BACTERICIDAL ACTION OF OLEIC ACID FOR TUBERCLE BACILLI[1]

I. Quantitative and Analytical Survey of the Action

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Several researches have been carried out on the inhibitory action of oleic and other homologous long-chain higher fatty acids on tubercle bacilli (Boissevain, 1926; Platonov, 1930; Bergström *et al.*, 1946; Davis and Dubos, 1946; Dubos and Davis, 1946; Dubos, 1947; Gray *et al.*, 1948; Franke *et al.*, 1949; Patnode, 1954), but the mode of action of these acids still remains obscure. The tubercle bacilli, in turn, are capable of utilizing "tween-80" as a carbon source (Minami *et al.*, 1954; Yamane *et al.*, 1954*a*, *b*), and their growth is effectively enhanced by this substance which, however, contains oleic acid. The favorable effect of tween is altered if the culture contains no serum albumin and if a low inoculum is used (Davis and Dubos, 1947; Dubos and Middlebrook, 1947, Minami *et al.*, 1955), and it has been assumed by these investigators that the alteration is caused by the oleic acid liberated from the tween molecule. This interesting contradictory relationship between tween and oleic acid has been studied, and in this paper several aspects of the effects of oleic acid on viability and metabolic activities of tubercle bacilli are reported and possible mechanisms are discussed.

MATERIALS AND METHODS

Mycobacterium avium (TAKEO strain) was used. Large amounts of the cells were obtained from the 3-day surface cultures of this strain on Sauton's medium. When viability of the organism was tested, a portion of the cell suspension was used as an inoculum into the following tween-containing medium: KH_2PO_4, 1.0 g; $Na_2HPO_4 \cdot 12H_2O$, 4.0 g; $(NH_4)_2SO_4$, 1.0 g; Na-glutamate, 1.0 g; Na-citrate, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.02 g; ferric ammonium citrate, 0.001 g; glycerol, 10.0 g; tween-80, 0.5 g; and distilled water, 1,000 ml (autoclaved at 120 C for 15 min). After 3 days' cultivation in this medium, the harvested cells were washed three times with phosphate buffer (pH 6.8, M/40), and then diluted to 10^5 times with the same buffer and viability determined. All the chemicals used were of reagent grade. Oleic acid was used as the sodium salt.

Colony counts were made by means of the usual pour plate method. The bacterial suspension (diluted 10^5 times, containing about 200 viable organisms per ml) was brought into contact with oleate, together with (or without) various test substances, at 37 C before it was inoculated into the tween-agar medium devised by Yamane *et al.* (1955). After incubation for 4 days, the colonies were counted directly on or in the tween-agar plate. An average colony number of duplicated or triplicated tests was calculated to indicate the number of colonies per ml of the suspension used.

Measurement of oxygen uptake was performed mostly in an ordinary Warburg vessel with one side-bulb. The main space of the vessel contained 0.5 ml of bacterial suspension, 0.5 ml of phosphate buffer (1/15 M, pH 6.8) and 0.5 ml of distilled water. The side-bulb contained 0.5 ml of substrate solution, and the central cup contained 0.2 ml of 20 per cent potassium hydroxide with a small strip of filter paper. The gaseous phase was air. The vessels were shaken at 120 strokes per min for 15 min at 37 C to ensure equilibration before admixing the substrate from the side-bulb.

Measurement of dehydrogenase activity was carried out in a usual Thunberg tube by recording the time required to reduce methylene blue. The main space of the tube contained 0.5 ml of bacterial suspension, 0.5 ml of phosphate buffer (1/15 M, pH 6.8), and 0.5 ml of substrate solution. The side-bulb contained 0.5 ml of methylene blue solution (1:2,500). The tube was evacuated by vacuum oil pump for 5 min and incubated at 39 C for 5 min before admixing the dye

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solution from the side-bulb. The time needed to reduce 90 per cent of the color was recorded.

