ELECTRON-SCATTERING GRANULES AND REDUCING SITES IN MYCOBACTERIA[1]

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Intracellular granules of mycobacteria were first revealed as discrete, dense, electron scattering bodies by Lembke and Ruska (1940) and by Wessel (1942). These investigators distinguished "Granula" (in diameter 300–350 mμ) and "Mikrogranula" (in diameter about 20 to 30 mμ). Subsequent work (Shinohara, 1955) has shown, however, that granules form a continuous series of dimensions, graded from a few to 400 mμ or more in diameter, and that size is influenced by cultural conditions, the presence of inhibitors, etc. (Meissner and Diller, 1953; Shinohara and Fukushi, 1955). The microgranules and many of the large granules may be volatilized by intense electron bombardment, and both may be reduced to sponge-like residues by ether-extraction (Lembke and Ruska, 1940).

Electron pictures of an avian strain of *Mycobacterium tuberculosis* were published by Knaysi *et al.* (1950), and the intracellular granules were interpreted as nuclei. This interpretation was reiterated by Knaysi (1952). Cogent reasons for rejecting the interpretation of the electron-scattering granules of mycobacteria as nuclei have been published by Mudd *et al.* (1951*b*), Werner (1951), Winterscheid and Mudd (1953), Brieger and Glauert (1953), Brieger *et al.* (1954), Takeya *et al.* (1954), and Kölbel (1955).

Mycobacterial granules were shown (Mudd *et al.*, 1951*b*) to be loci of enzymatic oxidative-reductive activities, to contain phospholipid, and to give in high dilutions of Janus green B the succession of colors characteristic for the staining of mitochondria. Cytoplasmic granules and nuclei were stained differentially in the same mycobacterial cells (Winterscheid and Mudd, 1953), cytoplasmic granules with oxidative-reductive indicators and metachromatically with methylene blue, and nuclei with the Feulgen and DeLamater nuclear staining procedures.

König and Winkler (1948) demonstrated that the electron scattering granules of diphtheria, tubercle and dysentery bacilli could be dissolved out under appropriate conditions by organic acids. Extracts from diphtheria bacilli were analyzed and found to contain calcium and phosphate in considerable amounts. Ruska *et al.* (1952) extracted the electron scattering granules of mycobacteria and demonstrated their metaphosphate content; in model experiments dried droplets containing metaphosphate were shown to imitate the granules of the bacteria in respect to electron scattering and volatilization by electron bombardment. The work of König and Winkler (1948), Werner (1951), Ruska *et al.* (1952), Winterscheid and Mudd (1953), Winkler (1953), and Takeya *et al.* (1954) associated the electron scattering granules with the metachromatically staining metaphosphate granules known as volutin. The subject is admirably reviewed by Winkler (1956).

Glauert and Brieger (1955) have stained groups of cells of *Mycobacterium phlei* for metaphosphate and chromatin and by reduction of triphenyl tetrazolium, and then prepared electron pictures of the same cell groups. The metachromatic, metaphosphate-containing granules were found to be identical with the electron-scattering granules. These granules alternated in position with the nuclei, as described earlier by Winterscheid and Mudd (1953). Concerning the relationship of metaphosphate granules to reduction sites Glauert and Brieger write: "In some of the organisms the reduction sites and the electron-opaque granules occupied the same positions but the stained granules were frequently much larger than the dense bodies. Sometimes the deposits of dye were unaccom-

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panied by any corresponding dense granules." Also: "in some areas dense bodies are seen with no accompanying stained granules."

