Lipase Activity of *Mycoplasma*

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SUMMARY

All of eight *Mycoplasma* strains tested were capable of hydrolysing tributyrin. The saprophytic *Mycoplasma laidlawii* strains showed the lowest lipolytic activity, and the parasitic *Mycoplasma gallisepticum* the highest. The properties of *M. gallisepticum* lipase were studied in some detail. The lipolytic activity of this organism was highest at the logarithmic phase of growth and declined steeply afterwards. The enzyme was not bound to the cell membrane and appeared in the soluble fraction of disrupted cells. The cell extract hydrolysed tributyrin much faster than trilaurin or triolein. Methyl oleate and 'Tween 80' were only slowly hydrolysed. Cholesteryl acetate and stearate were not hydrolysed by cell extract or by intact *M. gallisepticum*. Partial purification of the lipase was accomplished by ammonium sulphate fractionation of cell-extract proteins, followed by anion-exchange chromatography. The partially purified enzyme did not require inorganic ions for activity and its optimal pH value varied between 7.5 and 8.0, depending on the substrate tested.

INTRODUCTION

In a study of the nutritional requirements of *Mycoplasma* for long-chain fatty acids, *Mycoplasma laidlawii* was found to utilize methyl oleate or 'Tween 80' as a source for oleic acid. There was evidence pointing to the enzymic hydrolysis of Tween 80 by this organism (Razin & Rottem, 1963). The presence of lipases in *Mycoplasma* had long been suspected. During growth on horse-serum agar several *Mycoplasma* strains form a pearly film with little spots, found to consist of calcium and magnesium soaps; the film contained cholesterol and phospholipids (Edward, 1954). On media enriched with egg yolk, spots were formed by some additional strains which did not form them with horse serum. A clearing of egg-yolk medium by mycoplasmas was also noted. These observations prompted Edward (1954) to suggest that *Mycoplasma* strains contain lipases which break down phospholipids. These indications for an active lipid metabolism in *Mycoplasma* notwithstanding, very little was done to study the enzymes involved. Smith (1959) described cholesterol esterase activity in several *Mycoplasma* strains. One human strain was also found to contain a lipase which hydrolysed triglycerides. In view of the importance of lipids as essential nutrients and building blocks of the *Mycoplasma* cell membrane (Rodwell & Abbott, 1961; Smith, 1963; Tourtellotte, Jensen, Gander & Morowitz, 1963; Razin, Argaman & Avigan, 1963) it seemed worthwhile to examine the lipid metabolism of these organisms.

METHODS

Organisms. *Mycoplasma laidlawii* strain A (PG8), *M. laidlawii* strain B (PG9), *M. mycoides* var. *mycoides* (PG1) and *M. bovis genitalium* (PG11) were obtained from Dr D. G. ff. Edward (The Wellcome Research Laboratories, Beckenham, Kent, England). *M. mycoides* var. *capri* was provided by Dr E. Klieneberger-Nobel (The Lister Institute of Preventive Medicine, London). *M. gallisepticum* named (R) was obtained from Mrs Ruth Bernstein (Faculty of Agriculture, The Hebrew University, Rehovot, Israel). Strain s6 of *M. gallisepticum* was provided by Dr H. E. Adler (School of Veterinary Medicine, University of California, Davis, U.S.A.). *M. laidlawii* (oral strain) was isolated in our laboratory from the human oral cavity. Most of the work to be described was done with *M. gallisepticum* (R).

Washed suspensions. The organisms were grown in 1 to 3 l. volumes of a modified Edward medium (Razin, 1968) for 24–72 hr at 37°. The organisms were collected by centrifugation in a Sharples Supercentrifuge at 4°. The paste of organisms so obtained was resuspended in 0.25M-NaCl solution and centrifuged at 16,000g for 5 min. at 4°. The sedimented organisms were then washed in cold 0.02M-phosphate buffer (pH 7.5) and resuspended in similar buffer. The amount of organisms in suspension was expressed as mg. cell protein/ml. suspension. In suspensions of the main test organism, *M. gallisepticum* (R), cellular protein was determined by comparing the extinction at 500 m μ to a previously prepared standard curve, which related extinction and cellular protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Cell-free extracts. *Mycoplasma gallisepticum* organisms were disrupted in an M.S.E. ultrasonic disintegrator (60 W.). Seven ml. of a heavy suspension of the washed organisms in 0.02M-phosphate buffer (pH 7.5) were treated at 1.5 amp. for 2.5 min. Cell debris was removed by centrifugation at 34,000g for 30 min. and the supernatant fluid separated and kept at –20° until used.