RESULTS

Effect of the concentration of oleate and the time of contact on viability. The diluted cell suspension (10^5 dilution) was kept in contact with oleate for various periods and then the cells so treated were inoculated into the plate medium for the test of their viability. The results are indicated in table 1. The number of viable cells was reduced to 60 per cent by treating with 10^{-3} M oleate for a few seconds, and they were completely suppressed after 60 min. With 10^{-4} M, the viable cells were reduced to 75 per cent by the instantaneous treatment, and the cells were completely killed after 6 hr; whereas with 10^{-5} M they still remained alive to some extent even after 6 hr. Without oleate, the number of viable cells gradually increased after incubation.

Possible effect of minute amount of oleate which may be delivered into the plate medium together with treated cell suspension. This effect was examined by using both washed and unwashed cell suspensions after treating them with oleate. The unwashed cell suspension was only 6 per cent viable after treating with 10^{-4} M oleate for 60 min, and almost the same per cent was counted in the washed cell suspension. The concentration of oleate was varied from 10^{-3} to 10^{-5} M, and the test was carried out at 10 and 60 min. In all these cases almost no effect of washing was observed. Once a cell suspension had been exposed to oleate, subsequent washing did not restore viability.

Suspension density and bactericidal action of oleate. The cells at various concentrations were exposed to 10^{-4} M oleate for 6 hr. A cell suspension diluted 10^5 times and exposed to 10^{-5} M oleate for 6 hr had 12 per cent survival, but no survival was observed at 10 times (or more) concentrated cell suspension when the amount of oleate was correspondingly increased 10-fold (10^{-4} M). Only the most dense suspension, *i.e.*, the original suspension afforded some protection against the toxic effect of the oleate at 10^{-4} M.

Effect of adsorption of oleate to the bacterial cells. A cell suspension diluted 10^4 times was exposed to 10^{-3} M oleate for 60 min at 37 C and then it was filtered through a Seitz filter. To the filtrate, a newly prepared cell suspension was added to make the same cell concentration (10^4 dilution of the original suspension) and was incubated at 37 C for 60 min and viability estimated. Such an incubation-filtration procedure was carried out twice further, with the filtrate and viability being determined at each step of the procedure. The bactericidal action of 10^{-3} M oleate was eliminated only after two adsorptions by the 10^4 dilution of cell suspension and two filtrations. The bactericidal capacity of oleate, however, was considerably depressed merely by the filtration procedure, probably because of the adsorptive action of the filter itself.

TABLE 1

The bactericidal effect of oleate in relation to its concentration and the time of contact with it

Concentration of Oleate	Duration of Contact						
	A few sec	10 min	30 min	1 hr	6 hr	24 hr	48 hr
	Per cent survival						
M							
10^{-3}	60	2	0.2	0	0	0	0
10^{-4}	75	32	7	2.3	0	0	0
10^{-5}	100	100	99	90	14	6	3
0	100	100	100	100	96	Growth	Growth

Each 1 per cent corresponds to 2.1 colonies.

Survey of the substances which protect the bactericidal action of oleate. The bacterial suspension was brought into contact with 10^{-5} M oleate and various substances, as indicated in table 2, for 6 hr and was then examined for viability. About 16 per cent of the bacilli remained viable after the contact with 10^{-5} M of oleate in phosphate buffer. When charcoal or starch was added under these conditions, 86 per cent were viable. The toxic action of oleate was neutralized almost entirely by 5 to 5 $\times$ 10^{-4} per cent of tween-80. Similar effects were observed with "triton A-20" and saponin. The other nonionic surface-active agents, such as triton X-100, "span-20", span-80, and "carbowax-1500", were somewhat effective; *i.e.*, about 50 per cent of the bacilli survived. About 80 per cent of the cells were kept alive by the presence of lecithin or cholesterol. About 30 to 50 per cent of the cells could be left viable by the addition of glucose, glycerol, succinate, malate, acetate, glutamate, or aspartate, respectively. A slight protecting effect was also observed with coenzyme-A and biotin.

