The Supply of Cholesterol and Fatty Acids for the Growth of Mycoplasmas

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(Accepted for publication 13 May 1969)

SUMMARY

Mycoplasma strain γ , serologically and biochemically related to *Mycoplasma mycoides*, grew well in a medium containing unesterified fatty acids, cholesterol and some samples of fatty acid-poor bovine serum albumin. With other samples of serum albumin, growth was accompanied by cellular lysis. Heating albumin solutions, treating them with charcoal, or varying the ratio of fatty acids to albumin, the concentration of cholesterol, or the order in which the lipid components were added to the medium, all influenced the amount of lysis. These effects may be due to varying interaction between fatty acids and cholesterol and between fatty acids and albumin. When fatty acids are firmly bound to albumin, their interaction with cholesterol is prevented, resulting in a cholesterol deficiency. Lysis was prevented by the addition of a heat-stable defatted serum protein fraction (fraction C), or a pronase digest of fraction C, both of which dispersed cholesterol.

INTRODUCTION

Media of partially defined composition for growth of *Mycoplasma mycoides* strain γ 5, and *Mycoplasma* strain γ were described as media A and B (Rodwell, 1956, 1960; Rodwell & Abbot, 1961). These media contained a defatted serum protein fraction (fraction C). Deficiencies of glycerol, cholesterol or fatty acids resulted in marked morphological changes, cell death and lysis (Rodwell & Abbot, 1961). Medium C (Rodwell, 1967) containing defatted bovine serum albumin (BSA) and a pronase digest of BSA as undefined components, gave good growth of strain γ , and was used to study the fatty acid growth requirements. The problem of supplying fatty acids in a non-toxic form, and of cholesterol in an available form, is considered further in this paper.

METHODS

Organism. Strain γ , isolated from a goat (Laws, 1956), is serologically and biochemically related to *Mycoplasma mycoides*, but differs in that its growth is not improved by aeration.

Growth assays. Mycoplasmas for inoculating cultures were grown in BVFOS medium (Turner, Campbell & Dick, 1935). The cultures were centrifuged, and the mycoplasmas suspended in an equal volume of 0.02 M-tris-HCl buffer containing 0.01 M-MgSO₄ (TM). The suspensions were diluted 1/10 or 1/100 in TM and one drop of diluted suspension containing approximately 10⁷ or 10⁶ viable particles was

inoculated into 5 ml. volumes of test media in aluminium capped, 18 mm. diameter, optically matched test tubes.

Measurement of growth. Growth was measured at intervals during incubation at 37° by turbidity at 660 m μ . In some experiments, where it was suspected that turbidity might be an unreliable measure of growth, the synthesis of DNA was also measured by the incorporation of ³H thymidine as described by Byfield & Scherbaum (1966). Samples of the cultures (90 μ l.) were absorbed on discs of Whatman 3 MM filter paper, which were collected in cold 5 % (w/v) trichloroacetic acid solution, and processed together at the end of the experiment.

Cultures were examined microscopically by dark-field illumination to assess the effects of nutrition on cellular morphology. Experience has shown (Rodwell & Abbot, 1961) that extensive lysis (as revealed by electron microscopy) during growth of *Mycoplasma* cultures was always accompanied by a decreased turbidity, an increased viscosity—probably due to liberated DNA—and a tendency of the cells to aggregate into a sticky deposit.

Composition of media. Medium C was described previously (Rodwell, 1967).

