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Utilization of Palmitic Acid by *Mycobacterium avium*

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Mycobacterium avium accumulates ^{14}C-palmitic acid with saturation kinetics; the process is both temperature dependent and *p*H sensitive. The fatty acid is incorporated into triglyceride in vivo and the conversion is detectable within 5 min after exposure of the cells to ^{14}C-palmitic acid. The triglyceride is rapidly utilized because $^{14}CO_2$ evolution from it begins within 30 min after ^{14}C-palmitic acid accumulation. Data from silicic acid column chromatography of extracts of cultures that have divided many times in medium containing ^{14}C-palmitic acid indicate that a large proportion of the cell lipid is triglyceride, but the radioactivity is widely dispersed among the other lipids. It is estimated that about 5% of the cell dry weight is triglyceride in a postexponential culture.

The growth rate of most mycobacteria is stimulated if oleic acid is included in the medium. This feature was particularly emphasized by Schaefer and Lewis (15) and Hedgecock (7), who showed that growth of *Mycobacterium kansasii* was delayed 5 to 9 days if the organism was incubated in a defined medium containing only glycerol as the available carbon source. Oleic acid, alone, stimulated rapid growth which stopped when the fatty acid was depleted, but the inclusion of glycerol permitted a continuation of growth. Schaefer and Lewis (15) also demonstrated that *M. kansasii* accumulated globules when grown in a medium containing oleic acid.

M. avium B2900 produces a transparent colony variant that requires either oleic or palmitic acid for growth and that is pathogenic for chickens and mice (14). The opaque colony variant derived from it is nonpathogenic (14) and does not require a fatty acid. Pathogenicity and requirement for a fatty acid are therefore associated in this strain. The kinetics with which a transparent colony variant of *M. avium* B2900 takes up and utilizes ^{14}C-palmitic acid were determined as a first step in the elucidation of this organism's fatty acid requirement.

MATERIALS AND METHODS

Organism and medium. Stock cultures of *M. avium* B2900, serotype II, were prepared and maintained at −40 C as described previously (12). Minimal S medium consisted of S salts (12), 0.5% dialyzed albumin, 0.005% palmitic acid, 0.5% glucose, 0.5% glycerol, and 0.1% Tween 80 (Polysorbate 80, Atlas Chem. Ind., Inc.). *M. avium* is unable to hydrolyze Tween 80 or to use it as a source of fatty acid (17). This organism did not increase in viability when incubated in medium containing only Tween 80 as a carbon source (*unpublished observation*).

Chemicals. ^{14}C-Palmitic acid, uniformly labeled (640 mCi/mmole); 2,5 diphenyloxazole (PPO); and 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) were purchased from New England Nuclear Corp. (Boston, Mass.). The palmitic acid remained chromatographically pure if it was used within 4 weeks after purchase.

Accumulation experiments. The stock culture was diluted into minimal S medium and aerated at 37 C for 40 hr (1.5 doublings). The bacteria were harvested by centrifugation and washed with tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride buffer (0.05 M *p*H 7.0) which contained 0.5% dialyzed albumin and 0.1% Tween 80. The cells were then diluted into the same buffer, prewarmed to 37 C, which contained carrier, nonradioactive, palmitic acid and ^{14}C-palmitic acid at 0.01 or 0.02 μCi/ml. Samples of 0.5 ml were removed at intervals, filtered onto 0.45-μm pore-size filters (25 mm; Millipore Corp., Bedford, Mass.), and washed with cold S salts which contained 0.1% Tween 80. The filters were placed in scintillation vials, dried with an infrared heat lamp, and immersed in 5 ml of toluene with 4 g of PPO and 0.05 g of POPOP per liter. The vials were counted in a Nuclear-Chicago Mark I liquid scintillation counter. Values obtained for samples removed at the beginning of an experiment were subtracted from those of subsequent samples. Total radioactivity was assayed by spotting 50 μliters of the radioactive culture at the beginning of the experiment onto a filter; this was counted under the same conditions as the samples and represented the counts per minute per nanomole of palmitic acid in the experiment.