If the cell suspension was treated with 10^{-5} M oleate for 6 hr before it was brought into contact

TABLE 2
Protective effect of various substances on the bactericidal action of oleate

Substances Added		Per Cent Survival*
Type	Per cent	
None, at starting point†	—	100
None, after 6 hr.†	—	98
None, with 10^{-5} M oleate‡	—	16
Charcoal	0.1	86
Starch	0.1	80
	0.05	83
Lecithin	90 γ/ml	87
Cholesterol	0.1	73
	0.01	71
Tween-80	5	81
	0.5	94
	0.05	98
	0.005	100
	0.0005	80
	0.00005	41
Triton A-20	0.5	81
	0.05	95
	0.005	84
	0.0005	43
	0.00005	20
Triton X-100	0.05	8
	0.005	43
	0.0005	19
Span-20	0.05	37
	0.005	33
	0.0005	29
Span-85	0.05	42
	0.005	55
	0.0005	46
Carbowax-1500	0.5	55
	0.05	60
	0.005	48
Saponin	0.5	85
	0.05	98
	0.005	70
Glucose	0.5	52
	0.1	43
Glycerol	0.5	48
	0.1	49
Succinate	0.1	47
	0.05	54
Malate	0.1	36
	0.05	47
Acetate	0.1	32
	0.05	30
Glutamate	0.5	35
	0.05	33
Aspartate	0.5	28
	0.05	30
Biotin	0.5 (γ/ml)	42
	0.05 (γ/ml)	54
Coenzyme-A	10 (γ/ml)	32
	1 (γ/ml)	24

The bacilli were exposed to 10^{-5} M of oleate together with the surveyed substances at 37 C for 6 hr.

* Each 1 per cent corresponds to 1.98 colonies.

† Without oleate as a standard case.

‡ None of the surveyed substance was added and only oleate was in touch with the cells.

with the protecting substances such as charcoal, lecithin, or tween-80, the bactericidal action was not neutralized by these substances. Once the cells had been treated with 10^{-4} M oleate, no restoration of viability was observed by washing either with petroleum ether or distilled water.

The bactericidal action of 10^{-5} M oleate was also examined in the Thunberg tubes, of which gaseous phase was varied from air to 5 per cent CO_2 in N_2 mixtures. Carbon dioxide was without influence. The anaerobic circumstances seemed to accelerate the bactericidal action slightly.

Effect of concentration of oleate on oxygen consumption. The results are illustrated in figure 1. The oxygen uptake was accelerated remarkably by 10^{-4} M oleate, while with 10^{-3} M the oxygen uptake dropped lower than the endogenous level. Furthermore, its amount was not proportional to the substrate concentrations between the range from 10^{-6} to 10^{-4} M. At the latter concentration, it was found that the oxygen uptake was elevated conspicuously. A cell-free water extract was prepared by grinding the cell in a porcelain bowl and by centrifuging the resulting paste at 12,000 rpm for 20 min. The supernatant extract was used as an enzyme suspension, and the effect

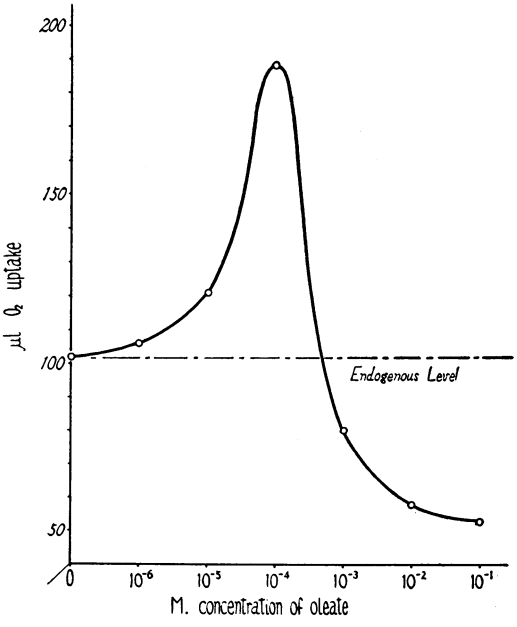

Figure 1. Effect of concentration of oleate on the oxygen uptake of the intact cell. Each Warburg vessel contained 10 mg (wet weight) of tubercle bacilli. Amounts of oxygen uptake at 90 min were plotted against the log concentration of oleate.

of concentration of oleate on the oxygen uptake was also observed (figure 2). The activity dropped generally, and a considerably different curve was obtained as compared with that of the intact cells. The oxygen uptake was increased almost proportionally to the substrate concentration between the range from 10^{-6} to 10^{-4} M, and none of the oxygen uptake dropped lower than the endogenous level, even at 10^{-3} M.

