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The Ohio State University, Ph.D., 1976
Microbiology

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1976
STUDIES ON MYCOPLASMA
GROWTH FACTORS IN SERUM

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

by
Leigh Rice Washburn, B.A., MT(ASCP)

* * * * *

The Ohio State University
1976

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To my parents, Ellen E. and Franklin T. Rice
for their faith in me;
to my husband, Bruce Alan Washburn,
for his patience and support;
and to my daughter, Erika Leigh Washburn,
with love ...
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My respect and gratitude extend to my adviser, Dr. Norman L. Somerson, for his time, efforts, and encouragement, and to the other members of my graduate committee, Drs. V.V. Hamparian, J.H. Hughes, F.A. Kapral, R.W. Lang, and J.D. Pollack, for their advice and assistance.

I also wish to express my appreciation to John P. Kocka, for his excellent technical assistance, and my thanks to John M. Gnau, who assisted with photomicrography.
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FIELD OF STUDY

Major Field: Medical Microbiology

Mycoplasmology. Mycoplasma nutrition -- isolation and characterization of mycoplasma growth factors in serum.
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INTRODUCTION

The mycoplasmas belong to the Class Mollicutes, Order Mycoplasmatales (12,13,87). This Order is further divided into two Families, Mycoplasmataceae and Acholeplasmataceae, containing the genera Mycoplasma and Acholeplasma (14), respectively. Three additional genera have been described, Thermoplasma (7,4), Ureaplasma (66), and Spiroplasma (64).

The rigid cell walls typical of bacterial and fungal cells are absent in the Mollicutes, and these organisms depend on the plasma membrane for maintenance of cellular integrity. A major portion of the mycoplasma membrane is lipid in nature (75,89,41). As much as 22 to 25% of the membrane lipid of Mycoplasma species consists of cholesterol (49,62,89), which these organisms cannot synthesize but must obtain from exogenous sources (11,46,48,79). Acholeplasmas, in contrast, do not require sterol but synthesize carotenoid pigments (55,71,83), which probably occupy those sites in the membrane where cholesterol is incorporated by Mycoplasma species (55,71). When cholesterol is supplied in the culture media, the acholeplasmas also incorporate it into their membranes (55,71,75). Smith and coworkers have suggested that the requirement of Mycoplasma species for cholesterol reflects a genetic defect in the production of enzymes for carotenoid synthesis (74,77,83).
The requirement of the so-called "pleuropneumonia-like organisms" for some component of serum has long been recognized. Approximately twenty years ago cholesterol was identified as one of the components of serum necessary for mycoplasma growth (11,48,78). Edward and Fitzgerald in 1951 (11) successfully grew several strains in medium which contained cholesterol, bovine serum albumin or starch, and an acetone-insoluble fraction of egg yolk (phospholipid) in place of serum. They concluded that both the cholesterol and the protein portion of the serum were required for growth. Rodwell in 1956 (48) reported that the agent of bovine pleuropneumonia (now known as *Mycoplasma mycoides* var. *mycoides*) also required sterol in the culture medium. Smith and Morton in the early 1950's (80,81) isolated by ammonium sulfate precipitation of bovine serum a protein fraction which contained all the growth supporting activity. Smith *et al.* (78) tentatively identified it as a lipoprotein and speculated that its function was to supply lipids required for mycoplasma growth.

Several compounds similar to cholesterol also possess growth promoting activity. Edward and Fitzgerald (11) indicated that cholestanol and stigmasterol were equally as effective as cholesterol. Through the efforts of Smith *et al.* (73,79,82) and Rodwell (49), the structural requirements determining growth supporting ability were elucidated. To promote mycoplasma growth, a sterol must be a planar molecule with an equatorial 3-OH group and an apolar side chain; the latter is probably the portion which adsorbs to the cell (82). Although sterols with other configurations may be incorporated into membrane lipids, no
growth occurs unless these conditions are met, probably because these other configurations do not allow proper movement of the sterol molecule in the membrane (73).

By 1961 Smith and Rothblat (82) and Rothblat and Smith (55) had proven that cholesterol was actually incorporated into mycoplasma cell membranes. However, its function remained obscure for some time. Smith and coworkers (55, 69, 70, 71, 72, 74) suggested a dual function, both as an actual structural component of the membrane and as a mechanism for transport of glucose and fatty acids through the membrane. Substantiating the transport hypothesis was the discovery of cholesterol esterase activity associated with mycoplasma membranes (55, 69, 89) and β-glucosidase activity in the membranes of both sterol-requiring and -nonrequiring species (21, 55). Cholesterol esters of volatile fatty acids (primarily butyric and acetic) were found in sterol-requiring non-glucose-fermenting mycoplasmas (70, 72), which oxidize fatty acids as a major source of energy (31, 75). Acetic acid (the end product of glucose fermentation) esters and steryl or carotenyl β-D-glucosides were detected in mycoplasmas utilizing glucose as their primary energy source (21, 55, 71).

Smith (72, 74) proposed a membrane model in which cholesterol or carotenoid molecules function in transport. Substrates such as glucose or fatty acids are coupled at the 3-OH site to the cholesterol or carotenoid molecule in the membrane. The resulting change in polarity causes a rotation of the molecule; the substrates are thus transported into the cell where they are enzymatically cleaved from their carrier. End products of metabolism are then coupled to the same site, causing a
second change in polarity. Rotation of the lipid molecule brings the product to the outside of the membrane where it is cleaved off. The lipid is then free to begin the cycle once more.

There was some disagreement as to whether cholesterol was actually modified in such a manner after incorporation into membranes; several workers reported finding no evidence of esterification or alteration of any kind (1,2,40,49,61). There was, however, considerable support for the notion that cholesterol formed an integral part of the membrane structure (1,2,39,40,49,53,56,62). Rodwell and Abbot in 1961 (53) suggested that cholesterol affected the morphologic and physical properties of the mycoplasma membranes. Studies by Rottem et al. (56,62) and DeKruyff et al. (8) concluded that cholesterol functioned as a critical regulator of membrane fluidity by preventing crystallization of membrane lipids at temperatures below phase transition and exerting a condensing effect above (56,8). Membrane fluidity in turn affects such properties as osmotic fragility, permeability, and certain enzyme functions (8,39,62).

A requirement for long-chain fatty acids was first suggested by Rodwell in 1956 (48). Smith and Boughton in 1960 (76) noted some enhancement of mycoplasma growth by sodium-oleate, as well as some incorporation into the cells. Rodwell and Abbot in 1961 (53), further investigating the role of fatty acids in mycoplasma nutrition, concluded that both saturated and unsaturated long-chain fatty acids were needed by Mycoplasma mycoides var. mycoides for maintenance of membrane integrity and for filamentous growth. In 1963 Tourtellotte et al. (89) reported that Mycoplasma gallisepticum also incorporated
long-chain fatty acids from the culture medium into its membrane lipids. Rodwell (50,51,52) and Rodwell and Peterson (54) reported a similar requirement for Mycoplasma species strain Y, which is thought to be similar to M. mycoides (51).

In 1963 Razin and Rottem (44) described an unsaturated, but not a saturated, fatty acid requirement for Acholeplasma laidlawii B. Later, it was found that this was not an absolute requirement but rather seemed to depend upon the medium and the strain of Acholeplasma tested (20). Some strains, including A. laidlawii B, can, in fact, grow without an added octadecenoic acid in complex delipidized culture media (20). Although acholeplasmas cannot synthesize unsaturated fatty acids de novo (20,44), at least one species (species KHS) appears to have some capacity for elongating monoenoic acid precursors to octadecenoic acids (20,34). Acholeplasma laidlawii A, in contrast, does possess an absolute need for preformed octadecenoic acid in the culture medium, since it is unable to effect elongation of precursors past sixteen carbon atoms (35,58).