Medium C1 had the same composition except that L-alanyl-L-alanyl-L-alanine (L-al₃) replaced the pronase digest of bovine serum albumin (BSA), and glutamic acid, aspartic acid, cystine, biotin, leucovorin and vitamin B₁₂ were omitted. It had the following composition: Na₂HPO₄ + NaH₂PO₄, pH 7.8, 0.14 M; KCl, 0.01 M; MgSO₄, 0.001 M; glucose, 0.04 M; glycerol, 5 mM; spermine, 0.1 mM; cholesterol, 0.08 mM; sodium oleate, 0.05 mM; sodium palmitate, 0.05 mM; 'fatty acid poor' bovine serum albumin (BSA), 0.8 g./l.; adenine, guanine and uracil, 10 mg./l. each; thymine 5.0 mg./l.; coenzyme A, riboflavin, nicotinamide and thiamine, 1.0 mg./l. each; DL α -lipoic acid, 0.1 mg./l.; L-asparagine, L-glutamine, L-arginine, L-lysine, L-histidine, L-leucine, L-isoleucine and L-proline, 1.0 mM each; DL-phenylalanine, DL-valine, DL-methionine, DL-serine, DL-threonine, DL-tryptophane and glycine, 2.0 mM each; L-cysteine, 1.3 mM; L-al₃, 0.3 mM. Glucose, riboflavin, BSA, glutamine and cysteine were added from sterile solutions to previously autoclaved medium. Glutamine and cysteine solutions were freshly prepared. Cholesterol was added as an aqueous dispersion prepared by the addition of 1 vol. of a solution in ethanol to 19 vol. stirred water at 65°. Coenzyme A was added before autoclaving from a 0.1 mg./ml. solution containing dithiothreitol 1.0 mg./ml. in phosphate buffer, pH 7.0.

In medium C2, L-alanine (1 mM) was substituted for L-al₃, the concentration of cholesterol was increased to 0.1 mM, 'fatty acid poor' BSA was replaced by charcoal treated BSA (BSA-Ch) and pronase digest of fraction C (PC) 0.4 g./l. was added.

Materials. Fraction C was prepared as described by Rodwell & Abbot (1961). Carbohydrate reacting in the anthrone reaction was not detected in the product. A washed lipid extract (Folch, Lees & Sloane Stanley, 1957) of one batch contained 0.38 μ g. atom of phosphorus and a total of 0.9 μ mole fatty acids (mainly palmitic, stearic and oleic acids) per g. fraction C. After extraction with chloroform + methanol + N-hydrochloric acid (13.3 + 6.7 + 1.0), the washed lipid extract contained a total of 2.6 μ moles fatty acids per g. fraction C, while after hydrolysis of fraction C in 6 N-HCl for 16 hr at 100°, 8.2 μ moles fatty acids per g. were extracted. If these were present in phospholipids, this would correspond to a total of approximately 0.3 % phospholipid,

most of which is firmly bound. The total phosphorus content was 12.0 μg . atom per g. fraction C.

Pronase digest of fraction C (PC) was made by incubating a solution of fraction C (100 mg.) at pH 7.5 with 1.0 mg. pronase (Calbiochem, Los Angeles, California) at 37° for 24 hr. The pH was readjusted to 7.5, another 1.0 mg. pronase added and incubation continued for a second 24 hr period.

Samples of human and bovine serum albumin were obtained from Calbiochem; Pentex Incorporated, Kankakee, Illinois; Armour Ltd., Eastbourne, England; and Sigma Co., St Louis, Missouri. 'Fatty acid poor BSA' was manufactured by Pentex Incorporated. All these were depleted of bound fatty acids either by Goodman's (1957) method or by charcoal treatment (Chen, 1967). The charcoal (Nuchar C190) was treated *in vacuo* in 10 N-HCl for 1 to 2 hr and washed with water. Charcoal could then be removed from albumin solutions by filtration. The treated solutions (BSA-Ch) were sterilized by filtration through a Seitz filter pad which had been extracted successively with chloroform + methanol, ethanol + acetic acid, ethanol and water. Protein was determined in the filtrate either by the method of Lowry, Rosebrough, Farr & Randall (1951) or by extinction at 280 m μ .