Chemicals. Tri-*n*-butyrin, di-*n*-butyrin, 1-monobutyryl and mono-olein were obtained from Eastman Organic Chemicals (Rochester 3, New York, U.S.A.). Tri-olein and trilaurin were the products of Mann Research Laboratories (New York 6, N.Y., U.S.A.). Methyl oleate, cholesteryl acetate and cholesteryl stearate were obtained from Nutritional Biochemicals Corporation (Cleveland, Ohio, U.S.A.). Aqueous emulsions of lipids were prepared by ultrasonic treatment. The lipid (about 200 μ moles) was dissolved in diethyl ether (1–2 ml.) and the solution injected with a syringe into 15 ml. of 0.02M-phosphate buffer containing 0.02M-2-hydroxymethyl-propane-1:3-diol (tris) buffer (pH 7.7; subsequently this will be referred to as phosphate tris buffer). The buffer solution contained in addition 200 μ mole sodium taurocholate. The suspension was immediately treated in an M.S.E. ultrasonic disintegrator at 1.5 amp. for 4 min. without cooling. During this treatment the temperature of the emulsion reached 40°, causing the ether to evaporate. These emulsions obtained remained stable for at least several days.

Determination of lipolytic activity

Qualitative assays. Edward medium plates containing 0.4% (w/v) tributyrin were prepared. Tributyrin dissolved in a small volume of acetone was added to basal

Edward medium containing Bacto-heart infusion broth, Bacto-peptone, NaCl and Bacto-agar (Razin & Oliver, 1961; Razin, 1963). The medium was autoclaved at 121° for 20 min., cooled to 50° and all the other ingredients (Razin, 1963) added. The medium, still liquid, was transferred to a sterile 50 ml. beaker and treated in the ultrasonic disintegrator at 1.5 amp. for 3 min. Twelve ml. volumes of this medium were then poured into sterile Petri dishes and allowed to solidify. In some experiments the tributyrin plates were covered with a thin film of polyvinyl formal ('Formvar', Shawinigan Ltd., London, E.C. 3). A solution of 0.5% (w/v) Formvar in chloroform was quickly poured on the surface of the agar, and allowed to drain off by inclining the plates. After evaporation of the chloroform a fine film of Formvar was produced on the agar surface. This film was freely permeable to medium nutrients, but prevented direct contact of *Mycoplasma* organisms placed on top of it, with the medium underneath. The organisms to be tested were grown in 5 ml. volumes of liquid Edward medium for 24–48 hr and washed as described above. The washed organisms obtained from a 5 ml. culture were suspended in 0.3 ml. of 0.02M-phosphate buffer (pH 7.5) and 0.02 ml. drops of these suspensions were placed on the tributyrin plates. The plates were incubated at 37° for 24 hr; clear zones which appeared in and around the drop areas indicated lipolytic activity.

A simple semi-quantitative method for estimating lipase activity in chromatographic fractions of *Mycoplasma* proteins was devised. Tributyrin, dissolved in a small volume of acetone, was added in a final concentration of 0.4% (w/v) to 0.02M-phosphate buffer (pH 7.5) containing 0.9% (w/v) Bacto-agar. The medium was autoclaved at 121° for 20 min., cooled to 50° and treated in the ultrasonic disintegrator at 1.5 amp. for 3 min. Twelve ml. volumes of the resulting suspensions were poured into sterile Petri dishes and allowed to solidify. Drops (0.02 ml.) of each of the chromatographic fractions were placed on the plates, which were then incubated at 37° for 24 hr. The presence and degree of lipolytic activity in these fractions could be then estimated by the size of the clear zones which appeared in the drop areas (Pl. 1, fig. 1).