In 1967 Pollack and Tourtellotte (38) discovered that long-chain saturated fatty acids could be synthesized from acetate by three Acholeplasma strains. This finding was confirmed for A. laidlawii by Rottem and Razin (60) and Rottem and Panos (59), and the presence of acyl carrier protein was confirmed by Rottem et al. in 1973 (57). In 1974 Herring and Pollack (22) reported that fifteen Acholeplasma species were capable of synthesizing membrane lipids from acetate while six Mycoplasma species were unable to do so, and on this basis, it was suggested that this capability was one useful criterion for
differentiating the two genera.

The hypothesis was set forth in 1961 by Rodwell and Abbot (53) that long-chain fatty acids supplied in the growth media affected mycoplasma membrane integrity. The dependence of mycoplasma and acholeplasma membrane structure and integrity on the proper quantity and balance of long-chain fatty acids was investigated by a number of workers (8,11,20,23,24,40,43,45,51,52,54,58,89). Cell membrane characteristics which depend on membrane elasticity were shown to be dependent upon the fatty acid content and composition of mycoplasma membrane lipids (8,23,24,40,43,45,54,58). These characteristics include osmotic fragility (23,40,45,58) and the ability to grow in long branching filaments; the latter requires not only the appropriate quantity but also the appropriate ratio of saturated to unsaturated fatty acids (40,43,44,45,53).

The role in nutrition of the protein and phospholipid, or other surface active agents, present in serum has also been explored. Edward and Fitzgerald (11) recognized that the protein portion of serum was required for growth in addition to the sterol. These workers demonstrated as well the growth enhancing properties of an acetone-insoluble fraction of egg yolk, which probably consisted mostly of phospholipid. Rodwell (48) cited the need for a protein fraction of serum to be added along with cholesterol and sodium-oleate for growth of M. mycoides. Smith and coworkers (76,79) postulated that the role of the surface active agents in serum, including phospholipids, was in the solubilization of cholesterol and that the protein functioned in the regulation of sterol uptake by the mycoplasma cells.
Another role for serum protein appears to be the supply of fatty acids to the cells (44), since fatty acids are carried in vivo by the albumin fraction of blood plasma (10,18,86). Long-chain fatty acids, although essential for growth, are, in free form, quite toxic to mycoplasma cells (11,48,53,76), even at concentrations which are suboptimal for growth (51). When added separately to culture media instead of as a component of serum, they must be combined with a defatted protein such as BSA or some other compound which prevents their lytic action and allows their slow release to mycoplasma cells (43,44,50,51,52).

Finally, serum protein may provide mycoplasmas with a source of peptides and amino acids (50). Peptide (30,50,90) and amino acid requirements have been demonstrated for both Mycoplasma and Acholeplasma species. Peptide function appears to be two-fold: (a) the detoxification of fatty acids and (b) a supply of amino acids necessary for growth (50,75,90).

For routine cultivation of mycoplasmas, serum remains the least expensive and most convenient source of essential lipids and protein. Unfortunately, commercial serum preparations used in culture media are inconsistent in quality, probably partly due to variations in concentrations of growth factors (25). Alternatives to such serum preparations as bovine serum fraction, prepared by the method of Smith and Morton (80), have not been extensively explored. The present study was undertaken to isolate from serum those components required for mycoplasma growth, characterize them, and determine their suitability as serum substitutes. Serum is only one of a number of
undefined constituents of mycoplasma culture media. Serum replacement with a well characterized product will aid in the eventual development of a defined medium for the culturally fastidious mycoplasmas.
Serum is an essential ingredient of culture media for many mycoplasmas, supplying essential protein (48,76,78) as well as lipids, such as cholesterol (11,48,79) and fatty acids (40,43,44,50,53). In 1951 Smith and Morton (80), using ammonium sulfate precipitation, isolated a mycoplasma growth factor from serum. This serum fraction was later reported to strongly resemble α-lipoprotein, and it was thought to serve as a source of lipids required for mycoplasma growth (78). Smith and Boughton (76) postulated that, while the sterol fraction of the lipoprotein was incorporated into mycoplasma cells, the phospholipid and protein moieties were required for solubilization and regulation of sterol uptake.

The process described by Smith and Morton for the isolation of this serum growth factor is employed in the commercial production of bovine serum fraction (BSF). This product is often used in mycoplasma culture media in place of whole serum, which occasionally contains inhibitory substances (80). However, the ability of commercial BSF to promote mycoplasma growth varies from lot to lot (25). Hughes et al. (25) separated BSF into three major components by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and observed that the concentration of one of these, a slowly migrating component
(SMC), appeared to be directly related to growth promoting activity. The ability of the SMC to take up lipid dyes suggested that it might contain lipoprotein. The other two components (components II and III) were not characterized but were present in approximately equal concentrations among different preparations of BSF. The purpose of this paper is to present our results on the isolation and further characterization of the mycoplasma growth factors in BSF.

MATERIALS AND METHODS

Culture Media. The SSR2 broth medium, previously described (9,37), is a buffered medium supplemented with BSF (PPLO Serum Fraction, Difco Laboratories, Detroit, Mich.), yeast extract (Grand Island Biological Company, Grand Island, N.Y.), glucose, phenol red, and Eagle's minimum essential medium with glutamine (Grand Island). When culturing Mycoplasma arthritidis, 0.5% L-arginine HCl was included. Since Mycoplasma pneumoniae is inhibited by arginine, it was omitted from media used to grow this organism. A second broth medium and an agar medium similar to the Chanock, Hayflick, and Barile formulation (5) were used with modifications. We included 1% glucose (w/v), 0.002% phenol red (in broth only), 5% BSF substituted for horse serum, and, for M. arthritidis, 0.5% L-arginine HCl.

The BSF used in this study came from two different commercial lots with similar growth promoting activities. One lot was used in media formulated with BSF. The other lot was chromatographed on Sephadex G150 columns: the resulting fractions, containing the SMC and components
II and III, were tested as substitutes for BSF in our studies on growth promoting activities of BSF components. In some experiments, crystalline fatty acid-free bovine serum albumin (BSA, Fraction V, Pentex, Kankakee, Ill.) was also included in the culture media. When we analyzed this BSA preparation by four-directional thin-layer chromatography, we detected no lipids (36).

All media received 500,000 units per liter of potassium penicillin G (E.R. Squibb and Sons, Inc., Princeton, N.J.) and thallous acetate (Matheson, Coleman and Bell, Norwood, Ohio) at a final concentration of 0.025% (w/v).

**Mycoplasma Stock Cultures.** *Mycoplasma pneumoniae* strain CI-8 was isolated at The Children's Hospital, Columbus, Ohio, and identified by immunofluorescence and complement fixation using specific reference reagents. It was cloned and subcultured on media containing BSF and by the eighth passage grew as a confluent layer of organisms attached to glass (84). After the thirteenth passage the glass-adherent mycoplasmas (GAM) were suspended in a solution containing seven parts broth medium and three parts 50% (w/v) sucrose, divided into 1.2 ml aliquots, and stored at -60 C. *Mycoplasma pneumoniae* strain 65-2161, originally isolated from a two-year-old child with pneumonia and supplied to us by Dr. Jose Canchola from Children's Hospital, Washington, D.C., was similarly identified, cloned, and subcultured on BSF-containing media and frozen after the nineteenth passage. *Mycoplasma arthritidis* strain PC27 was subcultured six times in BSF-containing broth media, and, after the seventh passage, was pooled and frozen as described for
M. pneumoniae.

Inoculation of Cultures for Growth Experiments. In preparing inoculum, we thawed the contents of a vial from one of the frozen pools and grew the organisms as a confluent layer on glass in a six-ounce Brockway prescription bottle containing twenty-five ml of liquid medium. M. pneumoniae cultures were incubated for forty-eight hours, after which the GAM were harvested by trypsinization (84). The GAM were suspended in one or two ml of fresh broth which did not contain any serum or serum component. Mycoplasma arthritidis cultures were incubated for twenty-four hours; the GAM were then suspended in one to two ml serum-free broth by shaking with sterile glass beads (85). Aliquots of 0.05 ml of these mycoplasma suspensions were used as inoculum for broth cultures.