Assays. The cell dry weight was determined by filtering a sample of the cells onto a tared filter (Solvinert, 0.25-μm pore size, 25 mm; Millipore Corp.); this was washed with water, dried at 105 C for 16 hr, and then weighed. Packed cell volume was determined by placing suspensions of cells in Wintrobe hematocrit tubes which were then centrifuged at 4,500 rev/min for 30 min; the supernatant fluid was removed by aspiration and the volume of the cells was recorded. Protein was estimated by the method of

Lowry et al. (11) with bovine serum albumin as standard; the assay was modified by heating the samples in 0.5 N NaOH at 90 C for 10 min prior to addition of the other reagents. Viability was estimated by the number of colonies formed on 7H10 agar (BBL) after 2 weeks of incubation at 37 C. A cell dry weight of 1 mg corresponded to 200 μg of protein or 1.5×10^9 viable units; the packed cell volume was 3.3 μliters/mg (dry weight). Lipid was determined as described by Amenta (1) with oleic acid as standard. Carbohydrate was assayed as described by Dubois et al. (4) with glucose as standard.

Thin-layer, column, and gas chromatography. Radioactive lipids, known standards, or unidentified mixtures were applied to thin-layer silica gel plates (Brinkmann Instruments, Inc.). The plates were developed with the double-solvent system of Freeman and West (6), then sprayed with sulfuric acid-dichromate solution, and charred to locate organic material. Either before or after charring, the plates were placed on Kodak No-Screen X-ray film, NS-2T (Eastman Kodak Co., Rochester, N.Y.), and, after 5 days of exposure, the film was developed to locate the radioactive compounds. These areas were scraped into scintillation vials containing 10 ml of the toluene-PPO-POPOP solution for radioactivity determinations.

Silicic acid column chromatography was used to separate lipids obtained from an extract of ^{14}C-palmitic acid-labeled cells. The bacteria for the extract were grown for 20 days at 37 C in 500 ml of minimal S in a 2,800-ml Fernbach flask; ^{14}C-palmitic acid was included at 0.01 μCi/ml. The culture was harvested by centrifugation, washed with 0.05 M Tris-hydrochloride, *p*H 7.0, which contained 0.1% Tween 80. The cells were then suspended in 5 ml of water and extracted two times with 10 ml of chloroform-methanol (2:1) for 30 min on a wrist action shaker. The chloroform-methanol-soluble material was the crude extract for silicic acid column chromatography. The silicic acid, Bio-Sil HA (Bio-Rad Laboratories, Richmond, Calif.), was packed into a column 2 cm in diameter. The column of 18 g of silicic acid with a volume of 30 ml was washed with 300 ml of petroleum ether (bp 60 to 110 C) under N_2 and then with 200 ml of benzene. The crude extract was dissolved in benzene and applied to the column. The solvent used in eluting the lipids from the column was benzene with increasing concentrations of methanol. Fractions of 5 ml were collected under N_2 and a 0.1-ml sample of each was placed in a scintillation vial and counted after addition of 5 ml of toluene-PPO-POPOP solution.

Gas chromatography (GLC) was used to identify the fatty acids in the lipid obtained from the silicic acid column. The material was refluxed for 18 hr in 20 ml of absolute methanol containing 10 mg of sodium methoxide. The methylated fatty acids were extracted with hexane and analyzed by GLC in a Barber-Colman Series 5,000 instrument with a glass column (6 ft) by using argon as the carrier at a flow rate of 80 ml/min. The methyl esters of the fatty acids were identified by their retention times, as compared to that of standard compounds after GLC over 15% diethylene glycol succinate on Anachrom ABS, at 175 C.

RESULTS

Accumulation kinetics. The rate of palmitic acid accumulation by *M. avium* is dependent upon the concentration of the fatty acid in the medium (Fig. 1), and the uptake is linear for 30 min with 5 to 100 μg (19.5 to 390 nmoles) of palmitic acid/ml and 60 to 250 μg of cell dry weight/ml. The data of Fig. 1 indicate that 78 nmoles of palmitic acid was taken up by 1 mg of cells in 30 min when incubated in the buffer with 195 nmoles (50 μg) of palmitic acid/ml. Since the organism has a packed cell volume of 3.3 μliters/mg (dry weight), this corresponds to 23.6 nmoles/μliter or an intracellular concentration of 2.4×10^{-2} M in contrast to 1.95×10^{-4} M in the medium. This calculation assumes that the isotope is totally within the cell; however, some may be located on the cell surface.