The existence in mycobacteria of particulate entities capable of oxidizing most or all of the Krebs cycle substrates, has recently been demonstrated in cell-free preparations by Millman and Youmans (1955). They have described a preparation of microscopic particles from an avirulent strain of *M. tuberculosis hominis*, which reduces tetrazolium and oxidizes the Krebs cycle intermediates, and apparently contains a full complement of cytochromes. Cytologically these particles varied from blown up rods 5 μ in width and 10 μ in length to small coccoid forms approximately 100 mμ in diameter. Yamamura *et al.* (1955) and Kusunose *et al.* (1956) prepared from *M. avium* particles from 50 mμ to 150 mμ in diameter which, stained with Janus green B, contained phospholipid and cytochromes and exhibited malic, succinic and formic dehydrogenase activities. Most recently Brodie and Gray (1955, 1956 and see Mudd, 1956) have isolated from *M. phlei* spherical particles which, under suitable conditions, exhibited oxidative phosphorylation, with the generation of high energy phosphate and incorporation of P32 into ADP. Dinitrophenol sensitive P/O ratios greater than one were obtained with substrates from the Krebs cycle.

The existence in mycobacteria of particulates which in functional activity are the equivalents of the mitochondria of higher forms has thus been documented first by cytochemical and later by biochemical methods. If then these enzymatically active granules are functional equivalents of mitochondria, are they also structural homologues of the mitochondria of higher plants and animals, whose internal organization has been found (Palade, 1953; Sjöstrand, 1953) to be so striking and characteristic? What is the relationship between these mitochondrial equivalents and the electron-scattering metaphosphate deposits of mycobacteria? It is to these two questions that the present investigation is directed.

MATERIALS AND METHODS

Bacterial strains used. Mycobacterium thamnopheos, *M. tuberculosis* var. *bovis*, strain BCG.

Culture methods. M. thamnopheos was incubated at 37 C for 16 or 24 hr on 3 per cent glycerol extract agar. BCG was grown for 6 days on American Trudeau Society medium at 37 C.

Staining procedures for reducing sites. Cells grown on the appropriate medium were suspended in 3 per cent glycerol extract broth containing 0.025 per cent blue tetrazolium or 0.05 per cent potassium tellurite. After incubation for an appropriate period, the cells in the suspension were pipetted onto agar blocks, fixed for 1 min over the vapors of a 2 per cent solution of osmium tetra-oxide, partially dried and impressed onto coverslips. The smears were treated for 10 min with 1 per cent phosphotungstic acid solution (Hale, 1953) and counterstained with Ziehl-Neelsen carbol-fuchsin solution diluted 1:10 with distilled water.

Staining procedures for metaphosphate granules. Cells were smeared directly onto coverslips and fixed for 1 min in the vapors of a 2 per cent solution of OsO_4. a) The cells of *M. thamnopheos* smears were stained with Neisser's acidified methylene blue stain for 8 min and those of BCG for 15 min, followed by 0.25 per cent malachite green in aqueous solution for 30 sec for the former and 2.0 per cent malachite green for 5 min for BCG as a counterstain. b) The lead sulfide stain of Wachstein and Pisano (1950) was also used. Smears were treated for 15 min with lead nitrate, rinsed well and placed in a 0.1 per cent aqueous solution of ammonium sulfide for 1 min, rinsed and counterstained with carbol-fuchsin solution. c) The smears were stained with Gram's crystal violet solution for 15 sec, partially destained for 1 min in 5 per cent citric acid solution, placed for 10 min in 1 per cent phosphotungstic acid solution (Hale, 1953) and counterstained with carbol fuchsin solution.

Microscopic examination. One hundred rods were examined on each smear and the numbers of granules stained by the method in question were counted. The length of 100 rods was also measured with a micrometer. The optical system was as previously described (Mudd *et al.*, 1951*b*).

Electron micrographs. Figures 1–3 of *M. thamnopheos* were prepared by L. C. Winterscheid as described in an earlier work (Mudd *et al.*, 1951*b*) and micrographed by Mrs. Jean Minkin through the courtesy of the Laboratories for Research and Development, the Franklin Institute, Philadelphia. Figures 4–7 and 13–14 of *M. thamnopheos* and 8–11 of BCG were prepared

and micrographed by K. Takeya with the Philips electron microscope of the Department of Pathology, University of Pennsylvania, through the courtesy of Professor Dale R. Coman. Figures 15 and 16 of BCG were micrographed at Kyushu University, Japan, by K. Takeya and M. Koike, using the Hitachi, type HU-V electron microscope. Figures 17 and 18 of *M. tuberculosis* var. *hominis* strain H37Rv were prepared by L. C. Winterscheid and micrographed by Dr. James Hillier at the RCA Laboratories, Princeton, N. J.