In preparing inoculum for solid media, the mycoplasmas were first grown in six-ounce bottles as described. After the appropriate incubation time, the GAM were suspended in twenty-five ml of serum-free broth by shaking with glass beads. This suspension was filtered through a 0.4 (for M. pneumoniae) or a 0.6 (for M. arthritidis) µm Nuclepore filter (Nuclepore Corp., Pleasanton, Calif.) and then diluted one to one hundred in serum-free broth; 0.10 ml of diluted filtrate was inoculated onto each agar plate.

Quantitation and Assessment of Mycoplasma Growth. Mycoplasma growth in culture bottles was assessed by measuring both the final pH of the medium and the quantity of GAM. The latter was determined by removing
the GAM from the bottles and measuring the total protein. Growth experiments were performed in three-ounce prescription bottles containing twelve ml of broth. We incubated *M. pneumoniae* cultures for seventy-two and *M. arthritidis* for forty-eight hours before assaying for mycoplasma growth. After the appropriate incubation period, the pH of the broth was determined. The GAM remaining in each bottle were rinsed four times with five-ml volumes of 0.15 M phosphate buffered saline, pH 7.3 (PBS), and were suspended in one ml of PBS by shaking the bottle with glass beads. The Lowry protein content of each suspension was determined (29), and the results were expressed as micrograms of GAM protein per bottle. The amount of GAM protein correlated directly with the extent to which the pH had declined within the pH range 7.6 to 6.0 for *M. pneumoniae* (correlation coefficient = 0.957) or risen within the range 7.6 to 8.4 for *M. arthritidis* (correlation coefficient = 0.894).

The mean values for GAM protein and the standard error of the mean (SEM) were calculated in most cases from four replicate cultures. When GAM protein values were derived from duplicate cultures, only the means were calculated; however, based upon statistical analysis of 121 sets of replicate cultures, we determined that, for *M. pneumoniae*, a difference in the means of replicate cultures of one hundred micrograms GAM protein should be considered significant (*P* < 0.05).

*Mycoplasma arthritidis* colonies on agar were photographed through a Nikon phase contrast microscope at a 50X magnification with Automatic Microflex AFM camera attachment.
Electrophoresis and Sephadex Column Chromatography. The methods described by Hughes et al. (25) were used for SDS PAGE and, with slight modifications, for Sephadex G150 column chromatography. Fractions were eluted from the Sephadex columns with 0.002 M NaCl, pH 7.8, at 5 C., and the bed volume of the column was approximately 200 ml. Five-ml samples of BSF were applied to the column.

In our immunoelectrophoresis studies, we used human serum fraction (HSF), which was prepared for us by the method of Smith and Morton (80) and donated by Difco Laboratories. HSF rather than BSF was chosen for analysis for lipoproteins by immunoelectrophoresis because of the ready availability of anti-human-lipoprotein antisera. Immunoelectrophoresis was performed according to the method of Schiedegger (65), using 1% Noble agar and barbital acetate buffer, pH 8.6, on 1 by 3 inch glass microscope slides. After electrophoresis, slides were developed with goat anti-human-serum, or by commercial (Behring Diagnostics, Woodburn, N.Y.) anti-human-α- or β-lipoprotein-sera or anti-human-albumin-serum produced in rabbits. After development, slides were photographed, then dried and stained with either Oil Red O or Crowle's Triple stain (6).

The Osserman immunoelectrophoresis technique (33) was used to detect lipoproteins in components of HSF obtained by Sephadex chromatography. Whole human serum was placed in a well in the middle of an agar-covered microscope slide and electrophoresed. Troughs were then cut above and below the well parallel to the direction of the electrophoresis; anti-α- or β-lipoprotein-serum was placed in the top trough and the HSF component to be tested in the bottom. The antiserum formed
a precipitin arc with the lipoprotein from the whole serum. If that specific lipoprotein was also present in the HSF component in the bottom trough, a precipitin line was produced which extended the length of the slide between the two troughs, forming a line of identity with the arc.

Microzone electrophoresis (Beckman, Spinco Division, Palo Alto, Calif.) of serum proteins on cellulose acetate strips was performed on BSF and HSF by the clinical chemistry laboratory at The Children's Hospital, Columbus, Ohio (26,93).

RESULTS

Sephadex G150 Chromatography and PAGE of BSF. Bovine serum fraction was separated into two major 280 nm-absorbing components by column chromatography (Fig. 1). We collected and pooled four three-ml fractions from the first peak, forming pool I. Pool II was obtained by pooling four fractions from the second peak. Pools I and II and BSF were then subjected to PAGE (Fig. 2).

Confirming the results of Hughes et al. (25), BSF separated into three major bands on SDS PAGE, the SMC and components II and III. Pool I contained a band corresponding to the SMC and small amounts of components II and III, and pool II contained only components II and III. In addition, pool I showed a small fourth band between the SMC and component II which was not observed on PAGE of BSF.

Growth Supporting Activities of Pools I and II in Broth Medium. To assess the growth promoting activities of these components of BSF, we
incorporated pools I and II into broth medium in place of BSF (Table 1).

Table 1. Effect of pools I and II on growth of *Mycoplasma pneumoniae* Cl-8 and *M. arthritidis* PG27 in SSR2 broth.*

<table>
<thead>
<tr>
<th>Serum component</th>
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<th>M. pneumoniae Gam protein (µg)</th>
<th>M. arthritidis Gam protein (µg)</th>
</tr>
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<tbody>
<tr>
<td>BSF</td>
<td>3</td>
<td>6.16 ± 0.12</td>
<td>8.14 ± 0.01</td>
</tr>
<tr>
<td>no serum</td>
<td>-</td>
<td>7.47 ± 0.01</td>
<td>7.64 ± 0.01</td>
</tr>
<tr>
<td>pool I</td>
<td>6</td>
<td>7.07 ± 0.01</td>
<td>7.89 ± 0.02</td>
</tr>
<tr>
<td>pool II</td>
<td>6</td>
<td>7.40 ± 0.01</td>
<td>7.70 ± 0.01</td>
</tr>
<tr>
<td>pool I + pool II</td>
<td>6 each</td>
<td>6.96 ± 0.01</td>
<td>8.19 ± 0.02</td>
</tr>
</tbody>
</table>

* Each value is the mean pH or GAM protein content of four broth cultures plus or minus one standard error (SEM).

Each pool was tested at a 6% final concentration. Pool I, despite its low protein content, 305 µg/ml, was considerably more active than pool II, containing 8.5 mg/ml protein. However, it was apparent that pool II also contained one or more growth factors, since mycoplasma growth increased significantly when pool II was added to pool I (P<0.001). No attempt was made in this initial experiment to determine the optimal concentrations or ratios of the growth factors in pools I and II, since our intention was only to determine which fractions showed growth supporting activity.

We performed one additional experiment to confirm that pool I contained the major growth factor of BSF. We showed that *M. pneumoniae* was able to survive twenty passes in SSR2 broth containing 6% pool I as the only serum constituent. Numerous attempts to pass these
mycoplasmas in broth supplemented with pool II alone failed.

The SMC concentration was previously reported to be growth limiting for M. pneumoniae (25). To determine if pool I, containing the SMC, had such an effect, we examined both M. pneumoniae and M. arthritidis growth in broth supplemented with pool I at concentrations ranging from zero to 25% (Fig. 3). There was a linear relationship between the amount of pool I added to the medium and the level of M. pneumoniae growth. For M. arthritidis, the amount of growth also depended on the pool I concentration, although statistically the relationship was not linear, and growth improved very little at pool concentrations above 15%.

Analysis and Comparison of Human Serum Fraction to BSF. Human serum fraction was also separated into two major components by Sephadex chromatography, and pools I and II were collected as described for BSF; these will be referred to as pools H-I and H-II. Electrophoretic separation patterns of HSF and pools H-I and H-II on polyacrylamide gels were similar to those observed with BSF. An SMC-like band appeared in pool H-I; pool H-II showed bands similar to components II and III, and all three bands were observed on PAGE of HSF. Growth promoting activity again appeared primarily in pool H-I, and growth could be improved by the addition of pool H-II.

