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Role of Bovine Serum Albumin in the Nutrition of *Mycobacterium tuberculosis*

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Bovine serum albumin promotes the growth of small inocula of *Mycobacterium tuberculosis* in media containing unesterified fatty acids. Albumin binds fatty acids present in concentrations toxic for the organisms. In the present study, additional roles of albumin were investigated. When present in a basal medium, fatty acid-free albumin could be utilized by *M. tuberculosis* as a sole source of carbon. Since albumin could not substitute for the amino acids in basal medium as a nitrogen source, it was concluded that the protein component in albumin was not utilized as a nutrient by the organisms. An ether extract of fatty acid-free albumin supported a small but significant amount of growth. Analysis of the lipids in fatty acid-free albumin by gas chromatography revealed the presence of 686 μg of fatty acid per g of albumin. Although a small amount of growth occurred when a lipid extract of albumin was present in the medium, growth stimulation was dependent in major part on the presence of undenatured albumin in the medium. Lipids, when bound to albumin, can serve as a nontoxic source of carbon and energy.

The addition of nonionic detergents to media for the cultivation of *Mycobacterium tuberculosis* facilitates growth in diffuse form not otherwise possible. The favored wetting agent has been Tween 80, a polyoxyethylene sorbitan monooleate preparation. However, in the concentration necessary to promote diffused growth, Tween 80 can be toxic to the organisms due to the presence of unesterified fatty acids (3).

In media containing unesterified fatty acids, the addition of albumin is required for the growth of small inocula of *M. tuberculosis* (B. D. Davis and R. J. Dubos, Fed. Proc. 5:246, 1946). The role of albumin in such media has not been completely defined. Davis and Dubos (4) associated a reduction in opalescence of oleic acid-containing media, after addition of bovine serum albumin (BSA), with the binding of fatty acids to albumin. Subsequently, Spector and coworkers (13) demonstrated that albumin binds with the carboxyl group of fatty acids. When bound to albumin, fatty acids are rendered more soluble in aqueous solutions.

In addition to the detoxifying action of albumin, Dubos (7) suggested that BSA fraction V contained heat-stable, nonprotein impurities that markedly increased the amount of growth. These nutritive impurities were not characterized further or identified. The present study has been devoted to further characterizing the role of BSA on the growth of *M. tuberculosis* in defined medium and the identification of the presumed nutrients or growth factors.

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MATERIALS AND METHODS

Organism. The strain of *M. tuberculosis* H37Rv used in this study was obtained from William A. Steenken, Jr., of the Trudeau Laboratory, Trudeau, N.Y., and had been maintained in basal medium supplemented with 2% Tween 80 and 0.5% albumin.

Media. The basal medium contained the following, in grams per liter: disodium phosphate, 3.5; monopotassium phosphate, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; $FeCl_3 \cdot 6H_2O$, 0.001; L-glutamic acid, 1.0; L-asparagine, 1.0; and Triton WR1339, 0.1. Triton (Rutger Chemical Co., Irvington-on-Hudson, N.Y.) was used as a non-nutritive wetting agent. Fifty-milliliter portions of medium were placed in 250-ml Nephelo flasks and autoclaved at 121°C for 15 min. Purified sodium oleate (Fisher Scientific Co., Fairlawn, N.J.), when used, was added to a concentration of 0.001% before autoclaving. BSA fraction V, lot no. 81-003, and fatty acid-free (FAF) albumin, lot no. 82-002 (Miles Laboratories, Kankakee, Ill.), were prepared as 25% (wt/vol) solutions in distilled water and were sterilized by membrane filtration. When used, 1 ml of the sterile albumin solution was added to 50 ml of cooled, autoclaved medium.

Cultivation. A 10-day-old culture was washed and starved in carbon-free basal medium for 24 h. The

culture was standardized to 0.3 optical density units at 550 nm with a spectrophotometer, and the flasks of medium were inoculated with 0.05-ml amounts of the standardized culture. The flasks were incubated in a stationary state at 37°C and were examined turbidimetrically at 550 nm periodically for 21 days. At the conclusion of experiments, the presence of a pure culture of mycobacteria was determined by acid-fast smears.