Quantitative assays. The reaction mixtures were usually composed of washed organisms, cell extracts or chromatographic fractions in 0.02M-phosphate buffer (pH 7.5). The lipid was added to the reaction mixture as a finely dispersed suspension prepared by ultrasonic treatment as described above. Thallium acetate, which did not affect lipolytic activity, was added to a final concentration of 0.04% (w/v) to prevent growth of contaminating organisms during long incubation periods. The total volume of the reaction mixture was 5–10 ml. Experiments with washed organisms or cell-free extracts were done in 100 ml. Erlenmeyer flasks shaken in a 37° water bath. Lipase activity in chromatographic fractions was assayed in test tubes incubated statically at 37°. Hydrolysis of tri-, di- and monobutyryl was determined by titration of freed butyric acid with 0.01N-NaOH, measured by a glass electrode. Hydrolysis of triglycerides and other esters of long-chain fatty acids was determined according to Dole (1956). The liberated long-chain fatty acids were eluted from the reaction mixture with heptane + water + isopropanol (36 + 28 + 36, by vol.) and titrated with 0.02N-NaOH, with Nile blue as indicator. Corrections were always made for titratable acidity of reaction mixtures containing boiled organisms, cell extracts or chromatographic fractions. One enzyme unit was defined as that amount which under the conditions of the test liberated 1 μ equiv.

of acid/6 hr. Specific activity of the lipase was defined as the number of enzyme units/mg. protein in the volume of reaction mixture titrated.

For measurement of tributyrin hydrolysis by cell extracts or chromatographic fractions, a rapid turbidimetric method was devised. A suspension containing 32 μ moles tributyrin/ml. in 0.02 M-phosphate buffer (pH 7.5) was prepared by ultrasonic treatment at 1.5 amp. for 3 min. Three ml. samples of cell extracts or chromatographic fractions in 0.02 M-phosphate buffer (pH 7.5) were incubated in a 37° water bath. Each sample received 0.2 ml. tributyrin suspension and the extinction was measured immediately in a 'Unicam SP 500' spectrophotometer at 625 m μ . Decrease in extinction of suspensions was determined after 10 and 20 min. of incubation at 37°. Corrections were made for the decrease of extinction in a control tube containing tributyrin suspended in 0.02 M-phosphate buffer (pH 7.5). The extinction in the control tube during the incubation period decreased only (slightly by not more than 0.03 unit scale reading at 625 m μ). One enzyme unit, according to the turbidimetric method, was defined as that amount which under the conditions of the test caused a decrease of 0.01 unit scale reading at 625 m μ after 20 min. incubation. Specific activity of the lipase was defined as the number of enzyme units/mg. protein in 1 ml. of reaction mixture.

Hydrolysis of cholesteryl esters was measured by titration of the free acid liberated according to Dole (1956) and by colorimetric determination of the free cholesterol formed. Free cholesterol was separated from its esters by thin-layer chromatography (Razin *et al.* 1963; Argaman & Razin, to be published) and estimated by the FeCl₃ reaction (Wycoff & Parsons, 1957).

Partial purification of lipase. Ammonium sulphate was added to cell extract of *Mycoplasma gallisepticum* (R) to 50 % saturation (31.3 g./100 ml.). The solution was left overnight at 4°; the resulting precipitate was centrifuged down at 25,000g for 1 hr in the cold, and discarded. More ammonium sulphate was then added to the supernatant fluid to 80 % saturation (24.8 g./100 ml.) and the solution kept overnight at 4°. This second precipitate was collected by centrifugation at 25,000g for 1 hr at 4° and dissolved in 0.02 M-phosphate buffer (pH 7.5). The solution was then dialysed at 4° for 15 hr against the same buffer with constant stirring. The buffer was changed several times during this dialysis. A granular precipitate which appeared in the dialysis bag was separated by centrifugation and discarded. The clear protein solution was then chromatographed on a DEAE-Sephadex A-50 (medium) column. Fractions were collected by a fraction collector and tested for lipase activity. The fractions containing lipase activity were combined, dialysed against de-ionized water overnight at 4° and freeze-dried.