Amino acids (A Grade) were obtained from Calbiochem; fatty acids and cholesterol from Applied Science Inc., Pennsylvania, and alanyl peptides from Yeda Research and Development Co., Rehovoth, Israel. Cholesterol-4 ¹⁴C was obtained from the Radiochemical Centre, Amersham, Bucks. and purified by thin layer chromatography (ITLC-SA, Gelman Instrument Co., Michigan; solvent isooctane + diethyl ether, 9:1). The main radioactive band was located by radioautography and eluted with ethanol.

Analytical methods. Cholesterol was determined by the method of Wycoff & Parsons (1957), phosphorus by that of Bartlett (1959) as modified by Marinetti (1962), and radioactivity with a Packard Tri-Carb liquid scintillation spectrometer in a dioxane system (Bray, 1960). Fatty acids were estimated by gas liquid chromatography as described previously (Rodwell, 1968) except that for the analysis of fatty acids in lipid extracts of fraction C, the methyl esters were prepared by trans-esterification with boron trifluoride in methanol at 64° for 2 hr.

Paper electrophoresis was performed in barbiturate buffer, pH 8.6, in an apparatus manufactured by LKB-Produkter, Sweden. The paper strips were stained for protein with brom phenol blue; ¹⁴C-cholesterol was located by radioautography.

RESULTS

Growth of Mycoplasma strain Y in medium C. Medium C gave excellent growth of this strain from an inoculum of 1 to 10 viable particles. The maximum yield, which was undiminished in serial subculture, was 0.35 mg. dry weight, and the maximum viable count was 6.0×10^9 colony forming units (c.f.u.) per ml. culture. Growth curves determined by viable counts, turbidity and by ³H thymidine incorporation are shown (Fig. 1). The mean doubling time as determined by all three methods was 1.6 to 1.7 hr. Thymidine was incorporated at an exponential rate and then ceased abruptly. The turbidity continued to increase (at a decreasing rate) for about 4 hr longer, perhaps because the mycoplasmas increased in mass after DNA replication ceased. In this experiment viable counts were discontinued after 24 hr incubation, but in other

experiments the count reached a maximum at about the same time as the turbidity. Increase in the number of viable particles after the cessation of DNA replication might be caused by the breaking up of filaments containing several 'DNA complements'. Medium C1 gave the same growth rate and yield, and was used in subsequent experiments.

Fig. 1

Fig. 2

Fig. 1. Growth of *Mycoplasma* strain Y in medium C. Medium C (100 ml.), modified by the substitution of ^3H thymidine (2 mg. l., specific activity $30 \mu\text{C.} \mu\text{mole}$) for thymine, was seeded with 2×10^5 colony forming units and dispensed in 5 ml. volumes in optically matched test tubes. Viable cell counts, ■; turbidity, ●; and ^3H thymidine incorporation, ▲, were determined on replicate tubes during incubation at 37° .

Fig. 2. Inhibition of oleate-induced lysis of *Mycoplasma* strain Y by bovine serum albumin (BSA). (a) Fraction C and pronase digest of fraction C (PC). (b) Phosphate buffer, 0.05 M , pH 7.4; sodium chloride, 0.25 M ; sodium oleate, $2.5 \times 10^{-6} \text{ M}$, and BSA, fraction C or PC were incubated at 37° for 10 min. in optically matched test tubes; cell suspension added and turbidity measured during incubation at 37° . (a) ●, No oleate or BSA; ○, oleate; Δ, oleate + BSA 0.08 mg. ml. (molar ratio 15:1); ▽, oleate + BSA, 0.2 mg. ml. (molar ratio 6:1). (b) ●, No oleate or fraction C; ○, oleate; Δ, oleate + 0.05 mg. ml. fraction C; ▽, oleate + 0.2 mg./ml. fraction C; □, oleate + 0.2 mg. ml. PC.