With 50 mg (wet weight) of the bacilli per vessel and 10^{-3} M oleate a higher oxygen uptake than endogenous was observed during the first 45 min, and then the activity was gradually weakened and the inhibition occurred clearly after 90 min. In every case with lower cell concentration—*i.e.*, with 10, 1, or 0.1 mg of the bacilli respectively—the oxygen uptake (with 10^{-3} M oleate) was less in amount than the endogenous respiration, even from the beginning of the measurements.

Effect of substrate concentration on the dehydrogenase activity. The dehydrogenase of the intact cells was compared with that of the ground cells, which were prepared by grinding the intact cells in a porcelain bowl for 1 hr by hand. The results are shown in figure 3. In the case of the intact cells the optimum concentration of substrate was 10^{-5} M. At 10^{-3} M, their activity was markedly depressed to a lower level than the endogenous. In contrast, in the case of ground cells, the decolorization time was shortened as a whole, and a considerably different pattern of curve was drawn, so that the optimum concen-

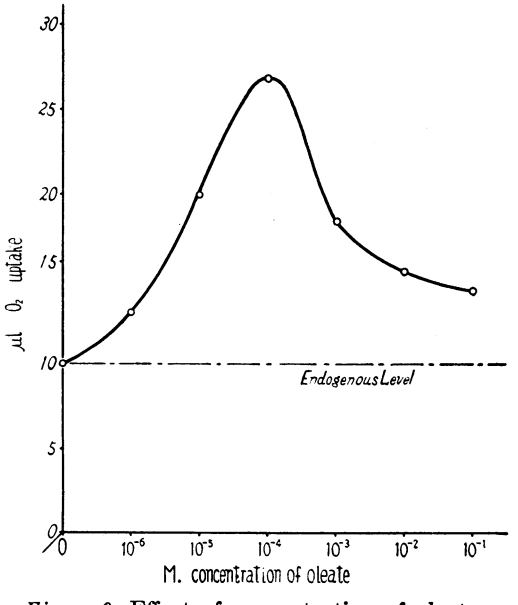

Figure 2. Effect of concentration of oleate on the oxygen uptake of the cell extract. Each Warburg vessel contained 0.5 ml of the cell extract per 2 ml of the medium. Amounts of oxygen uptake at 90 min were plotted against the log concentration of oleate.

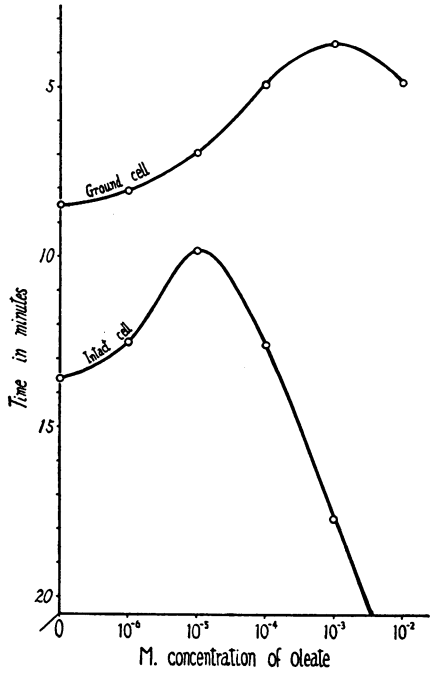

Figure 3. Effect of concentration of oleate on the dehydrogenase of the intact and ground cells of tubercle bacilli (methylene blue reduction). Each tube contained 25 mg (wet weight) of the intact or ground cells.