Analytical methods. Butyric acid liberated during hydrolysis of tributyrin was identified by paper chromatography (Block, Durrum & Zweig, 1958). The developing solvent was *n*-propanol + concentrated ammonia, sp.gr. 0.880 (70 + 30, by vol.). The paper was sprayed with an aqueous solution of bromophenol blue (0.05 %, w/v) acidified with citric acid (0.2 %, w/v). The ammonium salt of butyric acid appeared on the paper as a blue spot on a yellow background.

Degradation products of triglycerides containing long-chain fatty acids were identified by thin-layer chromatography. The chromatoplates were covered with silica gel G containing Rhodamine (Razin *et al.* 1963). The lipid to be chromatographed was dissolved in a small volume of benzene and applied to the plates as a

short band. The developing solvent was light petroleum (B.P. 40–60°) + diethyl ether (50 + 50, by vol.) and the running time was about 1 hr. After chromatography, the tri-, di- and monoglycerides were visible under ultraviolet radiation as pink-yellow fluorescent areas on a pale-green background.

RESULTS

Occurrence of lipolytic activity in various Mycoplasma strains

All the *Mycoplasma* strains tested were found to hydrolyse tributyrin when grown on Edward medium plates containing this substrate (Pl. 1, fig. 2). The saprophytic *Mycoplasma laidlawii* strains showed the lowest lipolytic activity and the parasitic *M. gallisepticum* strains the highest (Table 1). Most of the work was therefore carried out with *M. gallisepticum* strains.

Table 1. *Hydrolysis of tributyrin by various Mycoplasma strains*

Reaction mixtures consisted of washed organisms, 40 μ moles tributyrin and 20 μ moles sodium taurocholate in 5 ml. 0.01 M-phosphate buffer (pH 7.5). The results represent the mean values obtained in 3 to 6 experiments with different batches of organisms.

Organism	Specific lipase activity (μ equiv. acid liberated/mg. protein/6 hr)
<i>Mycoplasma gallisepticum</i> (S6)	3.20
<i>M. gallisepticum</i> (R)	2.60
<i>M. mycoides</i> var. <i>mycoides</i>	2.10
<i>M. mycoides</i> var. <i>capri</i>	1.60
<i>M. bovis genitalium</i>	1.60
<i>M. laidlawii</i> strain A	0.66
<i>M. laidlawii</i> strain B	0.54
<i>M. laidlawii</i> oral strain	0.42

Localization of lipase activity in the Mycoplasma organism

The hydrolysis of tributyrin in and around the growth areas on tributyrin plates (Pl. 1, fig. 2) suggested that lipases of *Mycoplasma* are soluble and not cell-bound. Tributyrin was also hydrolysed by organisms grown on a Formvar film which prevented direct contact between the organisms and the substrate in the growth medium. When *Mycoplasma gallisepticum* organisms were disrupted by ultrasonic treatment, the lipase activity was found in the fraction not sedimented by centrifugation at 34,000 g for 30 min. (Fig. 1). Lipolytic activity in this 'soluble' fraction was maximal after ultrasonic treatment for 1.5–2.5 min. Further treatment diminished enzymic activity, probably because of denaturation of proteins by the ultrasonic treatment (Hughes, 1961). Lipase activity in the cell-debris fraction was much lower than in the 'soluble' fraction. On centrifugation of the 'soluble' fraction at 100,000 g for 80 min. the lipolytic activity remained in the supernatant fluid; the sediment was practically lipase-free. Lipase activity of cell-free extracts of *M. gallisepticum* did not significantly decrease during 6 months storage at 4°.

Relation of lipase activity to age of culture

The lipolytic activity of *Mycoplasma gallisepticum* whole organisms and cell extracts was highest at the logarithmic phase of growth, declining steeply afterwards. This decline paralleled the rapid death of *M. gallisepticum* in the glucose containing Edward medium (Fig. 2).