Growth-promoting properties of serum albumin

It was concluded (Rodwell, 1967) that in medium C1, in which unesterified fatty acids are added in a total concentration of 0.1 mM , albumin is required to bind fatty acids. The concentration of BSA required for growth increased with increasing fatty acid concentrations, and albumin was not required when the concentration of fatty acids was decreased below a critical value as in medium D. *Mycoplasma* cells are rapidly lysed by long chain monoenoic fatty acids in concentrations lower than those required for optimum growth, and lysis is prevented by BSA (Fig. 2a). It was found subsequently that not all samples of serum albumin were equally effective in promoting growth of strain Y in medium C1. Two crystalline samples and six preparations of

fraction V were tested, some of them before and after fatty acid depletion by Goodman's procedure or by charcoal treatment. Of these, only two gave good growth (equivalent to growth of strain Y in best media), whereas all of the others gave growth accompanied by various degrees of cellular lysis. The amount of lysis was greater with increasing albumin concentration (i.e. with decreasing fatty acid-albumin ratios, Fig. 3), but not when the concentrations of fatty acids were increased in proportion. Increasing the cholesterol concentration to 0.1 mM or heating the albumin solutions at 56° for 30 min. decreased the amount of lysis and growth was now better at a lower fatty acid:albumin ratio (Fig. 3). Two samples of 'fatty acid poor BSA' which originally gave growth without appreciable lysis, gave growth with lysis after charcoal treatment.

Fig. 3

Fig. 4

Fig. 3. Effect of heating at 56° for 30 min. on the growth-promoting activity of bovine serum albumin (BSA) for *Mycoplasma* strain γ in medium C 1. ▲, BSA solution unheated, 1.0 mg./ml.; △, BSA solution unheated, 2.0 mg./ml.; ■, BSA solution heated, 1.0 mg./ml.; □, BSA solution heated, 2.0 mg./ml. ---, indicates lysis. The BSA was a crystalline sample; the molar ratio of fatty acids to BSA was 7:1 for a BSA concentration of 1.0 mg./ml.

Fig. 4. Effect of the order in which fatty acids and cholesterol dispersion were added to medium C 1 on the growth of *Mycoplasma* strain γ . In condition 1, cholesterol dispersion and sodium salts of fatty acids were added before autoclaving the medium; BSA-Ch solution was added after autoclaving. In condition 2, cholesterol dispersion was added before autoclaving the medium; the fatty acid salts were incubated with the BSA-Ch solution at 37° for 30 min., and the mixture added to the autoclaved medium. In condition 3, fatty acids were added as BSA-Ch fatty acid mixtures as in condition 2, but the cholesterol dispersion was autoclaved separately and added last. △, Condition 1, BSA-Ch 0.9 mg./ml.; ○, condition 1, BSA-Ch 1.8 mg./ml.; □, condition 2, BSA-Ch 0.9 mg./ml.; ×, condition 2, BSA-Ch 1.8 mg./ml.; ▲, condition 3, BSA-Ch 0.9 mg./ml.; ■, condition 3, BSA-Ch 1.8 mg./ml. ---, indicates lysis.

Experiments in which the order in which cholesterol and fatty acids were added to the medium was varied suggested that these results might be explained by varying degrees of interaction between fatty acids and cholesterol on the one hand, and between fatty acids and albumin on the other. It may be seen (Fig. 4) that when cholesterol and fatty acids were autoclaved in the medium (condition 1) there was growth with lysis; when cholesterol was autoclaved in the medium in the absence of

fatty acids (condition 2), there was almost no growth; but when the cholesterol dispersion was autoclaved in the absence of salts and added to the medium last (condition 3) there was good growth with little apparent lysis. Turbidity, proportional to the amount of cholesterol, developed during autoclaving in condition 2, and was presumably due to cholesterol precipitation.

Fraction C and pronase digest of fraction C (PC)

BSA could be replaced for growth of strain γ in medium C1 by the heat-stable, defatted serum fraction C, but growth was slower. Fraction C had about the same activity as BSA in preventing lysis of cells by oleate (Fig. 2*b*). When added before

Fig. 5

Fig. 6

Fig. 5. Effect of fraction C, pronase digest of fraction C (PC) and dialysed PC on the growth of *Mycoplasma* strain γ in medium C1 containing charcoal-treated bovine serum albumin (BSA-Ch). Fraction C (or PC), fatty acids and cholesterol were added before, and BSA-Ch after the medium was autoclaved. The cultures were incubated for 22 hr. $\circ$, Fraction C; $\square$, PC; $\triangle$, PC after dialysis. ---, indicates lysis.

