Short chain fatty acids plus 25 µg/ml thiamine pyrophosphate, added to either a basal medium or a medium containing 0.4% albumin, supported limited growth. The principle cellular fatty acids of *T. denticola* grown in an oleate medium were myristic, pentadecanoic, and palmitic acids. *Treponema denticola* appears capable of limited synthesis of cellular fatty acids from oleate.

Fifty percent of the total protein content of commercial alpha globulin was found to be albumin. The protein required for *T. denticola* growth was separated from the other alpha globulin proteins by Affi-Gel Blue (Bio-Rad Laboratories) affinity chromatography which selectively

adsorbed albumin. Serum albumin, added to a medium containing oleate, substituted for the alpha globulin protein required by these two treponemes. Trypsin destroyed the growth promoting activity of albumin. A weight ratio of albumin to sodium oleate of 50:1 (0.4% delipified albumin - 0.08 mg/ml oleate) supported optimal growth of T. denticola and T. vincentii. Starch, added to media containing oleate, could not replace albumin for optimal growth. Serum albumin solutions tightly bound added thiamine pyrophosphate (TPP). Optimal growth was achieved only when the TPP concentrations in albumin-oleate media were sufficient to provide excess TPP, unbound to albumin. Whole cells of T. denticola were shown to have proteolytic activity toward casein and alpha globulin proteins. Alpha globulin proteins were also found avidly attached to T. denticola cells that had been suspended in alpha globulin.