The accumulation of palmitic acid is both temperature sensitive and *p*H dependent, and the interrelationship of the two parameters is shown in Fig. 2. At 38 C a variation in *p*H from 6 through 7.5 causes negligible effect on accumulation of palmitic acid, but, at 26 C, *p*H 6.5 is optimum.

Utilization of palmitic acid. The intracellular fate of accumulated ^{14}C-palmitic acid was determined by thin-layer chromatography of cell extracts. The bacteria were incubated at 37 C in the presence of ^{14}C-palmitic acid, and 2-ml samples were withdrawn at intervals. The samples were caught on solvent-resistant filters and were rapidly washed with cold S salts; then they were extracted

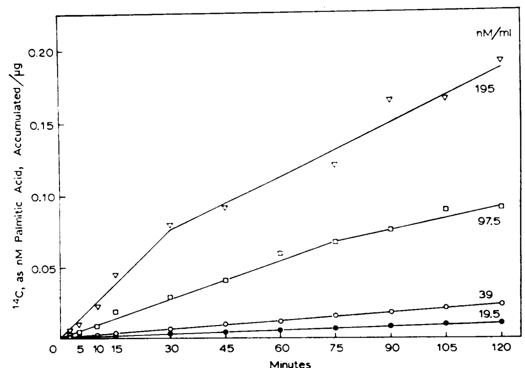

FIG. 1. *Rate of palmitic acid accumulation. Cells (238 μg/ml) were incubated with the concentration of palmitic acid indicated, and the accumulation was assayed. The concentrations of 19.5, 39, 97.5, and 195 nmoles/ml are equivalent to 5, 10, 25, and 50 μg of palmitic acid/ml, respectively.*

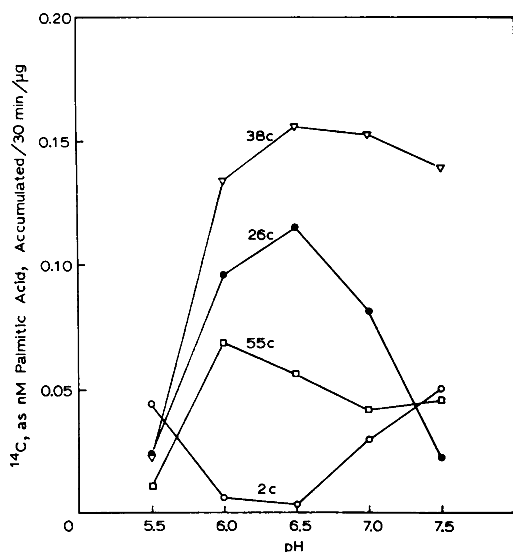

FIG. 2. *Effect of pH and temperature on palmitic acid accumulation. The cells (63 µg/ml) were incubated for 30 min with 50 µg (195 nmoles) of palmitic acid/ml, without aeration at the temperature indicated; 0.05 M phosphate buffers at the pH shown were used in place of the Tris-hydrochloride buffer.*

with chloroform-methanol (2:1). All of the solubilized material was applied to thin-layer silica gel plates along with 100 µg each of carrier oleic acid and triolein. The only radioactive areas were at the positions of the fatty acid and the triglyceride.

The palmitic acid that is taken up by the cells is converted to triglyceride within 5 min after exposure to the isotope and the amount of radioactive triglyceride increases with the length of the incubation (Fig. 3). Only a small amount is present intracellularly as free fatty acid, and this quantity remains at the same level throughout the incubation period of 60 min.

The utilization of palmitic acid was determined by measuring the $^{14}CO_2$ evolved from a culture that was pulsed with ^{14}C-palmitic acid for 30 min. The cells were washed free of the isotope and placed in medium containing no supplement, nonradioactive palmitic acid, glycerol, or both supplements.

Evolution of detectable quantities of $^{14}CO_2$ occurs within 30 min after incorporation of ^{14}C-palmitic acid into triglyceride has ceased. Initially the presence of glycerol or palmitic acid in the medium has little effect, and within 6 hr 0.4 to 0.5% of the incorporated ^{14}C-palmitic acid evolves as CO_2 (Table 1). However, over a long period of time, differences occur due to utilization of the supplements. After 67 hr of incubation, the viable cells incubated with palmitic acid as the only carbon source doubled and those in both palmitic acid and glycerol tripled; the latter yielded concomitantly the greatest quantity of $^{14}CO_2$ and the former evolved the least. The cells starved for palmitic acid neither replicated nor lost viability and they evolved an intermediate quantity of $^{14}CO_2$ (Table 1).