Fig. 6. Effect of pronase digest of fraction C (PC) on the distribution of cholesterol between hexane and aqueous phases after shaking with hexane, and on the turbidity of cholesterol dispersions after autoclaving in buffer solution: curve (a) cholesterol in hexane phase; curve (b) cholesterol in aqueous phase; curve (c) turbidity. For curves (a) and (b), 0.7 mmole $\text{Na}_2\text{HPO}_4 + \text{NaH}_2\text{PO}_4$ buffer, pH 7.8; aqueous dispersion of ^{14}C -labelled cholesterol, 1.0 μmole and PC (total volume 6.0 ml.) were incubated at 37° for 30 min. Hexane (2.5 ml.) was added, the tubes shaken vigorously and centrifuged at 35,000 rev./min. for 1 hr (Spinco Model L, rotor 40). Radioactivity was determined in the hexane and aqueous phases. For curve (c), 0.7 μmole $\text{Na}_2\text{HPO}_4 + \text{NaH}_2\text{PO}_4$ buffer, pH 7.8; aqueous cholesterol dispersion, 1.0 μmole and PC (total volume 6.0 ml.) were autoclaved at 10 lb. for 10 min. and the turbidity measured at 420 mμ in 1 cm. cuvettes.

autoclaving, it also prevented lysis in media containing BSA-Ch. Its ability to replace BSA for growth, and its fatty acid-binding activity, were largely destroyed by pronase digestion (Fig. 2*b*), but its ability to prevent lysis during growth in medium containing BSA-Ch was almost unaffected (Fig. 5). With BSA-Ch and either fraction C or PC, the order in which cholesterol and fatty acids were added made no apparent difference to growth.

Fraction C disperses cholesterol. It prevented the precipitation of cholesterol when aqueous dispersions were heated in the presence of salts. When cholesterol dispersions were mixed with fraction C and buffer solution, and the mixture shaken with hexane, the cholesterol remained in the aqueous phase. Cholesterol-dispersing activity, in contrast to fatty acid-binding activity, was little changed by pronase digestion. The distribution of cholesterol (determined by radioactivity measurements) in the hexane and aqueous phases when a ^{14}C -labelled cholesterol dispersion was mixed with buffer

Fig. 7. Chromatography of pronase digest of fraction C (PC) on a Sephadex G 50 column. PC (80 mg.) in 1.0 ml. 0.02 M-ammonium bicarbonate was applied to a column of 80 ml. bed volume. The column was eluted with 0.02 M-ammonium bicarbonate, 2.7 ml. fractions collected, and assayed for extinction at 280 $m\mu$ (a); growth-promoting activity in medium C 2 (b); and cholesterol-dispersing activity (c). Cholesterol-dispersing activity was measured by the effect on the distribution of cholesterol between hexane and aqueous phases under the conditions described in Fig. 6, cholesterol being determined colorimetrically in the hexane phases. The units of growth-promoting activity and cholesterol-dispersing activity were the amounts having activity equal to 1 mg. of PC.

and various amounts of PC and the mixtures then shaken with hexane is shown in Fig. 6 (curves *a* and *b*), and the effect of PC on turbidity when an aqueous cholesterol dispersion was autoclaved in buffer solution in Fig. 6 (curve *c*). In the presence of PC, only about 80 % of the radioactivity was recovered in the hexane and aqueous phases;

the remainder was probably present in an interfacial gel layer which was not analysed. The discrepancy and the amount of interfacial layer was even larger with fraction C. The turbidity after autoclaving without PC was less than with a low concentration of PC. Cholesterol precipitated in large aggregates after autoclaving without PC; with low concentrations of PC the mixtures were opalescent and with higher concentrations they were clear.