TABLE 3

Effect of washing and grinding on the decolorization time of methylene blue by the tubercle bacilli treated with 10^{-2} M oleate

Substrate	Absence	10^{-4} M Oleate
	min	*min*
Not treated with oleate		
Washed cell	10	5
Ground cell	6.5	5
Treated with 10^{-2} M oleate for 10 min		
Washed cell	18	18
Ground cell	11	5.5

Each tube contained 25 mg (wet weight) of the whole or ground cell.

tration rose to 10^{-3} M and stimulation took place even at 10^{-2} M.

The bacterial suspension was pretreated with 10^{-2} M oleate for 5 min before it was washed three times with distilled water. One portion of such a pretreated suspension was ground in a porcelain mill for 1 hr and the other portion was employed in intact form. The enzymatic activities of both pretreated suspensions were compared with those of non-pretreated ones (table 3). The activity of the suspension pretreated with 10^{-2} M oleate was not recovered by washing with distilled water. The enzymatic activity, however, revealed itself again after grinding the pretreated cells.

DISCUSSION

It has been indicated that the toxicity of oleic and other long-chain higher fatty acids against tubercle bacilli depends more on the ratio of the quantity of the fatty acid to the number of organisms present than on the calculated concentration of the fatty acids in the medium (Boissevain, 1926; Bergström *et al.*, 1946; Karlsson, 1954). While this relation was observed to some extent in measurements of respiration, such a quantitative relation was difficult to find in the results of the viable count, thus some other explanation might be expected.

If adsorption is strong enough the amount adsorbed onto the surface of an adsorbent is not affected by the concentration of the agent contained in the solution. In this case the adsorption of oleate to the cell must be very strong since the bactericidal action of oleate developed after only a few seconds of contact with oleate (table 1), and this action was not reversed by washing with distilled water or by adding such a protective substance as tween-80, lecithin, or charcoal. It was observed that the bactericidal activity of oleate solution decreased considerably after it was kept in touch with the tubercle bacilli; hence, oleate obviously gets adsorbed to the bacilli, as stated by Bergström *et al.* (1946).

The marked inhibitory action of 10^{-3} to 10^{-2} M oleate was observed both upon the oxygen uptake (figure 1) and upon the dehydrogenase activity (figure 3). Further, the conspicuous enhancement was shown for the oxygen uptake at 10^{-4} M, and for the dehydrogenase activity at 10^{-5} M. But these circumstances were turned into very different ones in the experiment carried out with the cell-free crude extract or ground cell (figures 2 and 3). Furthermore, the whole cells had entirely lost their ability to reduce methylene blue after treating with 10^{-2} M oleate, while, in contrast, the cells disrupted by grinding in a mill showed a discernible activity whether or not they had been treated with oleate (table 3). These observations lead to the assumption that the action of oleate is concerned with the surface structure of the cell. This view is further in accord with data from electron microscopy (Minami, 1957).

The bactericidal activity of oleate was found to be neutralized or reduced by the simultaneous presence of a surface-active agent such as tween-80, triton A-20, and saponin (table 2). Yet, it was concluded by Davis and Dubos (1946, 1948) that the growth-inhibitory action of tween-80, if it occurred, depended on the action of oleic acid which might be either contained in commercial tween-80 or released from it by the action of the tubercle bacilli. Thus, Dubos and Middlebrook (1947) employed serum albumin to protect against its inhibitory action and to ensure the bacterial growth. This would seem to contradict the data on the action of tween-80 cited above. However, since tween-80 is not only toxic by itself for the bacilli but is metabolized by them vigorously (Minami *et al.*, 1954; Yamane *et al.*, 1954*a*, *b*; Minami and Yamane, 1954) it would be possible for tween-80 to protect against the adsorption of oleate to the cell surface and subsequently the inhibitory or bactericidal action of oleate itself, if tween-80 were adsorbed selectively onto the cell surface. The irreversibility of the oleate adsorption to the cells might be assumed from the fact that the viability of the cells which had been treated with oleate before the addition of tween-80 was not recovered by the subsequent addition of tween-80.