Dialysis studies. To study the effects of dialyzable substances in the culture medium and albumin solutions, lengths of 1-inch-wide dialysis tubing (Fisher Scientific Co.) were soaked and boiled for 15 min three times in large volumes of distilled water. The tubing was then suspended in a Nephelo flask with a piece of glass tubing (8-mm outer diameter) partially inserted in one end. A foam plug was fitted around the glass rod and the other free end of the dialysis tubing. A small plug was placed in the glass tube at the top. After filled culture flasks were autoclaved and cooled, the plug could be removed and albumin could be introduced aseptically into the dialysis tubing. Empty sacs were used as controls. Passage of peptides through the dialysis sac into the basal medium was determined by measuring the protein content in the medium by the Lowry et al. modification (8) of the Folin-Ciocalteu color reaction.

Extraction of lipids from albumin. A modification of the procedure of Dole and Meinertz (5) was used to extract the fatty acids from albumin. One milliliter of a 25% solution of albumin was shaken with 5 ml of an extraction mixture containing isopropyl alcohol–pentane–1 N H_2SO_4 (40:10:1, vol/vol/vol) in a 30-ml glass-stoppered test tube. After 15 min at room temperature, 3 ml of pentane and 2 ml of 0.1 N H_2SO_4 were added, and the tube was again shaken. The upper phase was then transferred to a second test tube containing 1 ml of 0.01 N H_2SO_4. After shaking, the upper phase containing the extracted fatty acids was added to basal medium.

Determination of fatty acids in FAF albumin. A sample of FAF albumin was saponified in 1 N KOH in 50% ethanol for 90 min. The non-saponifiable material was extracted with redistilled petroleum ether (bp 30 to 60°C) and discarded. The alkaline hydrolysate was acidified strongly with 15 N HCl. The fatty acids were extracted three times with redistilled pentane and evaporated under a stream of nitrogen. The fatty acids were methylated by the method of Metcalf and Schmitz (9). Gas chromatographic analyses were performed with a Bendix 2600 gas chromatograph (Bendix Corp., Ronceverte, W.Va.) equipped with dual hydrogen flame ionization detectors, paired 15% diethylene glycol succinate 3-ft (about 0.9-m) glass columns, and on-column injection. Fatty acids were determined by comparing triangular areas under the peaks of the sample with that of an internal methyl pentadecanoic fatty acid standard.

RESULTS

Detoxifying effect of albumin. To study the interaction between fatty acids and albumin in growth media, two sets of basal medium containing 0.001% oleate, one with and one without FAF albumin, were inoculated with 0.05 ml of a standardized suspension of *M. tuberculosis*. Addition of albumin to oleate-containing medium promoted growth not attainable without albumin (Fig. 1). In the absence of albumin, organisms recovered from oleate-containing medium displayed loss of acid fastness, indicating the toxicity of the medium (1).

Utilization of albumin as a sole source of carbon. To examine the nutritive role of albumin, organisms were inoculated into basal medium containing (i) no albumin, (ii) FAF albumin in a dialysis sac suspended in the medium, and (iii) FAF albumin in the medium. In addition, one set of flasks contained basal salts and FAF albumin, but no glutamic acid or asparagine. Increases in turbidity associated with growth were not observed in medium lacking albumin or in medium containing albumin but no glutamic acid or asparagine (Fig. 2). These data indicated that the basal medium lacking albumin was devoid of a utilizable source of carbon and that albumin could not substitute for the amino acids in basal medium as a source of nitrogen. Therefore, the protein component in albumin was not utilized as a nutrient by the organisms. The presence of FAF albumin in the medium allowed an amount of growth comparable to that obtained with FAF albumin and oleate (Fig. 1), indicating that the FAF albumin preparation could be utilized by *M. tuberculosis* as a sole source of carbon. The suspension of a

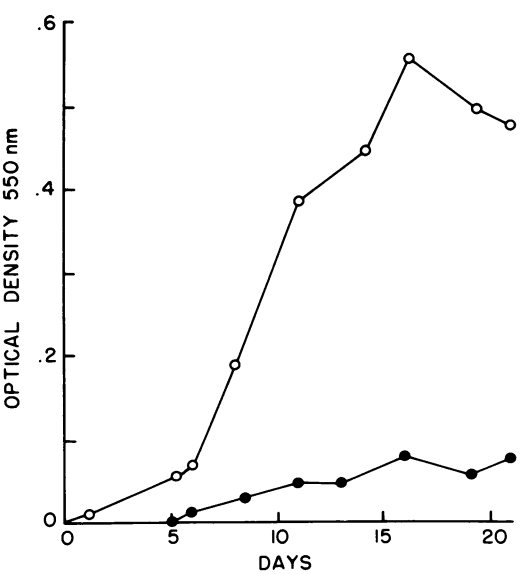

FIG. 1. *Effect of FAF albumin on the growth of M. tuberculosis H37Rv in basal medium supplemented with 0.001% sodium oleate. Symbols: ●, no albumin; ○, 0.5% FAF albumin.*