Microzone electrophoresis revealed that BSF and HSF were composed primarily of albumin, 84% and 95%, respectively. In addition, immuno-electrophoresis of samples of pools H-I and H-II revealed that albumin was present in both pools and apparently was the major constituent of
of pool H-II (Fig. 4). Although we could not detect any lipoprotein in pool H-I by immunoelectrophoresis, with the Osserman technique we demonstrated the presence of β-lipoprotein (Fig. 5A); no α-lipoprotein was detected (Fig. 5B). We observed no lipid-containing substances in pool H-II. Thus albumin was the major constituent of both human and bovine serum fractions, and almost all of it apparently eluted from the Sephadex column in the second peak.

**Growth Supporting Activity of Albumin in Broth Media.** Albumin, as the major constituent of pool II, was thought responsible for its growth enhancing properties, and, to confirm this, we tested the growth promoting activity of fatty acid-free crystalline BSA substituted for pool II. Broth media were prepared without serum or BSF and were supplemented with 6% (final concentration) pool I. Quantities of BSA ranging from zero to 3.3% (w/v) were added to the media and assessed for their ability to enhance the growth of two strains of *M. pneumoniae* (Fig. 6A) and strain PG27 of *M. arthritidis* (Fig. 6B). Media with a BSA content of 0.8% permitted significantly better growth of both *M. pneumoniae* strains (P<0.001) and *M. arthritidis* (P<0.01) than media without BSA. *M. pneumoniae* strain Cl-8 actually grew as well with a combination of pool I and BSA as with BSF (Fig. 6A). BSA levels above 0.8% were significantly inhibitory for *M. pneumoniae* (P<0.001) and for *M. arthritidis* in SSR2 broth (P<0.01). Like pool II, BSA in the absence of pool I showed very little activity for either species.

To determine if altering the pool I content of the medium would affect the optimal concentration of BSA, we varied the concentrations...
of both pool I and BSA in broth (Table 2). In this and in additional

Table 2. Effect of alteration of the pool I level in broth on the optimal BSA concentration.

<table>
<thead>
<tr>
<th>pool I concentration (%)</th>
<th>6</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA concentration (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.40</td>
<td>450</td>
<td>540</td>
<td>570</td>
</tr>
<tr>
<td>0.80</td>
<td>515</td>
<td>658</td>
<td>620</td>
</tr>
<tr>
<td>1.25</td>
<td>292</td>
<td>445</td>
<td>520</td>
</tr>
<tr>
<td>1.70</td>
<td>202</td>
<td>269</td>
<td>292</td>
</tr>
</tbody>
</table>

* Each value is the mean GAM protein content (µg) calculated from duplicate cultures.

experiments, we observed optimal growth of M. pneumoniae at 0.8% BSA regardless of the pool I concentration. In contrast, several experiments with M. arthritidis indicated that BSA ceased to have any effect on growth when pool I levels were raised to 10% or higher, and concentrations of BSA as high as 3.3% were not inhibitory.

Growth Supporting Activities of Pools I and II and BSA in Agar. Pools I, II, and BSA were incorporated separately and in combinations into agar in place of BSF to determine if they affected mycoplasma colony formation on solid medium just as observed with GAM growth in bottle cultures. M. pneumoniae failed to grow on agar plates with either pool II or BSA alone. Colonies on media containing pool I or pool I combined with either pool II or BSA were similar in size and morphology to those on BSF-containing plates. However, due to the small size and
nondistinctive morphology of these colonies, even under the best of conditions, it was difficult to distinguish differences in growth promoting activity among these serum preparations with *M. pneumoniae* as test organism.

Differences were much more striking with *M. arthritidis* (Fig. 7). Once again, no growth occurred on plates supplemented with pool II or BSA alone. Small colonies appeared on plates containing pool I alone (Fig. 7A), and the addition of pool II (Fig. 7B) or BSA (Fig. 7C) to agar containing pool I resulted in the appearance of large characteristic "fried egg" colonies similar in size and morphology to those growing on BSF (Fig. 7D).

**DISCUSSION**

We obtained only two major peaks by Sephadex G150 chromatography of BSF, in contrast to the three observed by Hughes and coworkers (25). Our failure to separate components II and III may have been due to differences in flow rate or quantity of BSF applied to the column. However, our results with PAGE (Fig. 2B) showed that pool II was comprised of both components II and III. Their failure to separate on Sephadex columns was unimportant, since both could be replaced by BSA in culture media.

A small fourth band appearing between the SMC and components II and III of PAGE is of minor interest. It may be a breakdown product of the SMC or possibly a component of BSF which was too dilute to be detected in unchromatographed BSF.
In the complex and undefined culture media used to grow *Mycoplasma pneumoniae*, BSF supplies at least two growth factors. One of these, contained in pool I, is probably lipid in nature, possibly in the form of lipoprotein. The other, in pool II, is in all likelihood albumin. These two serum components functioned as growth factors for two metabolically diverse mycoplasmas, *M. pneumoniae* and *M. arthritidis*, suggesting that our results are probably applicable to a number of *Mycoplasma* species.

*M. pneumoniae* responded much more dramatically than *M. arthritidis* to changes in the serum constituents of broth. However, the level of growth of *M. arthritidis* in pool I plus BSA was never as high as in BSF. This may indicate that some nutrients in BSF, for instance fatty acids, which are used by nonfermenting mycoplasmas as a major energy source (31,75), are present in suboptimal concentrations in pool I. Fatty acids are carried by the albumin fraction of blood plasma (10, 18,86). This may explain the inability of fatty acid-depleted BSA to improve *M. arthritidis* growth as dramatically as pool II. Also, the fact that there was light growth of *M. arthritidis* through one subculture in broth media containing pool II or BSA alone could indicate that these organisms store other nutrients from the previous passage.

The quantity of pool I in broth was growth limiting for both mycoplasmas; however, growth in pool I alone, even at concentrations as high as 25%, never reached the level of growth in BSF. This was not due to dilution of the growth factor in pool I during the chromatography process, because pool I's growth promoting activity was
greatly enhanced by the addition of pool II or BSA. This phenomenon strongly suggests that pool I lacked sufficient protein to promote optimal growth. In fact, the ability of pool I to support even a moderate amount of growth was probably due to the presence of a small quantity of protein supplied either by pool I itself or by the basal medium. Our results agree with reports by other workers on the need for serum protein in the culture media (11,48,76).

The sterol-requiring mycoplasmas are unable to synthesize long-chain fatty acids but require them for growth (40,43,44,50,53). However, these nutrients by themselves are quite toxic to mycoplasma cells. When fatty acids are supplied in serum, they are complexed to albumin and thus detoxified. When they are added separately to culture media, they are often added in combination with a protein such as BSA, which allows their slow release and prevents the accumulation of toxic levels (43,44,48,50,51,53). The BSA in our media may function to detoxify fatty acids or other surface active agents in pool I or in other medium constituents (48,76).

However, we observed that levels of BSA above 0.8% in media were inhibitory to mycoplasma growth (Fig. 6). This is in accord with a report by Rodwell (51) that some samples of BSA which had been extensively extracted of lipids were growth inhibiting for Mycoplasma strain Y. He speculated that these fatty acid-poor BSA samples bound fatty acids in the medium too firmly to permit their normal interaction with the mycoplasma cells. Spector et al. (86) proposed a model for albumin-fatty acid interaction which tends to support Rodwell's hypothesis. They suggested that BSA possesses six high energy binding sites for
fatty acids, as well as a number of weak sites. It is possible that extensive lipid extraction of BSA frees these high energy sites, thus allowing fatty acids in the growth media to bind so tightly that they can no longer function as nutrients. This may explain why higher levels of our fatty acid-free BSA were toxic to M. pneumoniae and M. arthritidis.

Another possible function for BSA is the regulation of sterol uptake by mycoplasma cells (76,78). Pool I is poor in protein, and we demonstrated a lipoprotein in pool H-I only with difficulty. If pool I lacks sufficient protein, the BSA may indeed have some effect on cholesterol uptake. Finally, BSA may also serve as a source of peptides (50), and some mycoplasmas have shown proteolytic activity against serum proteins (91).