To eliminate the toxic substances from the culture medium, charcoal was employed by Hirsch (1954), Whalen and Mallmann (1955), and Yamane *et al.* (1955), and starch by Drea (1948). The protective action of these substances was also confirmed to some extent in the present studies (table 2). This protective effect may be due to decreasing the quantity of oleate in the medium by its adsorption to these substances, since the cells are not protected by these substances if added after the treatment of oleate. The effects of lecithin and cholesterol on the growth and respiration of *Micrococcus pyogenes* var. *aureus* have been studied by Wynne

and Foster (1950), who found that these substances were also effective for tubercle bacilli, and their effect would be due probably to their action on the bacilli in a manner similar to that of tween-80.

The bactericidal activity of oleate was somewhat diminished by the addition of an energy-supplying substance such as glucose, glycerol, succinate, malate, acetate, glutamate, or aspartate. This action could be due to the reduction of oleate concentration by the metabolic process mobilized by the addition of such energy-supplying substances, since oleate seems to be metabolized by the bacilli (Minami and Yamane, 1954, 1955).

The recovering action of biotin would be an interesting problem in its association with the function of carbon dioxide and oleate (Miura, 1955; Schaefer *et al.*, 1955), although any favorable effect of carbon dioxide on the reversion of the bactericidal action of oleate was not observed in the present experiment.

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SUMMARY

The effects of oleic acid on viable numbers, oxygen uptake, and dehydrogenase activity were studied with *Mycobacterium avium* (TAKEO strain).

The bactericidal activity of oleate depended upon its concentration and upon the length of time the cells were exposed to it, but was not dependent upon cell density.

Various substances protected against this bactericidal action as follows: tween-80, triton A-20, saponin, lecithin, cholesterol, charcoal, and starch. Effective to a small extent were, respectively: glucose, glycerol, succinate, malate, glutamate, aspartate, biotin, etc. When the cells were once exposed to oleate, however, its bactericidal effect was not reversed either by washing them with distilled water, or by addition of tween-80, lecithin, and charcoal, respectively.

On the other hand, the oxygen uptake was increased vigorously by 10^{-4} M oleate, whereas it was markedly inhibited by 10^{-3} M. Similar effects were also observed in the dehydrogenase activity. These inhibitory phenomena disappeared by grinding the cells, but were not restored by washing them with distilled water. From these facts, it might be assumed that the surface structure of the cells would be concerned with these inhibitions, and some possible mechanisms of the oleate action were discussed.

REFERENCES

BERGSTRÖM, S., THEORELL, H., AND DAVIDE, H. 1946 Effect of some fatty acids on the oxygen uptake of *Mycobact. tubercul. hum.* in relation to their bactericidal action. Nature, **157**, 306–307.

BOISSEVAIN, C. H. 1926 The action of unsaturated fatty acids on tubercle bacilli. Am. Rev. Tuberc., **13**, 84–89.

DAVIS, B. D. AND DUBOS R. J. 1946 Interaction of serum albumin, free and esterified oleic acid and lipase in relation to cultivation of the tubercle bacilli. Arch. Biochem., **11**, 201–203.

DAVIS, B. D. AND DUBOS, R. J. 1947 The binding of fatty acids by serum albumin—a protective growth factor in bacteriological media. J. Exptl. Med., **86**, 215–228.

DAVIS, B. D. AND DUBOS, R. J. 1948 The inhibitory effect of lipase on bacterial growth in media containing fatty acid ester. J. Bacteriol., **55**, 11–23.

DREA, W. F. 1948 Growth of some mycobacteria: (a) in the depth of Long's liquid synthetic culture medium, (b) on the surface of Long's medium plus agar. J. Colorado, Wyoming Acad. Sc., **3**, 15.

DUBOS, R. J. 1947 The effect of lipids and serum albumin on bacterial growth. J. Exptl. Med., **85**, 9–22.

DUBOS, R. J. AND DAVIS, B. D. 1946 Factors affecting the growth of tubercle bacilli in liquid medium. J. Exptl. Med., **83**, 409–423.

DUBOS, R. J. AND MIDDLEBROOK, G. 1947 Media for tubercle bacilli. Am. Rev. Tuberc., **56**, 335–345.