The triglyceride of postexponential cultures. The total triglyceride present in a stationary phase culture was then estimated. The cells were grown in 500 ml of minimal S medium to 5×10^8 viable units/ml; they were harvested, washed, and dried with acetone. The dry cells (73.3 mg) were extracted two times by shaking for 30 min with chloroform-methanol-water (8:4:3). The phases were separated by centrifugation and the lower phases were pooled, concentrated, and then applied to a silica gel plate; 20 µg of triolein was applied to the same plate in a separate lane and the plate was developed with the double-develop-

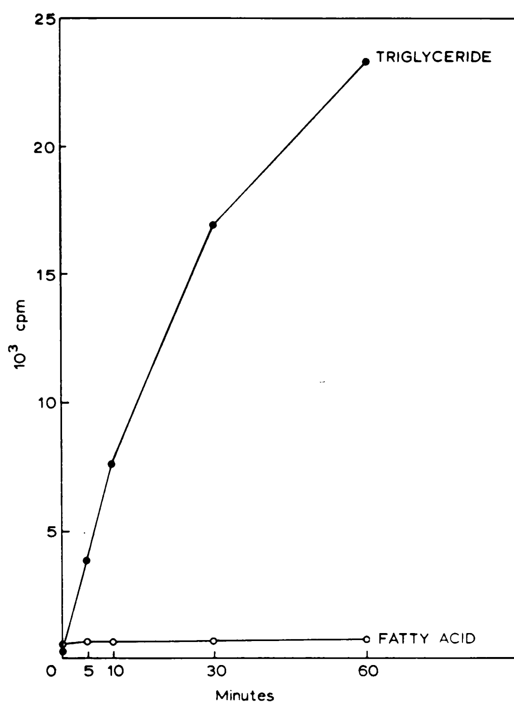

FIG. 3. *Triglyceride synthesis in vivo. Cells (400 µg/ml) were incubated with 195 nmoles of palmitic acid/ml (50 µg/ml) and ^{14}C-palmitic acid. Samples of 2 ml were removed at the times shown and these were immediately extracted. The extracts were applied to thin-layer silica gel plates and, after development, the triglyceride and fatty acid areas were assayed for radioactivity. Palmitic acid accumulated at 5, 10, 30, and 60 min was 0.03, 0.06, 0.16, and 0.26 nmoles/µg, respectively.*

TABLE 1. $^{14}CO_2$ *evolution from cells "pulsed" with* ^{14}*C-palmitic acid*[a]

Medium supplement	Cumulative $^{14}CO_2$ evolved[b]						
	0.5 hr	1.0 hr	1.5 hr	2.0 hr	2.5 hr	6.0 hr	67 hr
Palmitic acid	0.010	0.024	0.098	0.158	0.225	0.521	1.70
Palmitic acid plus glycerol	0.033	0.112	0.211	0.270	0.270	0.389	10.75
Glycerol	0.083	0.142	0.177	0.254	0.301	0.360	4.15
No glycerol, no palmitic acid	0.013	0.084	0.219	0.238	0.238	0.379	6.25

[a] Bacteria were prepared as usual and then placed in S salts containing 0.5% dialyzed albumin (12), 50 μg of palmitic acid/ml, 0.01 μCi of ^{14}C-palmitic acid/ml, and 0.1% Tween 80. They were aerated for 30 min, and during this time they accumulated 0.108 nmole of palmitic acid/μg of cell dry weight. Then the radioactive cells were chilled and washed twice in S salts containing the albumin and Tween 80. They were diluted 1:2 into the same medium (7×10^7 viable units/ml) with no supplement, 50 μg of palmitic acid/ml, 0.5% glycerol, or with both palmitic acid and glycerol. They were incubated at 37 C and aerated by gentle sparging with compressed air; evolved CO_2 was trapped in 8 ml of phenethylamine-methanol (1:1) (9). A sample of 4 ml of the trapping solution was mixed with 8 ml of toluene-2,5-diphenyloxazole-1,4-bis-2-(5-phenyloxazolyl) benzene and counted in a scintillation counter.