Mycoplasma culture media are varied and complex, and currently used media invariably contain undefined constituents, including animal products. Variations in BSF's complement of cholesterol and fatty acids, and probably other materials as well, are probably reflected in growth supporting activity. In support of the findings of Hughes and coworkers (25), it appears that the concentration of the SMC, present in pool I, directly determines the growth supporting activity for a particular lot of BSF. However, activity of the SMC-containing fraction also depends on the presence of a protein-rich component which is mostly albumin and which can be completely replaced by BSA. Unlike the SMC, slight variations in albumin concentration in BSF are probably insignificant, but albumin is a requirement for mycoplasma growth.
Figure 1. Separation of bovine serum fraction into two components by Sephadex G150 column chromatography. The column effluent was scanned continuously at 280 nm. Four three-ml fractions were collected from each peak, forming pools I and II.
Figure 2. Polyacrylamide gel electrophoresis of (A) bovine serum fraction and (B) pools I (----) and II (--.--.--). Protein bands were stained with Coomassie blue dye, and the gels were scanned at 610 nm.
Figure 3. Effect of pool I concentration on growth of *M. pneumoniae* Cl-8 and *M. arthritidis* PG27 in SSR2 broth. Each point is the mean of duplicate cultures.
Figure 4. Immunoelectrophoresis of pool H-II derived from Sephadex column chromatography of human serum fraction. The slide was developed with anti-human-serum (top trough) and anti-human-albumin-serum (bottom trough). Arrows indicate albumin precipitin arcs.
Figure 5. Drawings of Osserman immunoelectrophoresis test to detect lipoproteins in pool H-I. Whole human serum was electrophoresed. Pool H-I was then placed in the bottom trough of both slides. Anti-\(\beta\)-lipoprotein-serum was placed in the top trough of slide A and anti-\(\alpha\)-lipoprotein-serum in the top trough of slide B. The presence of a line continuous with the \(\beta\)-lipoprotein precipitin arc on slide A indicates the presence of \(\beta\)-lipoprotein in pool H-I. No \(\alpha\)-lipoprotein was present, since there is no Osserman line on slide B.
Figure 6. Effect of bovine serum albumin on mycoplasma growth in broth supplemented with 6% pool I. Each point represents the mean of four to eight replicate cultures and the vertical lines, one standard error to either side of the mean. *Mycoplasma pneumoniae* (A) strain Cl-8 was tested in SSR2 broth (-----) and strain 65-2161 in Modified Hayflick broth (o-----o). *Mycoplasma arthritidis* strain PG27 (B) was tested in both SSR2 (-----) and Modified Hayflick (-----) broth. (a) Growth in media supplemented with BSF. (b) Growth in media supplemented with 0.8% final concentration BSA as the only serum component.
Figure 7. Effect of pools I, II, and BSA on colony formation of *M. arthritidis* on agar. Mycoplasmas were grown on agar medium supplemented with 6% pool I (A), 6% pool I + 6% pool II (B), 6% pool I + 0.8% BSA (C), and 5% BSF (D).
Members of the genus Mycoplasma require sterol and fatty acids for growth and membrane synthesis (11,44,46,48,53,79,89). These components are usually provided by serum in the culture media. For some Mycoplasma species serum can be replaced by purified cholesterol combined with a solubilizing agent (27,40,46,51,76) along with fatty acids complexed to a defatted serum protein or some other detoxifying substance (48,50,52,53). However, for routine cultivation of mycoplasmas, serum is the least expensive and most convenient source of these nutrients. Plasma lipoproteins, which solubilize and transport cholesterol in vivo, have also been reported to serve as sources of sterol in mycoplasma culture media (78,79) and are capable of donating cholesterol to mycoplasma membranes (67). Unfortunately, growth factors such as lipoproteins are not always present in the same quantity in all commercial serum preparations, resulting in serious inconsistencies in culture media. For instance, bovine serum fraction (BSF), prepared commercially by the method of Smith and Morton (80) and often used in mycoplasma culture media in place of whole serum, varies widely in growth supporting activity, apparently due to differences in the concentration of a lipoprotein-like component (25).
The purpose of our present study was to isolate and further characterize the growth factors of serum and BSF. Additional information about these substances should eventually permit the production of a serum product which is both well-defined and consistent in quality. In an initial study (Part I), we demonstrated that two metabolically different mycoplasmas, *Mycoplasma pneumoniae* and *Mycoplasma arthritidis*, required for growth two components derived from BSF. One of these was identified as albumin. The other, although not fully characterized, was adjudged to contain lipoprotein. In the work presented here, we isolated by ultracentrifugation lipoproteins and other lipid-containing components of BSF and serum, partially characterized them, and compared their growth supporting activities.

**MATERIALS AND METHODS**

**Culture and Assessment of Growth.** The SSR2 and Modified Hayflick broth, the agar medium, and the source of our stock cultures of *Mycoplasma pneumoniae* strain Cl-8 and *M. arthritidis* strain PC27 have been described in Part I. The method of preparing inoculum and of assessing mycoplasma growth by measuring the glass-adherent mycoplasma (GAM) protein have also been previously described (Part I). In the present study, all tests with *M. pneumoniae* were performed in SSR2 broth, and with *M. arthritidis*, except for studies on agar, in Modified Hayflick broth.
Serum Preparations. Two lots of BSF (PPLO Serum Fraction, Difco Laboratories, Detroit, Mich.) with similar growth supporting activities were used throughout this study. Both lots were stored at -20 C. prior to use. One was used in ultracentrifugation studies, and the components obtained from this procedure were tested for growth-supporting activity as BSF substitutes. The other was used in media formulated with BSF. Horse serum and mycoplasma-free calf serum were purchased from Flow Laboratories, and one lot of each was used throughout this study. The horse serum was stored at -20 C. and the calf serum at 4 C.

Fresh bovine sera were obtained from The Ohio State University Veterinary Hospital, courtesy of Dr. Glen Hoffsis. Cows were bled aseptically from the jugular vein into sterile five hundred ml vacuum bottles. The blood was allowed to clot at 4 C. On the following day, the serum from each animal was separated by centrifugation and stored at 4 C. prior to fractionation by ultracentrifugation.

Human serum was obtained on two separate occasions from a twenty-eight-year-old male volunteer. The second sample was collected approximately one hour after the subject had eaten. In both instances, samples were subjected to ultracentrifugation immediately after collection.

Fractionation of BSF and Serum by Ultracentrifugation. The Hatch and Lees modification (19) of the method of Lindgren et al. (28) was used for the fractionation of serum and BSF. Ultracentrifugation of the BSF and serum samples was carried out in a Beckman Model L ultracentrifuge with type 40 rotor, using 1/2 by 2 1/2 inch cellulose nitrate
tubes (six ml capacity) with adapters. This technique isolates serum lipoproteins on the basis of their densities and involves four major ultracentrifugation steps (Table 3). On the first step a four-ml

Table 3. Sequential ultracentrifugation for isolation of serum lipoproteins.

<table>
<thead>
<tr>
<th>step and component no.</th>
<th>lipoprotein species to be isolated</th>
<th>density of solution added</th>
<th>density of top one ml</th>
<th>length of spin</th>
<th>force x g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spin I (comp. 1)</td>
<td>chylomicrons</td>
<td>1.006</td>
<td>1.006</td>
<td>1/2 hr</td>
<td>26,000</td>
</tr>
<tr>
<td>Spin II (comp. 2)</td>
<td>very low density lipoproteins (VLDL, density less than 1.007)</td>
<td>1.006</td>
<td>1.006</td>
<td>16 hrs</td>
<td>114,000</td>
</tr>
<tr>
<td>Spin III (comp. 3)</td>
<td>low density lipoproteins (LDL, density 1.007-1.063)</td>
<td>1.182</td>
<td>1.062</td>
<td>20 hrs</td>
<td>114,000</td>
</tr>
<tr>
<td>Spin IV (comp. 4)</td>
<td>high density lipoproteins (HDL, density 1.063-1.21)</td>
<td>1.478</td>
<td>1.20</td>
<td>40 hrs</td>
<td>114,000</td>
</tr>
</tbody>
</table>

sample was layered with two ml of NaCl solution, density 1.006, and centrifuged for one half hour at 26,000 x g. The top two ml, containing the chylomicrons, are removed. On each succeeding step the top one ml was collected, the second ml discarded, and the remaining four ml mixed with NaCl or NaCl-NaBr solutions of increasing densities. Each of the NaCl and NaCl-NaBr solutions also contained $10^{-4}$ M EDTA. Samples collected after all four steps were designated components 1 - 4; the pellet remaining after step IV was also saved. Occasionally the
procedure was initiated with step II, omitting the separation of the chylomicrons from the very low density lipoproteins (VLDL).