RESULTS

Reducing sites and metaphosphate deposits. Surface growth of *M. thamnopheos* after 24 hr was removed with a platinum loop, dispersed by grinding and suspended in 3 per cent glycerol extract broth; this suspension was inoculated onto the surface of 3 per cent glycerol extract agar. After 0, 3, 7, 24 and 72 hr of incubation the bacilli were stained for metaphosphate granules and for reduction of tetrazole or potassium tellurite. The numbers of granules in *M. thamnopheos* staining metachromatically and the numbers showing reducing activity, as functions of age of culture, are shown in table 1. The numbers of microscopically visible metachromatic granules per rod are seen to decrease up to 7 hr after subculture, to increase to 24 hr and to be much decreased at 72 hr; the 72-hr values are not regarded as very reliable, however (see footnote to table 1). The numbers of granules showing reducing activity were approximately proportional to rod length. The ratio of reducing sites to rod length remained nearly constant at approximately 1 site per μ of rod length. The absolute numbers of visible reducing sites exceeded the numbers of visible metachromatic deposits in a ratio of about 1.5–3 to 1 during the 24 hr period when counts were reliable.

M. thamnopheos was grown on 3 per cent glycerol agar for 24 hr, inoculated onto 0, 0.5, 3.0 and 10 per cent glycerol agar, and incubated at 37 C. Metaphosphate deposits were stained by Neisser's acidified methylene blue and reducing sites by blue tetrazolium. The results are shown in table 2. Again the numbers of metachromatic granules decreased with time to a minimum near 6 hr and increased to 24 hr. The ratio of metachromatic granules to rod length decreased to a minimum and then increased to 24 hr. The ratio of metachromatic granules to rod length increased with the glycerol content of the agar medium. Meanwhile the ratio of reducing sites to rod length remained nearly constant at slightly greater than 1 reducing site per μ of rod length, and again the absolute numbers of visible reducing sites exceeded the

TABLE 1

Variation in numbers of metachromatic and of reducing granules in Mycobacterium thamnopheos with age of culture

Method of Staining	Mean Number of Granules per Rod after Incubation for				
	0 hr	3 hr	7 hr	24 hr	72 hr
Neisser	1.5	1.2	1.0	2.2	0.4*
Lead sulfide	1.4	1.2	1.1	2.4	0.3*
Crystal violet	1.5	1.2	1.0	2.4	0.2*
Blue tetrazolium	2.6	2.9	3.1	3.3	1.9
Potassium tellurite	2.7	3.1	3.3	3.4	1.9
Mean length of rod μ	2.3	2.8	3.2	3.5	1.6

* Most of the rods were stained very poorly and granules were also not distinct.

TABLE 2

Variation in numbers of metachromatic and reducing granules in Mycobacterium thamnopheos with glycerol content of medium

Glycerol Contained in Medium	Metaphosphate Granules					
	Mean number of Neisser's granules per rod after incubation for			Mean number of Neisser's granules per unit length (μ) of rod after incubation for		
	0 hr	6 hr	24 hr	0 hr	6 hr	24 hr
%						
0	1.2	0.6	2.3	0.60	0.15	0.51
0.5	1.2	0.9	2.1	0.60	0.23	0.50
3.0	1.2	1.0	1.9	0.60	0.31	0.53
10.0	1.2	1.2	1.6	0.60	0.40	0.66
	Reducing Sites					
	Mean number of blue tetrazolium granules per rod			Mean number of blue tetrazolium granules per unit length (μ) of rod		
0	2.4	5.0	4.8	1.2	1.2	1.1
0.5	2.4	4.8	4.6	1.2	1.3	1.1
3.0	2.4	3.9	3.4	1.2	1.2	1.0
10.0	2.4	3.5	2.8	1.2	1.2	1.2