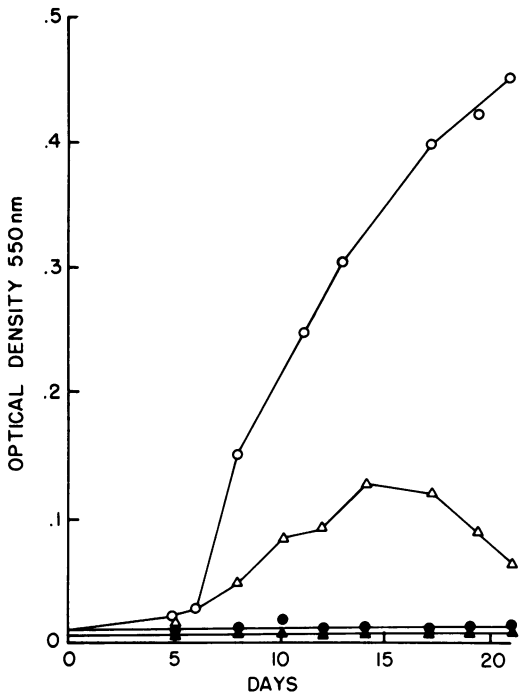

FIG. 2. *FAF albumin as a source of carbon or nitrogen for M. tuberculosis H37Rv. Symbols: ●, basal salts + glutamic acid + asparagine; ▲, basal salts + FAF albumin; △, basal salts + glutamic acid + asparagine + FAF albumin in a dialysis sac; ○, basal salts + glutamic acid + asparagine + FAF albumin.*

dialysis sac containing albumin in basal medium supported a small but significant amount of growth, which suggested that the albumin contained a dialyzable nutritive component. Separation of albumin from the growth produced the coarsely granular type of growth observed when albumin was absent from the oleate-containing medium.

To determine the effect of FAF albumin in the dialysis sac on the growth medium, a dialysis sac containing FAF albumin was suspended in uninoculated basal medium containing 0.001% sodium oleate. The concentrations of protein and fatty acid in the dialysis sac and the growth medium were determined at 0 and 72 h. These analyses revealed a sixfold concentration of oleate within the sac due to the binding by albumin and the movement of approximately 1% of the Lowry-reactive protein from the sac into the medium. Since the protein component of albumin was not used as a nutrient (Fig. 2), some other carbon-containing nutrient must also have moved through the dialysis sac into the medium.

To determine whether the growth-supporting components were of a lipid nature, (i) FAF albumin, (ii) an ether extract of BSA fraction V, and (iii) an ether extract of FAF albumin were added to basal medium and autoclaved. Upon autoclaving, the albumin was visibly precipitated and denatured. The flask used as spectrophotometric blank contained autoclaved albumin in basal medium but was not inoculated. Similar amounts of growth were obtained in media containing autoclaved FAF albumin and an ether extract of FAF albumin (Fig. 3), indicating that the ether extract of presumably FAF albumin was responsible for supporting growth in both flasks. Autoclaving albumin apparently released the tightly bound lipids in albumin into the medium. The ether-extracted FAF albumin residue did not support growth, further demonstrating that the protein components in albumin were not utilized by the organisms as a source of carbon and energy. An ether extract of BSA, not treated with charcoal, stimulated growth to a greater extent than did the extract of FAF albumin, probably due to its higher lipid content.

To identify and quantitate the fatty acids present in albumin, FAF albumin was saponified and the fatty acids were extracted. Gas chromatographic analysis of the extractable fatty acids demonstrated the presence of 686 μg of fatty acid per g of FAF albumin (Table 1).