Components 3, 4, and the pellet were dialyzed for twenty-four hours against one thousand volumes of a solution containing 0.15 M NaCl, 0.001 M EDTA, and 500,000 units per liter potassium penicillin G. All components were refrigerated until used and, before incorporation into mycoplasma culture media, were dialyzed against two hundred volumes 0.15 M NaCl.

When these components were examined for growth promoting activity, a starting concentration of 3% was chosen, since this is the serum concentration routinely used in our culture media, although several concentrations were ultimately tested.

**Electrophoresis.** Polyacrylamide gel electrophoresis for the detection of lipoproteins was carried out according to the method of Frings et al. (17). The electrophoresis was performed in a Buchler Polyanalyst Cell in 3.8% polyacrylamide gels. Twenty to fifty microliters of prestained sample were applied to each gel; samples were then electrophoresed in Tris-glycine buffer, pH 8.3, for forty-five minutes at 4 mA per gel. Lipoprotein electrophoresis on agarose strips of serum components obtained by ultracentrifugation of BSF and human serum was performed by The Ohio State University Hospital's clinical chemistry laboratory by the method of Noble (32).

**Analysis of the Protein and Lipid Constituents of Serum and BSF.** Protein content was determined by the Lowry method (29). Total cholesterol was
measured by the method of Rudel and Morris (63), utilizing the o-phthalaldehyde color reagent of Zlatkis and Zak (94). Prior to analysis by thin-layer chromatography (TLC) lipids were extracted from 0.25 ml quantities of BSF and serum components by the Folch method (16). The lipids were resolved by the four-directional TLC technique of Pollack et al. (36) on 8 by 8 inch Silica-Gel-G-coated glass plates. Additionally, free cholesterol and fatty acids were resolved by unidirectional chromatography in benzene and ethyl acetate (100:20) (3) and esterified cholesterol in heptane and benzene (60:40) (36). Resolved components were visualized by spraying the plates with dichromate and $H_2SO_4$ and charring at 110 C. for approximately twenty minutes.

Lipids were extracted by the Folch method from larger quantities of BSF component 3 for the purpose of testing growth supporting activity of the extract in combination with fatty acid-free bovine serum albumin (BSA, Fraction V, Pentex, Kankakee, Ill.). The chloroform extract was dried, the remaining lipids were suspended in a few drops of propylene glycol and the mixture was diluted to the original volume with distilled water. This procedure resulted in a reduction in the protein content from 2080 µg/ml to 100 µg/ml (95%) and a 30% reduction in total cholesterol to 268 mg%.

Addition of BSA to Culture Media. Previous work (Part I) had shown that a protein-poor component of BSF, isolated by Sephadex chromatography, supported optimal mycoplasma growth only when extra protein, such as BSA, was added to the growth media. Preliminary experiments in the present study indicated that the components of BSF and serum
isolated by ultracentrifugation also required added BSA for optimal activity. No statistically significant differences in activities were noted for BSA in final concentrations of 0.4, 0.8, and 1.2% in media. Unless otherwise stated, all media except those supplemented with whole serum, uncentrifuged BSF, or pellet were prepared with BSA at a final concentration of 0.4%.

RESULTS

Electrophoresis and Cholesterol Assay of BSF and Serum Components. Each fraction of serum and BSF obtained by ultracentrifugation was analyzed by lipoprotein electrophoresis and for cholesterol content (Table 4). In BSF, only one lipoprotein was detected by electrophoresis in both acrylamide and agarose gels. This was an α-lipoprotein, or HDL, which appeared in component 4. Although we detected no lipoprotein in component 3, it consisted of a large amount of white buttery material which dissolved on treatment with chloroform-methanol (2:1). Most of the cholesterol also appeared in component 3.

By electrophoresis of commercial calf, horse, and fresh bovine sera, we found two lipoproteins, a β-lipoprotein (LDL) in component 3 and an α in component 4. Additionally, the two fresh bovine sera contained traces of α-lipoprotein in component 3. In contrast to BSF, the cholesterol contents of component 3 from all these sera were low; much larger amounts of this lipid appeared in component 4. No pre-β-lipoprotein (VLDL) was observed in any of the bovine or horse sera and virtually no cholesterol was present in components 1, 2, or the pellet.
Table 4. Lipoprotein and cholesterol contents of BSF, serum, and their components isolated by sequential ultracentrifugation.

<table>
<thead>
<tr>
<th>component no.</th>
<th>BSF calf fresh bovine sample A</th>
<th>fresh bovine sample B</th>
<th>horse</th>
<th>human</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 and 2</td>
<td>(density &lt;1.007)</td>
<td>none detected</td>
<td>chylo-microns + pre-β</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>(density 1.007-1.063)</td>
<td>β, trace α</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>β, trace α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>(density 1.063-1.21)</td>
<td>α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pelleted</td>
<td>(density &gt;1.21)</td>
<td>none detected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>uncentrifuged serum</td>
<td>none detected</td>
<td>α, β, α</td>
<td>β, α</td>
<td>α</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>total cholesterol (mg%)b.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 and 2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>pellet</td>
</tr>
<tr>
<td>uncentrifuged serum</td>
</tr>
</tbody>
</table>

a. All sera and components were electrophoresed in polyacrylamide gels; in addition, BSF and human serum and their components were electrophoresed in agarose gels with identical results.

b. Cholesterol values for BSF components 1 and 2 and all calf serum components were obtained by averaging values for 3 samples each, for BSF components 3 and 4, 4 samples each, and for the rest, 1 sample each.
Unlike BSF, components 3 and 4 from these sera both contained lipoprotein, and the majority of the cholesterol appeared in the high density fraction, component 4.

Human serum samples collected on two occasions were also fractionated by ultracentrifugation. As expected, electrophoresis revealed three lipoproteins, pre-β in component 2, β in component 3, and α and traces of β in component 4. Additionally, chylomicrons probably constituted the turbid material which appeared in combined components 1 and 2 from the second sample. Analysis of the second sample showed almost twice as much cholesterol in component 3 as compared to component 4, as well as a small amount in the VLDL-chylomicron fraction.

**Protein Assay of BSF and Serum Components.** The Lowry protein contents of components 3 and 4 from five serum samples as well as combined components 1 and 2 from human serum are listed in Table 5. There was a wide

<table>
<thead>
<tr>
<th>Component No.</th>
<th>BSF Lowry Protein Content (mg/ml)</th>
<th>Calf Serum: 1.10-4.00</th>
<th>Fresh Bovine Sera A and B: 0.44-0.54</th>
<th>Horse Serum: 0.32-0.42</th>
<th>Human Serum: 0.32-2.30</th>
<th>1 + 2: 0.56</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The range of values from four different samples of BSF components, three of calf serum, and one each of the others are shown.
range of values for different samples of BSF components 3 and 4. In all instances component 3 of whole serum contained considerably less protein than component 4.

**Thin-layer Chromatography.** The cholesterol-rich fractions of BSF and calf serum (components 3 and 4) were chosen for further analysis by four-directional TLC. The major lipid constituents appeared to be cholesterol and cholesteryl esters. In addition, component 3 of BSF and component 4 of calf serum contained triglycerides.