Fig. 1

Fig. 2

Fig. 1. Lipase activity of extract and debris of *Mycoplasma gallisepticum* (R) organisms treated ultrasonically for various periods of time. Cell debris was separated from cell extract by centrifugation at 34,000g for 30 min. Lipase activity was determined in the fractions obtained, with tributyrin as substrate. Cell extract (○); cell debris (●). —, lipase activity; ---, protein content of cell fraction.

Fig. 2. Lipase activity of *Mycoplasma gallisepticum* (R) harvested at different phases of growth. Lipase activity was determined as described in Table 1, with tributyrin as substrate. Lipase activity of washed organisms (△) and of cell-extracts (○). Number of viable particles (●) determined according to Butler & Knight (1960).

Specificity of Mycoplasma gallisepticum lipase towards various substrates

The cell extract of *Mycoplasma gallisepticum* hydrolysed glycerides of short- and long-chain fatty acids. Tributyrin was hydrolysed at a much higher rate than was trilaurin or triolein. The oleic acid esters, methyl oleate and 'Tween 80', were also hydrolysed, but only slowly (Table 2). The optimal pH value for hydrolysis varied with the nature of the substrate; thus mono-, di- and tributyrin were best hydrolysed at pH 7.5, trilaurin and triolein at pH 8.0. The rate of hydrolysis of methyl oleate and 'Tween 80' was about the same at pH 7.5 and 8.0 (Table 2). During the hydrolysis of triolein and trilaurin the corresponding di- and monoglycerides appeared in the reaction mixture, as shown by thin-layer chromatography. Cholesteryl acetate and stearate were not hydrolysed by washed organisms or extracts of *M. gallisepticum* (R) or by washed organisms of *M. gallisepticum* (S6). During the incubation no free fatty acids were liberated from the cholesteryl esters (Table 2), and the amount of free cholesterol did not increase, as shown by thin-layer chromatography. Hydrolysis of cholesteryl esters was tested at various pH values ranging from 6.5 to 9.0.

Partial purification of Mycoplasma gallisepticum lipase

The lipase of *Mycoplasma gallisepticum* was precipitated between 50 and 80 % saturation with ammonium sulphate. The precipitated proteins were chromatographed on a DEAE-Sephadex A-50 column. Fractions 6 to 10 eluted from the column showed lipase activity, rising to a sharp peak in fractions 7 and 8 (Fig. 3). Table 3 summarizes the steps involved in the purification of the lipase, the degree of purification and the yield of enzyme.

Table 2. *Substrate specificity of Mycoplasma gallisepticum lipase*

The reaction mixture consisted of 5 ml. *M. gallisepticum* cell extract in 0.02M-phosphate tris buffer, containing 20 μ moles sodium taurocholate and substrate in the amounts indicated in the table.

Substrate	pH value of reaction mixture	μ equiv. acid liberated/mg. protein in	
		6 hr	24 hr
Tributyrim (20 μ moles)	7.5	6.52	9.20
	8.0	4.54	6.90
Dibutyrim (30 μ moles)	7.5	4.23	—
	8.0	2.82	—
1-Monobutyrim (60 μ moles)	7.5	3.04	—
	8.0	2.39	—
Trilaurin (20 μ moles)	7.5	0.07	0.21
	8.0	0.42	0.96
Triolein (20 μ moles)	7.5	0.06	0.16
	8.0	0.32	0.62
Methyl oleate (60 μ moles)	7.5	0.10	0.23
	8.0	0.12	0.24
Tween 80 (1 %, w/v)	7.5	0.22	0.52
	8.0	0.26	0.52
Cholesteryl stearate (20 μ moles)	7.5	0	0
	8.0	0	0

Table 3. *Partial purification of Mycoplasma gallisepticum lipase*

Step	Preparation	Volume (ml.)	Protein (mg./ml.)	Specific activity*	Degree of purification	Yield (%)
1	Crude cell extract	20.0	3.45	20.4	1.0	100.0
2	Supernatant fluid obtained after 50 % saturation with $(\text{NH}_4)_2\text{SO}_4$	29.0	1.04	33.0	1.6	69.4
3	Precipitate formed at 80 % saturation with $(\text{NH}_4)_2\text{SO}_4$	2.2	8.70	45.0	2.2	61.8
4	Chromatography on DEAE-Sephadex A-50					
	Fraction 7	4.0	0.052	594.0	27.6	25.6
	Fraction 8	4.0	0.050	495.0	24.1	
	Fraction 9	4.0	0.036	450.0	22.0	

* Determined by the turbidimetric method.