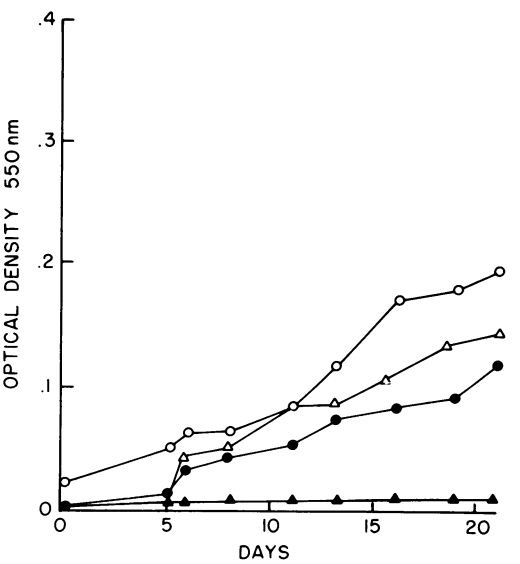

FIG. 3. *Effect of autoclaved FAF albumin or ether extracts of albumin on the growth of M. tuberculosis H37Rv in basal medium. Symbols: ●, ether extract of FAF albumin; ○, ether extract of BSA fraction V; ▲, residue of ether-extracted FAF albumin; △, autoclaved FAF albumin.*

TABLE 1. *Fatty acids extracted from FAF albumin*[a]

Fatty acid	% of sample	μg/g of albumin
Palmitic (16:0)	36.37[b]	249[b]
Stearic (18:0)	31.58	217
Oleic (18:1)	17.70	121
Linoleic (18:2)	14.35	99
Total	100.00	686

[a] Fatty acids were extracted and methylated from 500 mg of FAF albumin as described in the text. The fatty acid methyl esters were solubilized in pentane, and gas chromatographic analysis was performed under the following conditions: injector temperature, 250°C; detector temperature, 250°C; oven temperature, 170 to 190°C, 5°C/min; flow rate, 20 ml/min, N_2.

[b] ±5.5%.

DISCUSSION

The addition of albumin to liquid media containing toxic fatty acids has been known to facilitate the growth of small inocula of *M. tuberculosis* (Davis and Dubos, Fed. Proc. **5**:246, 1946). Albumin binds with carboxyl groups of fatty acids (13) and thereby reduces their toxicity. However, binding and subsequent detoxification of fatty acids does not fully account for the effect of albumin. The work by Hirsch (7) demonstrated that although charcoal could bind fatty acids more efficiently than albumin, the addition of a charcoal-oleate complex did not stimulate growth as well as albumin-oleate. This observation supports the hypothesis that albumin contributes more to growth media than detoxification of oleic acid.

In this study, it was observed by visual inspection that the addition of albumin to medium containing Triton WR1339 led to a more dispersed type of growth. Albumin, in close association with the cells, enhances the wetting effect (11), exposing a greater cellular surface to nutrient. The albumin exposes the cells to nutritive fatty acids in a manner not toxic to the cells. Separation of albumin from the cells by a dialysis membrane or denaturation by autoclaving eliminates the wetting action of albumin and decreases the stimulatory effect.

A growth-enhancing factor in albumin has been postulated to be heat stable and nonprotein (6). BSA fraction V, even though being characterized as fatty acid free, still contains a significant amount of fatty acid that can be utilized by *M. tuberculosis* as a sole source of carbon. It was previously claimed by Brezina et al. (2) that *M. tuberculosis* can utilize albumin as a sole source of carbon, but Tween 80, a known nutrient for mycobacteria (10, 14), was used as a wetting agent in all their experiments, rendering their conclusions equivocal.

FAF albumin contained as much as 686 μg of fatty acid per g of albumin. Similar concentrations of fatty acid were found by others after BSA was freed of fatty acid by charcoal treatment (12). At the concentration of albumin used in these experiments, 3×10^{-4}% fatty acids were contributed to the medium. Youmans and Youmans (15) showed that *M. tuberculosis* would grow in a medium containing 1.5×10^{-5} to 2.5×10^{-4}% of these fatty acids.

Albumin has a dual role in promoting growth of mycobacteria. It binds fatty acids that would otherwise be toxic to the organisms. When so detoxified, the fatty acids are rendered utilizable for the organisms. Extraction and subsequent addition of tightly bound lipids to media allows a small amount of growth due to the nutritive fatty acids. However, the lipids may be somewhat toxic to the organisms, resulting in less growth than would be obtained when detoxified by the albumin protein.

The possibility that the organisms were utilizing the albumin protein as nutrient was tested by omitting glutamic acid and asparagine from the basal medium. If albumin protein were to be utilized as a source of carbon, then the glutamate, aspartate, and amino residues in albumin should have provided an adequate source of nitrogen. Since no growth was then obtained, the protein components in albumin did not serve as a source of nutrient for the organisms.

These results indicate that even highly purified albumin contributes nutrients to media to which it is added as a detoxifying agent.

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