To detect cholesterol, cholesteryl esters, and fatty acids in these and the other components of BSF and calf serum, we also used unidirectional TLC in two solvent systems, benzene-ethyl acetate and heptane-benzene (Fig. 8). Neither cholesterol or fatty acids were detected in components 1 and 2; the pellets appeared to contain large quantities of free fatty acids, which was expected, since fatty acids are carried by the albumin fraction of blood plasma (10,18,86). However, besides the unfracionated sera, only components 3 and 4 contained both free and esterified cholesterol and free fatty acids. Components of one of the fresh bovine sera were also tested and, although not shown, results were identical to those obtained with calf serum.

**Growth Supporting Activities of BSF Components in Broth.** Growth supporting activities of BSF components were assessed by incorporating them into broth media at a final concentration of 3% (Fig. 9). The cholesterol-rich fraction, component 3, proved to be significantly more
active for both *M. pneumoniae* and *M. arthritidis* than component 4, the HDL-containing fraction. This finding was confirmed for *M. pneumoniae* by testing serial two-fold dilutions of components 3 and 4 for growth supporting activity (Fig. 10). Nearly all of component 4's activity was lost when it was diluted 1:4 (final concentration in broth of 0.75%); component 3, on the other hand, remained quite active at a dilution of 1:8 (final concentration 0.375%). Although there was no advantage to increasing the concentration of component 3 above 3%, growth of *M. pneumoniae* in concentrations ranging from 0.75% to 6% was as good as or better than growth in 3% BSF.

Statistical analysis of the data from these and additional experiments indicated a direct, statistically significant correlation for *M. pneumoniae* between the cholesterol concentration in each BSF component and its growth supporting ability in terms of µg of GAM protein (P<0.05). However, we no longer observed this relationship when the quantity of cholesterol in BSF components added to broth exceeded 600 µg. An example of this can be seen in Figure 10. Raising the component 4 level from 0.375% (24 µg cholesterol) to 6% (655 µg) and the component 3 level from 0.375% (89 µg) to 3% (713 µg) led to corresponding increases in mycoplasma growth, while increasing the concentration of component 3 above 3% had no such effect.

**Growth Supporting Activity of Lipid Extract of Component 3.** Since component 3 contained some protein in addition to a large amount of lipid, we wished to determine if the protein portion also contributed to its growth promoting ability. We prepared a lipid extract of component 3
and incorporated it into broth media at a final concentration of 0.75%; it was tested both in the presence and absence of BSA (Table 6). With

Table 6. Growth promoting activity of component 3 of BSF after reduction of protein.*

<table>
<thead>
<tr>
<th>BSF component</th>
<th>M. pneumoniae</th>
<th>M. arthritidis</th>
</tr>
</thead>
<tbody>
<tr>
<td>unextracted component 3 with BSA</td>
<td>585</td>
<td>692±52</td>
</tr>
<tr>
<td>lipid extract of component 3 without BSA with BSA</td>
<td>4±1</td>
<td>110±16</td>
</tr>
<tr>
<td>BSF</td>
<td>619±14</td>
<td>1142±42</td>
</tr>
</tbody>
</table>

* Both component 3 preparations were tested at a final concentration of 0.75% in broth. BSF was added at 3%. Mean GAM protein values were calculated from sets of four replicate cultures. For M. pneumoniae, the value for unextracted component 3 was obtained from duplicate cultures, both of which contained 585 µg GAM protein.

M. pneumoniae as test organism, removal of most of the protein from component 3 resulted in total loss of activity. Growth promoting activity was fully restored when BSA was included in the medium. Similarly, for M. arthritidis the protein-poor extract was considerably less active than unextracted component 3; activity returned on the addition of BSA. The importance of the protein constituents in growth promoting activity of component 3 was therefore demonstrated for both mycoplasmas.

Growth Supporting Activities for M. arthritidis of BSF Components in Agar.

For M. arthritidis, growth supporting activities of BSF components in
agar medium were very similar to those in broth (Fig. 11). No growth occurred with components 1 and 2. Colonies grown on component 3 and on the lipid extract of component 3 with added BSA were similar in size and "fried egg" morphology to those grown on BSF. Slight differences in detail in the photographs of these colonies are probably due to differences in the plane of focus. Although no quantitation was attempted, colonies on component 4 appeared somewhat smaller, a finding which we had anticipated from results obtained with component 4 in broth.

**Growth Supporting Activities of Components of Calf, Fresh Bovine, Horse, and Human Serum.** Two calf serum lipoproteins were isolated by ultracentrifugation, an LDL in component 3 and an HDL in component 4. Growth promoting activity of calf serum was confined to these two fractions (Fig. 12). In contrast to BSF, the HDL-containing fraction, component 4, was considerably more active than component 3. Also, component 4 exhibited much greater growth promoting activity than whole calf serum. As with BSF, those components lacking cholesterol -- components 1, 2, and the pellet -- were relatively inactive.

Similar results were obtained with a fresh bovine serum (sample B) and horse serum (Fig. 13). Again, growth was insignificant with components 1, 2, and the pellet, and activity was confined to the lipoprotein-containing fractions. The HDL fractions were more active than both the LDL and the whole serum samples from which they were derived. We tested two different concentrations of each component and of whole serum. When the whole serum concentrations were raised from 12% to 20%, the amount of growth changed very little, and in no instance did the amount of
growth in fresh bovine serum exceed 100 µg GAM protein, or in horse serum, 250 µg. However, growth improved dramatically when levels of components 3 and 4 were increased from 3 to 5%. Another sample of fresh bovine serum (sample A) gave a similar growth pattern, although the activities of components 3 and 4 were lower than with sample B. This was probably due to the overall lower cholesterol content of this specimen. The important point is that in every sample of bovine and horse serum tested, activity was limited to the lipoprotein fractions and was greatest in the HDL-containing components.

In further studies, samples of calf, fresh bovine, and horse sera were examined for growth supporting activity at concentrations in broth ranging from 3 to 20%. Increasing the concentrations of unfractionated calf and horse sera had no effect on *M. pneumoniae* growth, and only very slight improvement was observed with two fresh bovine sera at levels greater than 10%. All showed poor activity at all concentrations tested.

Human serum, containing three lipoproteins, exhibited activity in all components except the pellet (Fig. 14). However, in contrast to the other sera tested, human serum component 3, the LDL-containing fraction, was much more active than component 4. This sample of whole human serum was also quite inhibitory to *M. pneumoniae*.

Table 7 summarizes our findings for the HDL- and LDL-containing components isolated from bovine, horse, and human sera. Except for human serum, the HDL-containing fractions have higher cholesterol and protein concentrations than the LDL fractions, and they exhibit greater growth supporting activity for *M. pneumoniae* when incorporated into
Table 7. Cholesterol and protein content and growth supporting activities of HDL and LDL fractions of bovine, horse, and human sera.

<table>
<thead>
<tr>
<th>Lipoprotein and source</th>
<th>cholesterol (mg%)</th>
<th>protein ( yg/ml)</th>
<th>growth supporting activity ( yg GAM protein)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>component 3 (LDL, density 1.007-1.063)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>39</td>
<td>420</td>
<td>112±6</td>
</tr>
<tr>
<td>Horse</td>
<td>42</td>
<td>320</td>
<td>60±6</td>
</tr>
<tr>
<td>Human</td>
<td>248</td>
<td>2300</td>
<td>569±13</td>
</tr>
<tr>
<td>component 4 (HDL, density 1.063-1.21)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>170</td>
<td>2400</td>
<td>344±8</td>
</tr>
<tr>
<td>Horse</td>
<td>106</td>
<td>2400</td>
<td>259±29</td>
</tr>
<tr>
<td>Human</td>
<td>106</td>
<td>5200</td>
<td>194±3</td>
</tr>
</tbody>
</table>

* All components were tested at 3% final concentration in broth medium. GAM protein values were calculated from sets of four replicate cultures.

broth medium at a 3% final concentration. For human serum, the LDL-containing component, although poorer in protein than the HDL, was richer in cholesterol and exhibited considerably more growth supporting activity.