TABLE 3

Metachromatic and reducing granules in washed cells of Mycobacterium thamnopheos surviving under various metabolic conditions

No. and Contents	Metaphosphate Granules						Reducing Granules (K-tellurite)	
	Index of metachromasy			Mean number of granules per rod			Mean number of granules per rod	
	6 hr	24 hr	48 hr	6 hr	24 hr	48 hr	24 hr	48 hr
1. Control (water)	90	65	30	1.4	0.9	0.4	1.9	1.0
2. Control (phosphate + KCl)	105	75	55	1.4	1.0	0.7	1.8	0.8
3. Glucose + water	65	90	70	0.9	1.0	0.5	1.5	1.2
4. Glucose + phosphate + KCl	85	110	115	1.1	1.1	1.0	1.8	0.9
5. Glycerol + water	95	85	85	1.5	1.3	1.3	2.0	1.3
6. Glycerol + phosphate + KCl	120	135	150	1.5	1.4	1.5	1.8	1.0
7. Malate + water	110	125	175	1.6	1.7	1.9	1.4	0.8
8. Malate + phosphate + KCl	120	160	190	1.7	1.8	2.0	1.7	1.1

For all of the systems, the index of metachromasy at time 0 was 105 and the mean numbers of granules per rod 1.4, both with the metachromatic and with the K-tellurite stains. The final volume in each system was 4 ml, the final concentrations of glucose 0.1 M, of glycerol 4 per cent, of malate 0.1 M, and of tris buffer pH 7.0, 0.05 M.

TABLE 4

Size distribution of metaphosphate granules in experiment summarized in table 3

No. and Contents	Percentage of Cells Having Various Sizes of Granules														
	6 hr					24 hr					48 hr				
	0	1	2	3	IM*	0	1	2	3	IM*	0	1	2	3	IM*
1. Control (water)	20	70	10		90	40	55	5		65	75	20	5		30
2. Control (phosphate + KCl)	15	65	20		105	35	55	10		75	50	45	5		55
3. Glucose + water	45	45	10		65	50	20	20†	10‡	90	70	5	10‡	15‡	70
4. Glucose + phosphate + KCl	40	40	15	5	85	40	25	20†	15‡	110	45	15	20‡	20‡	115
5. Glycerol + water	15	75	10		95	20	75	5		85	20	75	5		85
6. Glycerol + phosphate + KCl	15	55	25	5	120	20	35	35†	10†	135	25	20	35†	20†	150
7. Malate + water	15	65	15	5	110	15	55	20†	10†	125	20	15	35†	30†	175
8. Malate + phosphate + KCl	15	55	25	5	120	15	25	45†	15†	160	15	15	35†	35†	190

* IM = index of metachromasy.

† Neisser-granules were stained violet and the cytoplasm remained blue.

‡ Not only Neisser-granules but also the cytoplasm stained violet.

At time 0 the distribution of granules for all systems was 15 per cent 0, 65 per cent 1, 20 per cent 2 and 0 per cent 3; the index of metachromasy was 105.

In the presence of glucose, many of the cells lost Neisser-staining granules, but some of the cells retained large granules stained violet and had violet stained cytoplasm.

numbers of visible metachromatic granules by several fold.

A study by Sall *et al.* (1956) has shown that metaphosphate deposits can be caused to accumulate in surviving cells of *Corynebacterium diphtheriae* by suspending the cells in media containing orthophosphate, potassium ion, and a suitable oxidizable substrate, and can be caused to disappear under conditions of metabolic demand. An experiment of similar pattern was set up with *M. thamnopheos*. Cells grown on 3 per cent glycerol agar for 16 hr were collected and washed by centrifugation 3 times with 0.2 M tris (tris(hydroxymethyl)aminomethane)

buffer of pH 7.0 and suspended homogeneously in tris buffer. Suspensions were prepared as in tables 3 and 4.