[b] The CO_2 evolved is given in per cent of incorporated palmitic acid. The values are cumulative totals for the time.

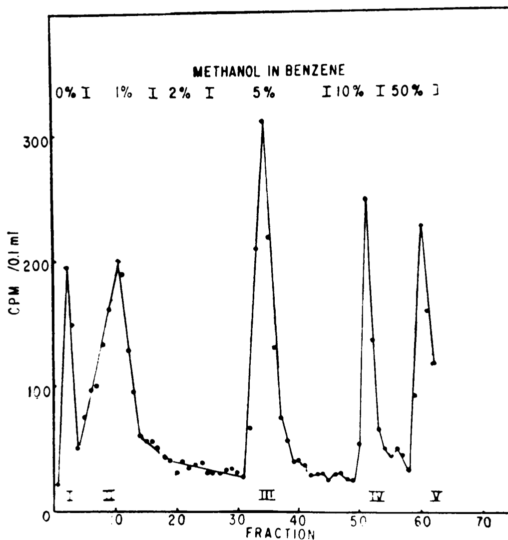

FIG. 4. *Silicic acid column chromatography of chloroform-methanol extract of* ^{14}*C-palmitic acid-labeled cells. The crude extract was applied to a silicic acid column and it was eluted sequentially with 50 ml of each of the solvents indicated, except 100 ml of 5% methanol in benzene was used to elute peak III. Fractions of 5 ml were collected under nitrogen gas and then 0.1 ml was removed from each for radioactivity determinations. The column was finally washed with 50 ml of chloroform-methanol, 2–1, to remove residual material.*

ent system (6). It was stained with iodine and the area that corresponded to triglyceride was extracted three times with 5 ml of chloroform-methanol-water (6:4:1) and, after evaporation to dryness, with water-saturated ethyl ether to remove the lipid from residual silica gel. The dry weight of the triglyceride recovered was 3.8 mg or 5% of the total cell dry weight.

An extract was then prepared from cells that had grown, in the presence of ^{14}C-palmitic acid, from a small inoculum to a fully grown culture (2×10^8 viable units/ml). The crude extract was adsorbed to a silicic acid column and fractions were eluted with benzene containing increasing amounts of methanol. Five peaks were obtained as detected by radioactivity measurements (Fig. 4).

The distribution of carbohydrate, lipid, and radioactivity among the five component fractions is shown in Table 2. The peak II fraction is triglyceride, when compared with triolein on thin-layer silica gel plates developed with any of three solvent mixtures: the double-development solvents (6), hexane-ethyl ether-acetic acid (90:10:3), chloroform-methanol-50% acetic acid (100:9:1). From the GLC data, the fatty acids that occur in greatest concentration in the triglyceride fraction are palmitic, palmitoleic, and oleic acids, but fatty acids of both higher and lower molecular weight are also present. Fraction II consists of 60% of the lipid, but only 26% of the radioactivity of the total lipid applied to the column (Table 2); the specific activity of this material is therefore low in terms of counts per minute per milligram of lipid.

DISCUSSION

It is evident from these experiments that *M. avium* has an avidity for palmitic acid and is able

TABLE 2. *Analyses of silicic acid column fractions*

Fraction	Lipid		Radioactivity		Lipid (counts per min per mg)	Carbohydrate (μg)
	Milligrams	Per cent	Counts/min	Per cent		
I	0.258	1.85	11,740	6.62	43,350	15.8
II	9.234	66.24	46,170	26.02	5,000	75.1
—[a]	0.483	3.46	8,770	4.94	18,130	20.6
III	1.135	8.14	43,950	24.77	38,700	136.5
IV	0.841	6.03	23,570	13.28	28,000	53.3
V	0.676	4.85	25,840	14.56	38,220	141.9
CM[b]	1.314	9.43	17,390	9.80	13,230	41.7

[a] Material eluted from column with 2% methanol in benzene produced no peak having radioactivity (*see* Fig. 4).
[b] Material eluted from column with chloroform-methanol (2:1).

to transport it rapidly. Since free fatty acids are inhibitory for bacterial enzymes involved in lipogenesis (5), nonesterified palmitic acid within the cell conceivably would be toxic. The transport process is linked, therefore, to another in which the fatty acid is immediately incorporated into nontoxic triglyceride. Walker et al. (16) have shown that the triglycerides of *M. bovis* BCG are esterified at position 1 principally with C_{18}-related fatty acids, at position 2 with C_{16} fatty acids, and at the third position are 76% esterified with C_{20} to C_{33} fatty acids. It is not known how this asymmetry is achieved nor if the triglycerides of *M. avium* are similar to those of *M. bovis*. However, *M. avium* should prove useful in an investigation of triglyceride biosynthesis since it must have an active enzyme system which esterifies the fatty acid to diglyceride.