DISCUSSION

The importance of sterol in mycoplasma growth has been recognized since the early 1950's (11,48,78) and the need for fatty acids since
the early 1960's (43,53,89). The roles of cholesterol and fatty acids in the maintenance of mycoplasma integrity have been extensively investigated (36,40,43,45,53,82). Unfortunately, the serum preparations routinely added to culture media as the sources of these essential nutrients are often inadequate for optimum growth of the most fastidious mycoplasmas, probably partly because of low levels of these critical growth factors. The concept of serum lipoproteins as carriers of lipid growth factors in mycoplasma culture media was advanced by Smith, Lecce, and Lynn in 1954 (78); however, until very recently, isolated serum lipoproteins had not been further tested as practical serum substitutes in culture media. Slutzky et al. (67) have recently reported that human serum lipoproteins added to growth media along with BSA and elaidic acid, can function as sole sources of cholesterol for M. hominis. In our study, we examined bovine, horse, and human serum lipoproteins, as well as an extract of bovine serum lipids, as substitutes for serum in mycoplasma culture media. Several of these lipid-containing components were satisfactory substitutes for whole serum.

M. pneumoniae is a culturally fastidious organism and therefore a very sensitive species for nutritional studies. While M. arthritidis does not respond as dramatically to changes in serum constituents in broth media as M. pneumoniae, it nevertheless provided us with information on the requirements of a species metabolically quite different from M. pneumoniae. We obtained similar findings with both organisms, suggesting that the results of this study, as with Part I, may be applicable over a broad range of Mycoplasma species.
In the ultracentrifugation procedure we used for fractionating sera, each step is designed to concentrate a lipid-containing component of a specific density (19). However, the most active lipoprotein component obtained from each whole serum sample gave greater growth than would be predicted from simple concentration of the component. Unfractionated serum samples probably contain inhibitory substances which are eliminated with the pellet via the ultracentrifugation process. Therefore, ultracentrifugation is an effective means of isolating active growth components even when whole serum is growth inhibiting.

In medium supplemented with BSA, the protein portion of each component did not appear to be a critical determinant of the level of its growth promoting ability. In any case, any differences in protein content were probably obviated by the large amounts of exogenous BSA included in the media. The variations in protein content of BSF components 3 and 4 (Table 5) probably resulted from contamination of the top lipid layers with protein from underlying fractions.

The cholesterol content of each component was probably the most important determinant of the component's growth promoting activity. In fact, we have shown that growth supporting ability and cholesterol content of BSF components were statistically related; similar correlations were noted as well for the three bovine and the horse sera. All the active components of BSF and bovine serum contained cholesterol, cholesteryl esters, and free fatty acids. Thus, the cholesterol concentration of each component may be indicative of the relative concentration of other lipids, several of which are probably involved in growth (48, 53, 76, 78, 79).
The major lipoprotein species in cattle (92) and horses (47) is HDL; in humans, it is LDL (15). We observed that the most active growth promoting component isolated from each animal serum we tested carried the major serum lipoprotein of that animal (Table 7). However, results with BSF were somewhat different. Besides HDL, BSF carried a large amount of lipid which was not associated with an intact lipoprotein and which was obtained on the third step of the ultracentrifugation procedure, the step designed to isolate LDL. No LDL was present in our BSF, but the lipid in BSF component 3 most likely came from a deteriorated lipoprotein. This component possessed far more growth promoting potential than component 4, which contained HDL. Additionally, for M. pneumoniae, a chloroform extract of component 3 retained all the growth potential when combined with BSA. This indicates that growth promoting activity of serum does not depend on the presence of intact native lipoproteins.

Ultracentrifugation for the isolation of serum lipoproteins may be used to obtain components which will substitute for serum in mycoplasma culture media. Such materials are much less complex than whole serum and lack inhibitory substances such as antibodies. Lipid extracts such as the one we prepared from component 3 of BSF may also have potential as serum substitutes when combined with BSA. With such a preparation, control of the composition and quantity of serum lipids and protein of culture media may be possible, and an active serum product with controlled protein, cholesterol, and fatty acid content may aid in the development of a defined culture medium for M. pneumoniae and other culturally fastidious mycoplasmas.
Figure 8. Thin-layer chromatograms of lipids of BSF and calf serum fractions obtained by ultracentrifugation. Lipids were resolved on Silica-Gel-G-coated glass plates with solvents (A) benzene-ethyl acetate (100:20) and (B) heptane-benzene (60:40). Free fatty acids appear as long spindle-shaped spots on plate A.
Figure 9. Effect of components of BSF isolated by sequential ultracentrifugation on growth of *M. pneumoniae* and *M. arthritidis*. Each component was incorporated into broth at a final concentration of 3%. Mean GAM protein values were calculated for from four to eight replicate cultures. The vertical lines represent one standard error to either side of the mean.
Figure 10. Effect of serial two-fold dilutions of BSF components 3 and 4 on growth of M. pneumoniae. Each component was also tested undiluted (3%) and at twice the normal concentration (6%). BSF was tested at a final concentration of 3%. Each GAM protein value was calculated from duplicate cultures.
Figure 11. Effect of BSF components 3 and 4 on M. arthritidis colony formation on agar. Mycoplasma arthritidis was grown on agar medium supplemented with 3% component 3 (A), 1.5% of a lipid extract of component 3 (B), 3% BSF (C), and 3% component 4. Plates A, B, and D received in addition BSA at a final concentration of 0.4%.
Figure 12. Effect on growth of M. pneumoniae of calf serum and its components isolated by sequential ultracentrifugation. All components, whole calf serum, and BSF were incorporated into broth at a final concentration of 3%. Mean GAM protein and SEM values were calculated from triplicate cultures.
Figure 13. Effect on growth of M. pneumoniae of bovine and horse sera and components 3 and 4 isolated from them by sequential ultracentrifugation. Components 3 and 4 were each tested at final concentrations in broth of 3% and 5%; whole sera were added at both 12% and 20%, and BSF at 3%. Mean GAM protein and SEM values were calculated from sets of four replicate cultures.
Figure 14. Effect on growth of *M. pneumoniae* of human serum and its components isolated by sequential ultracentrifugation. All components and whole serum were incorporated into broth at final concentrations of 0.75% and 3%, represented by the first and second bars of each pair, respectively. BSF was added at 3%. Mean GAM protein values and standard errors were calculated from sets of four replicate cultures.
SUMMARY

Bovine serum fraction separated into two 280-nm-absorbing components by Sephadex G150 column chromatography. One component (pool I) was low in protein but appeared to contain lipid; the other (pool II) was very rich in protein, with albumin as its major constituent. Most of the growth supporting activity for *Mycoplasma pneumoniae* and *Mycoplasma arthritidis* appeared in pool I. Growth in pool I was stimulated by either pool II or fatty acid-free crystalline bovine serum albumin. Therefore, both the lipid and protein constituents of BSF were important for growth.

Lipoproteins were isolated by ultracentrifugation from BSF and whole serum. Activity was confined to the lipoprotein-containing components of whole serum. The greatest activity appeared in the HDL-containing fractions of bovine and horse and the LDL-containing fraction of human sera. As with pool I, the growth supporting components obtained by ultracentrifugation required additional BSA in the media for optimal activity.

An HDL was also isolated from BSF, but most of the growth supporting activity appeared in a fraction which contained a considerable quantity of lipid and some protein but no lipoprotein. A lipid extract of this fraction, combined with BSA, retained all of the fraction's growth supporting activity for *M. pneumoniae*.
The lipid constituents of the active fractions appeared to be the major determinants of their growth supporting abilities. All contained various quantities of cholesterol, cholesteryl esters, and free fatty acids, and there was a direct, statistically significant correlation between a fraction's growth supporting ability and its cholesterol content.

It appears that serum fractions with high growth supporting activities for mycoplasmas can be obtained through a procedure designed to separate serum lipoproteins. Their growth supporting abilities can be predicted by measuring their lipid constituents and they will substitute for serum or BSF in mycoplasma culture media.