The granules staining for metaphosphate and those showing tellurite-reducing activity were enumerated at the various time intervals, and in addition the approximate size distribution of metachromatic granules was recorded and the "index of metachromasy" calculated for each experimental condition (tables 3 and 4). A scale, established by the use of a micrometer, assigned values to metachromatic granules of the following diameters: 4 = 1 μ diameter; 3 = ¾ μ diameter; 2 = ½ μ diameter; 1 ≦ ¼ μ diameter.

The index of metachromasy is the sum of the products obtained by multiplying the values corresponding to the estimated diameters of the metachromatic granules by the estimated percentages of cells containing granules of these sizes (table 4).

Tables 3 and 4 show that metaphosphate accumulated in *M. thamnopheos* in the presence of orthophosphate and potassium with either malate or glycerol as oxidizable substrate; metaphosphate disappeared in dilute buffer, or buffer with phosphate and potassium but without substrate. This is in general conformity with the pattern previously observed for *C. diphtheriae* (Sall *et al.*, 1956). In the presence of glucose the results are less clearly interpretable. From table 4 it may be seen that many of the cells suspended in buffered glucose lost their metachromatic granules in 24 and 48 hr. A minority of cells still showed large metachromatic granules; however, these cells stained atypically and may well have been abnormal cells incapable of utilizing the stored metaphosphate for synthesis of RNA (Sall *et al.*, 1956). The ratio of reducing sites to rod length remained as before essentially constant under the various metabolic conditions (table 3).

A similar experiment was performed with BCG. A 6-day growth on American Trudeau Society medium was ground with mortar and pestle to make a homogeneous suspension in 0.2 M tris buffer of pH 7.0. The cells were washed 3 times by centrifugation in tris buffer and resuspended in tris buffer with the several sub-

TABLE 5

Neisser-staining and reducing granules in washed cells of BCG surviving under various metabolic conditions

No. and Contents	Mean Number of Granules per Rod					
	Neisser granules				Reducing granules (K-tellurite)	
	1 day	3 days	5 days	8 days	1 day	8 days
1. Control (water)	0.9[b]	0.5[a]	0.4[a]	0.3[a]	1.9	0.9*
2. Control (phosphate + KCl)	0.9[b]	0.5[a]	0.4[a]	0.3[a]	1.8	1.0*
3. Glucose + water	1.0[b]	1.1[b]	1.2[b]	1.3[d]	1.7	1.4*
4. Glucose + phosphate + KCl	0.9[b]	1.0[b]	1.2[b]	1.7[d]	1.7	1.1*
5. Glycerol + water	0.8[b]	0.5[b]	1.7[c]	2.6[c]	1.6	1.4*
6. Glycerol + phosphate + KCl	0.9[b]	0.4[b]	1.6[c]	2.8[c]	1.8	1.3*
7. Malate + water	1.0[b]	0.9[b]	1.2[c]	†	2.0	†
8. Malate + phosphate + KCl	0.8[b]	1.0[b]	1.2[c]	2.8[c]	2.2	0.9*

After making the suspension of cells by grinding at room temperature in tris buffer the numbers of granules reducing tetrazolium were markedly reduced; they were considerably recovered after incubation for 1 day.

For all systems the mean number of Neisser-staining granules per rod at time 0 was 0.8.

[a] Most of the granules were stained dark blue.

[b] Many of the granules were stained dark blue.

[c] Some of the granules were stained dark blue.

[d] Many of the rods which contained violet granules had also violet protoplasm.

* As these granules were stained rather faintly, the number is not reliable.

† Contamination.

strates. Preparations at 0, 1, 3, 5 and 8 days were stained by Neisser and potassium tellurite.

The pattern of response with BCG was similar to that with *M. thamnopheos*, except that all responses occurred at a slower tempo (table 5). The index of metachromasy increased notably in the presence of orthophosphate and potassium with either malate or glycerol as substrate; the index of metachromasy fell in dilute buffer or buffer with phosphate and potassium but without substrate. The index rose slightly in the presence of glucose, but again many of the rods which contained metachromatic granules had also violet-staining protoplasm, suggesting abnormality. The ratio of granules reducing tellurium to rod length again showed little change. These sequences of change, which in *M. thamnopheos* had occurred in 2 days, in BCG required 8 days, in general conformity to the relative growth rates of the two strains.