Properties of the partially purified lipase

The hydrolysis of the various triglycerides increased at about the same rate during the purification procedure (Table 4). Tributyrin was used as substrate in testing the properties of the partially purified lipase. Optimal temperature for activity was 37° and pH 7.5 was optimal (Fig. 4). The partially purified lipase was completely inactivated by heating at 65° for 10 min. (Fig. 5), and was rapidly inactivated when

Fig. 3. Partial purification of *Mycoplasma gallisepticum* lipase by anion-exchange chromatography of cell-extract proteins. Two ml., containing 12 mg. protein obtained by ammonium sulphate precipitation, were placed on a DEAE-Sephadex A-50 (medium) column (1.4 × 14 cm.) equilibrated with 0.02M-phosphate buffer (pH 7.5). Elution was performed by successive addition of 20 ml. volumes of NaCl solution of increasing molarity (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6M-NaCl in 0.02M-phosphate buffer, pH 7.5). Flow rate was about 20 ml./hr and fraction volume 4 ml. Hydrolysis of tributyrin by all fractions was tested by the acid titration (●) or by the turbidimetric (×) method. Protein concentration in fractions (×).

Fig. 4. Effect of pH value on the hydrolysis of tributyrin by the partially purified lipase of *Mycoplasma gallisepticum*. Lipase activity was determined by the turbidimetric method. The reaction mixtures contained 0.02M-phosphate tris buffer.

Table 4. *Comparison of the hydrolysis rate of various triglycerides by the partially purified lipase and cell extract of Mycoplasma gallisepticum (R)*

Reaction mixtures as in Table 2. Hydrolysis of tributyrin was tested at pH 7.5 and that of trilaurin and triolein at pH 8.0.

Substrate	Specific lipase activity (acid titration units)	
	Partially purified lipase	Cell extract
Tributyrin	55.20	2.80
Trilaurin	4.21	0.24
Triolein	3.90	0.15

incubated in highly acid or alkaline solutions (Fig. 6). The divalent cations magnesium, calcium and manganese did not stimulate lipase activity. Prolonged dialysis of the enzyme solution, or addition of ethylenediaminetetra-acetic acid (EDTA) in a final concentration of 0.025 M did not affect enzymic activity.

Fig. 5

Fig. 6

Fig. 5. Heat inactivation of *Mycoplasma gallisepticum* lipase. The partially purified enzyme solution was heated for 10 min. at the temperatures indicated in the figure. Hydrolysis of tributyrin by the heated solutions was tested turbidimetrically.

Fig. 6. Inactivation of *Mycoplasma gallisepticum* lipase in acid or alkaline solutions. The pH value of the partially purified enzyme solution in 0.02 M-phosphate buffer was adjusted with 0.1 N-HCl or 0.1 N-NaOH to the values indicated in the figure. The enzyme solutions were incubated at 37° for 80 min. The ability of these solutions to hydrolyse tributyrin was tested after re-adjustment to pH 7.5.

DISCUSSION

The hydrolysis of tributyrin by all eight of the *Mycoplasma* strains tested indicated the widespread occurrence of lipolytic activity in this group of organisms. The saprophytic *Mycoplasma laidlawii* strains showed a much lower lipolytic activity than the parasitic strains. The saprophytic strains have already been found to differ from the parasitic strains in their nutritional requirements (Razin, 1962), lipid composition (Rothblat & Smith, 1961; Razin, Argaman & Avigan, 1963) and osmotic fragility (Razin, 1963). The lipases of mycoplasmas seem to resemble lipases of other micro-organisms in being soluble and not cell-bound (Davies, 1954). This was clearly shown for the lipase of *M. gallisepticum*. However, lipase activity of a human *Mycoplasma* strain was found to be associated with the cell membrane by Smith (1959).