Although palmitic acid is required for cell division, omission of the compound does not cause death of *M. avium* as it does for a mutant of *Escherichia coli* that requires fatty acid (8). This is probably because the eubacteria are devoid of triglycerides (10) and the mycobacteria are not (3, 13). Long-term experiments, in which the organism is permitted to undergo many divisions in medium containing ^{14}C-palmitic acid, do not provide accurate information concerning the intracellular fate of the compound because of turnover of the ^{14}C-triglyceride and redistribution of the radioactive label among other cell components.

In a fully grown culture of *M. avium*, nonstarved for fatty acid, the level of intracellular triglyceride may approximate 5% of the cell dry weight. It has been shown that *M. tuberculosis* accumulates lipid during postexponential growth (2); presumably this is triglyceride although it was not identified (2). The storage of such compounds constitutes a tremendous energy reserve for the mycobacteria, and the factors that regulate the catabolism of triglycerides should present an interesting focus of attention in future studies.

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LITERATURE CITED

1. Amenta, J. S. 1964. A rapid chemical method for quantification of lipids separated by thin-layer chromatography. J. Lipid Res. 5:270–272.
2. Antoine, A. D., and B. S. Tepper. 1969. Environmental control of glycogen and lipid content of *Mycobacterium tuberculosis*. J. Bacteriol. **100**:538–539.
3. Bloch, H., J. Defaye, E. Lederer, and H. Noll. 1957. Constituents of a "toxic-lipid" obtained from *Mycobacterium tuberculosis*. Biochim. Biophys. Acta **23**:312–321.
4. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
5. Ferdinandus, J., and J. B. Clark. 1969. Selective inhibition of bacterial enzymes by free fatty acids. J. Bacteriol. **98**: 1109–1113.
6. Freeman, C. P., and D. West. 1966. Complete separation of lipid classes on a single thin-layer plate. J. Lipid Res. **7**: 324–327.
7. Hedgecock, L. W. 1968. Nutritional characteristics of the atypical mycobacteria. J. Bacteriol. **96**:306–313.
8. Henning, U., G. Dennert, K. Rehn, and G. Deppe. 1969. Effects of oleate starvation in a fatty acid auxotroph of *Escherichia coli* K-12. J. Bacteriol. **98**:784–796.
9. Krulwich, T. A., and J. C. Ensign. 1969. Alteration of glucose metabolism of *Arthrobacter crystallopoietes* by compounds which induce sphere to rod morphogenesis. J. Bacteriol. **97**:526–534.
10. Lennarz, W. J. 1966. Lipid metabolism in the bacteria. Advan. Lipid Res. **4**:175–225.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
12. McCarthy, C. 1970. Spontaneous and induced mutation in *Mycobacterium avium*. Infec. Immun. **2**:223–228.
13. Michel, G. 1957. Composition des cires A de *Mycobacterium marianum*. C. R. Acad. Sci. Paris **244**:2429–2432.
14. Schaefer, W. B., C. L. Davis, and M. L. Cohn. 1970. Patho-

genicity of transparent, opaque, and rough variants of *Mycobacterium avium* in chickens and mice. Amer. Rev. Resp. Dis. **102**:499–506.

15. Schaefer, W. B., and C. W. Lewis, Jr. 1965. Effect of oleic acid on growth and cell structure of mycobacteria. J. Bacteriol. **90**:1438–1447.
16. Walker, R. W., H. Barakat, and J. G. C. Hung. 1970. The positional distribution of fatty acids in the phospholipids and triglycerides of *Mycobacterium smegmatis* and *M. bovis* BCG. Lipids **5**:684–691.
17. Wayne, L. G. 1962. Differentiation of mycobacteria by their effect on Tween 80. Amer. Rev. Resp. Dis. **86**:579–581.