ELECTRON MICROSCOPY

The building-up and depletion of metachromatic granules. The numbers of granules staining metachromatically in cells of *M. thamnopheos* freshly transferred to glycerol extract agar were shown in table 1 to pass through a minimum near 7 hr after transfer and to increase to 24 hr; reducing sites, on the contrary, remained nearly constant at 1 reducing site per μ of rod length. Cells of *M. thamnopheos* 6 hr after subculture on collodion overlying 5 per cent glycerol agar[3] are shown in figure 1. Three cells appear in which no intensely electron-scattering granules may be seen, and two in which only very small electron-scattering granules are discernible. The granule indicated by an arrow seems to be in a circumscribed area of greater density than the rest of the cytoplasm.

In figure 2 are shown cells of a 26-hr culture of *M. thamnopheos*. The collodion mount had broken with resulting compression of the bacterial cells. The ovoid contours of the electron-scattering particles at the edge of the collodion attest to their density and resistance to deformation.

In figure 3 are cells of a 72-hr culture of *M. thamnopheos* on collodion overlying 5 per cent on glycerol agar containing triphenyl-tetrazolium chloride in slightly inhibitory concentration. The electron-scattering particles are reduced in size and in several cases appear within areas of density greater than that of the surrounding cytoplasm (arrows).

In tables 3 and 4 metaphosphate granules are shown to build up in cells of *M. thamnopheos* incubated in buffer containing orthophosphate and potassium and either malate or glycerol as oxidizable substrate. Cells from this same experiment after incubation for 48 hr were washed 3 times by centrifugation in distilled water and mounted on a formvar film on copper mesh. Electron micrographs taken at an accelerating voltage of 80 kv are shown in figures 4–7. In figure 4, with malate, almost all cells contained evenly contoured electron-scattering particles up to 450 mμ in diameter. Very small electron-scattering granules were few. In figure 5, with glycerol, evenly contoured granules up to 400 mμ in diameter were formed; many small electron-scattering granules may be seen throughout the cytoplasm.

In presence of glucose (tables 3 and 4), some cells lost all microscopically visible metachromatic granules, and some retained large metachromatic granules but stained atypically. The corresponding picture is shown in figure 6. Cells with few electron-scattering granules are seen, and other swollen cells containing large, electron-scattering granules, some of which are irregularly shaped. When cells were incubated without oxidizable substrate (tables 3 and 4), they were progressively depleted of their metachromatic deposits. The corresponding picture is shown in figure 7. Electron-scattering particles are few and relatively small. Particular attention is directed to those very small granules indicated by arrows which seem to lie in circumscribed areas of density greater than the surrounding cytoplasm; these circumscribed areas are approximately 0.25 μ in diameter.

In table 5, the pattern of response to metabolic conditions of cells of BCG is shown to be similar to that of *M. thamnopheos*, but at a slower tempo. Cells of BCG after incubation for 8 days in the solutions indicated in table 5 were washed 3 times by centrifugation in distilled water and mounted on formvar films on copper mesh. Electron micrographs were taken at 80-kv accelerating voltage, and are shown in figures 8–11.

[3] The cells in the experiments recorded in tables 1 and 2 were not grown under quite the same cultural conditions as those micrographed in figures 1–3.