FRANKE, W., LEE, L., AND KIBAT, D. 1949 Zum Stoffwechsel der säurefesten Bakterien. II. Über das Verhalten der ungesättigten Fettsäuren. Biochem. Z., **316**, 313–334.

GRAY, C. T., GORDON, L. E., AND BIRKELAND, J. M. 1948 Effect of fatty acids and their esters on the respiration of tubercle bacilli.

Soc. Am. Bacteriologists, Proc. 48th General Meeting, pp. 84–85 (abstracts).

HIRSCH, J. G. 1954 Charcoal media for the cultivation of tubercle bacilli. Am. Rev. Tuberc., **70**, 955–976.

KARLSSON, J. L. 1954 Intracellular conjugation and detoxification of palmitic acid by *Mycobacterium tuberculosis*. J. Bacteriol., **64**, 456–459.

MINAMI, K. 1957 Bactericidal action of oleic acid for tubercle bacilli. II. Morphological response. J. Bacteriol., **73**, 345–352.

MINAMI, K., YAMANE, I., AND YASUI, T. 1954 Tween 80 as a metabolite for tubercle bacilli. I. On the metabolism of Tween 80 with the resting cells of tubercle bacilli. Fukushima J. Med. Sci., **1**, 95–103.

MINAMI, K. AND YAMANE, I. 1954 The metabolic process of Tween 80 by the tubercle bacillus. Symposium on Enzyme Chem., Tokyo, **10**, 93–102.

MINAMI, K. AND YAMANE, I. 1955 Tween 80 as a metabolite for tubercle bacilli. IV. The investigation of the metabolic process of Tween 80 by means of the adaptive cells of tubercle bacilli. Fukushima J. Med. Sci., **2**, 13–17.

MINAMI, K., TAKAHASHI, Y., AND YAMANE, I. 1955. Effect of the size of inoculum on the growth of tubercle bacilli in Tween-containing medium. Fukushima J. Med. Sci., **2**, 185–188.

MIURA, H. 1955 Growth of tubercle bacilli in serum of healthy guinea pigs—observation with slide culture method. Med. J. Osaka Univ. (Japanese Ed.), **7**, 31–42.

PATNODE, R. A. 1954 Tissue fatty acids and their possible relationship to the natural resistance of rabbits to infection with human-type tubercle bacilli. Am. Rev. Tuberc., **69**, 710–723.

PLATONOV, G. 1930 The influence of unsaturated fatty acids on the virulence of tubercle bacilli. Am. Rev. Tuberc., **21**, 362–369.

SCHAEFER, W. B., COHN, M. L., AND MIDDLEBROOK, G. 1955 The roles of biotin and carbon dioxide in the cultivation of *Mycobacterium tuberculosis*. J. Bacteriol., **69**, 706–712.

WHALEN, J. W. AND MALLMANN, W. L. 1955 Charcoal agar media for the cultivation of *Mycobacterium tuberculosis*. Am. Rev. Tuberc. Pulm. Diseases, **71**, 382–389.

WYNNE, E. S. AND FOSTER, J. W. 1950 Studies on the effect of C-18 unsaturated fatty acids on growth and respiration of *Micrococcus pyogenes* var. *aureus*. J. Infectious Diseases, **86**, 33–37.

YAMANE, I., MINAMI, K., AND YASUI, T. 1954*a* Tween 80 as a metabolite for tubercle bacilli. II. Culture medium containing Tween 80 as a sole source of carbon. Fukushima J. Med. Sci., **1**, 105–112.

YAMANE, I., MINAMI, K., AND YASUI, T. 1954*b* Un milieu simple pour la culture rapide et homogène du bacille tuberculeux, renfermant le tween 80 comme la seule source de carbone. Compt. rend. soc. biol., **148**, 769–768.

YAMANE, I., HYODO, S., HONDA, T., AND KADOYA, K. 1955 Tween agar medium for the clinical detection of tubercle bacilli (preliminary report). Fukushima J. Med. Sci., **2**, 35–37