The steep decline in the lipolytic activity of *Mycoplasma gallisepticum* at the end

of the logarithmic phase of growth may be explained in several ways. Activity may decline owing to oxidation or digestion of the lipase by autolytic enzymes (Sierra, 1957). The enzyme may be denatured by the high acidity developed in the growth medium; the pH value of Edward medium containing glucose (0.5 %, w/v) decreases to pH 5.2–5.4 during the decline phase of growth. At this low pH value the partially purified lipase of *M. gallisepticum* lost most of its activity (Fig. 6). The soluble lipase might also diffuse out of dying organisms. We detected no lipase activity in the growth medium after removal of the organisms. This may, however, be due to the relatively low activity of the lipase and its dilution in the growth medium.

The cell extract and the partially purified lipase of *Mycoplasma gallisepticum* hydrolysed triglycerides of short- and long-chain fatty acids, as well as methyl oleate and Tween 80. The rate of hydrolysis of trilaurin or triolein was only 1–10 % of that of tributyrin. The optimal pH value for hydrolysis also varied with the nature of the substrate. Dependence of the rate of hydrolysis and the optimal pH value on the nature of the substrate is a well-known characteristic of many lipases (Goldman & Rayman, 1952; Nashif & Nelson, 1953; Bier, 1955). Hence one enzyme only may be responsible for the hydrolysis of the various substrates by *M. gallisepticum*. The unchanged substrate specificity following purification of the lipase supports this assumption. The inability of *M. gallisepticum* organisms and cell extracts to hydrolyse cholesteryl esters has also been observed when radioactive cholesteryl oleate was used. *M. gallisepticum* incorporated this ester from the growth medium, but did not hydrolyse it (Argaman & Razin, to be published).

Formation of a film and spots in the growth medium due to degradation of lipids serves as a criterion for the classification of *Mycoplasma* (Edward, 1954; Freundt, 1958). In view of our results strictly defined conditions should be prescribed for this test. *Mycoplasma laidlawii*, for example, which is classified as a 'non-film and spots' producer (Freundt, 1958), will form a film and spots when Tween 80 and CaCl_2 supplement the Edward medium (Razin & Rottem, 1963).

The role of lipases in the physiology of *Mycoplasma* is yet to be elucidated. The lipases may hydrolyse lipids in the growth medium and liberate long-chain fatty acids required for growth of *Mycoplasma* (Rodwell & Abbott, 1961; Tourtellotte *et al.* 1963; Razin & Rottem, 1963). On the other hand, the liberated long-chain fatty acids may have a deleterious effect on the organisms when they accumulate above a certain concentration in the growth medium. The mycoplasmas, having only a thin lipoprotein membrane, are very susceptible to lysis by surface-active substances, including long-chain fatty acids (Edward & Fitzgerald, 1951; Rodwell, 1956; Smith & Boughton, 1960; Razin & Rottem, 1963).

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EXPLANATION OF PLATE

Fig. 1. Detection of lipolytic activity in fractions collected during anion-exchange chromatography of *Mycoplasma gallisepticum* proteins. Plates contained 0.4 % (w/v) tributyrin dispersed in 0.02M-phosphate buffer (pH 7.5) and 0.9 % (w/v) Bacto-agar (purified). Drops (0.02 ml.) of the chromatographic fractions were placed on the plate, and lipolytic activity determined after incubation at 37° for 24 hr.

Fig. 2. Hydrolysis of tributyrin by various *Mycoplasma* strains. Drops (0.02 ml.) of washed suspensions of organism were placed on Edward medium plates containing 0.4 % (w/v) tributyrin; incubation at 37° for 48 hr. 1 = *Mycoplasma laidlawii* strain A; 2 = *M. laidlawii* strain B; 3 = *M. mycoides* var. *capri*; 4 = *M. gallisepticum* (R); 5 = boiled organisms of *M. gallisepticum* (R); 6 = 0.02 ml. crude pancreatic lipase solution (1 mg./ml.).