The growth-promoting activity of PC was almost unchanged after dialysis (Fig. 5). PC was fractionated on a column of Sephadex G50 and the fractions assayed for material absorbing at $280\text{ m}\mu$ (Fig. 7*a*), for growth-promoting activity (Fig. 7*b*), and for their effect on the distribution of cholesterol between hexane and aqueous phases (Fig. 7*c*). Most of the growth-promoting activity, and the cholesterol-dispersing activity, were eluted in the void volume with about a twofold increase in specific activity.

Further purification of fraction C was obtained by iso-electric precipitation. After two precipitations at pH 4.8, all of the growth-promoting activity was recovered in the fraction insoluble at pH 4.8 (fraction D), with about a twofold increase in specific activity. Much of the inactive material absorbing at $280\text{ m}\mu$ in the fractionation of PC (Fig. 7) was thus probably derived from inactive material present in fraction C. Fraction D contained about 75 % of the fatty acids present in fraction C.

Fraction D gave a broad diffuse band extending about 2 cm. from the origin after paper electrophoresis. A mixture of fraction D in 0.1 M-phosphate buffer, pH 7.4, and ^{14}C -cholesterol dispersion was incubated at 37° for 30 min. After paper electrophoresis of the mixture, the cholesterol remained in a narrow band at the origin, and was almost entirely separated from the protein.

DISCUSSION

The provision of cholesterol in an assimilable form and of fatty acids in a non-toxic form for the growth of mycoplasmas presents a problem. On the one hand, unesterified fatty acids cause rapid lysis by interacting with the cell membranes, and on the other, a fatty acid deficiency results in lysis during growth in an otherwise complete medium, attributable to impaired membrane synthesis. The problem is more difficult in the case of strains producing high yields of organisms for which fatty acids and sterol must be provided in relatively high concentrations. Strain Y, for example, incorporates about $60\text{ }\mu\text{moles}$ fatty acids and $30\text{ }\mu\text{moles}$ cholesterol per l. of culture during growth in medium C2.

Medium C contained BSA to bind fatty acids (Rodwell, 1967). It is likely that, in this medium, cholesterol is dispersed by an interaction with fatty acids. Some samples of BSA gave growth accompanied by cellular lysis, and other samples which originally gave growth without lysis, gave growth with lysis after charcoal treatment. These samples may bind fatty acids more firmly, thus preventing their interaction with cholesterol. The poor growth and lysis may therefore be due to a cholesterol deficiency. The availability of fatty acids for growth may also differ with different albumin preparations. Kessler, Demeny & Sobotka (1967) showed a twofold difference in the rate at which palmitate was taken up by tissues from fatty acid + albumin complexes formed by two different methods.

Smith, Lecce & Lynn (1954) described a defatted serum lipoprotein for growth of some strains of sterol-requiring mycoplasmas, and Smith & Boughton (1960) postu-

lated that it was required for cholesterol incorporation. Rodwell (1956) described a heat-stable defatted serum protein fraction (fraction C) and suggested that fraction C, cholesterol and fatty acids might interact to provide a non-toxic source of fatty acids for growth of *Mycoplasma mycoides*. Rodwell & Abbot (1961) later suggested that fraction C might also be required for cholesterol incorporation. Evidence that it binds fatty acids and disperses cholesterol is presented in this paper.

Fraction C is thought to consist largely of a defatted serum lipoprotein. Cholesterol was bound by the apoprotein of human high-density serum lipoprotein only when phospholipids were also bound (Sodhi & Gould, 1967). Fraction C contains small amounts of firmly bound lipid, but whether this is essential for the cholesterol interaction, the nature of the interaction, and whether fraction C is related to the apoprotein of a serum lipoprotein, were not determined.

I wish to thank Mr B. Zwolak for his capable technical help.

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