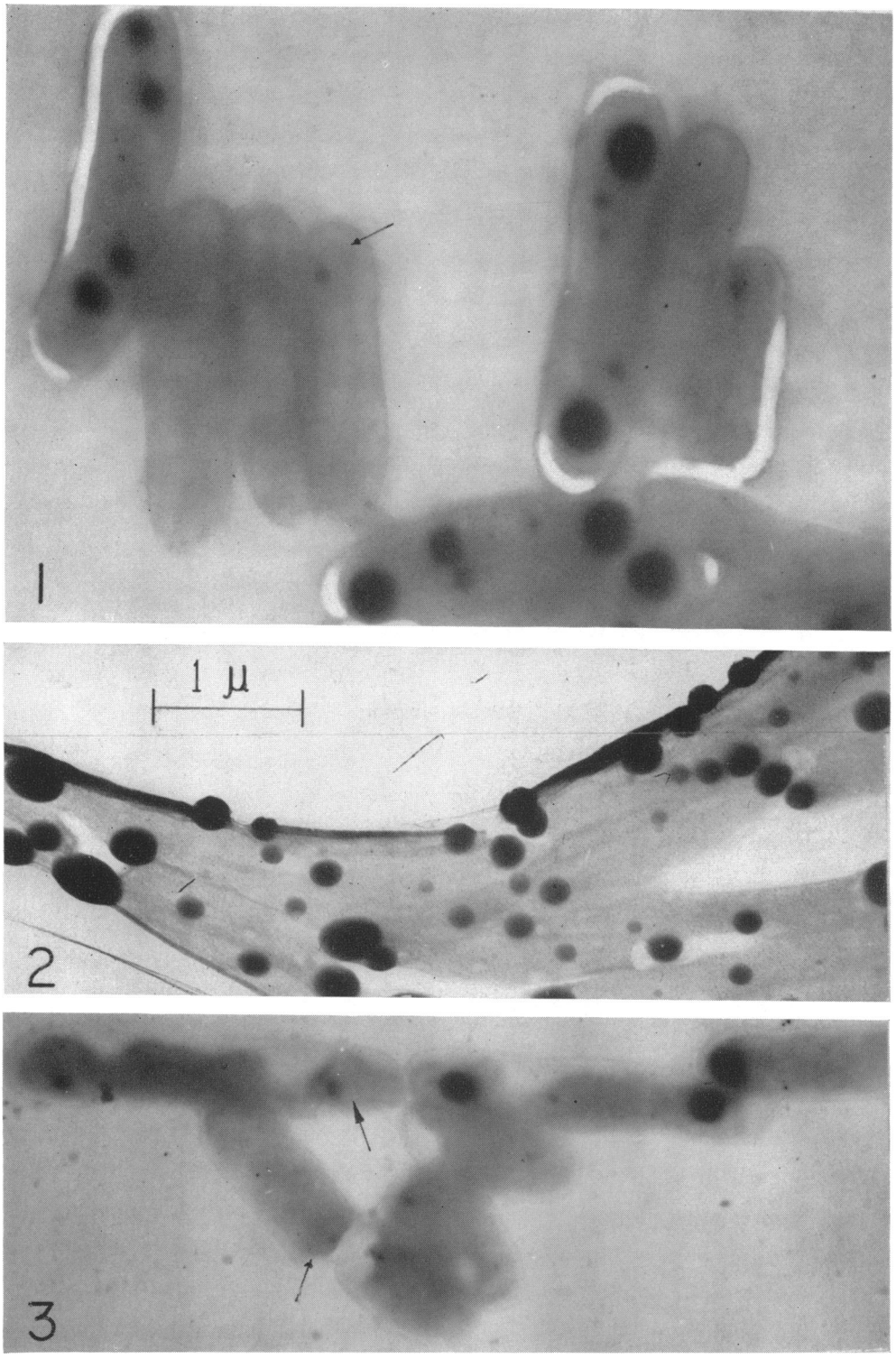


Figure 1. Cells of *Mycobacterium thamnopheos* 6 hr after subculture on surface of collodion film overlying 5 per cent glycerol agar. Unfixed except for desiccation. × 22,000.

Figure 2. Mycobacterium thamnopheos 26 hr after subculture on collodion film overlying 5 per cent glycerol agar. Unfixed except for desiccation. × 22,000.

Figure 3. Mycobacterium thamnopheos 72 hr after subculture on collodion overlying 5 per cent glycerol agar containing 0.4 per cent triphenyltetrazolium chloride. Unfixed except for desiccation. × 22,000.

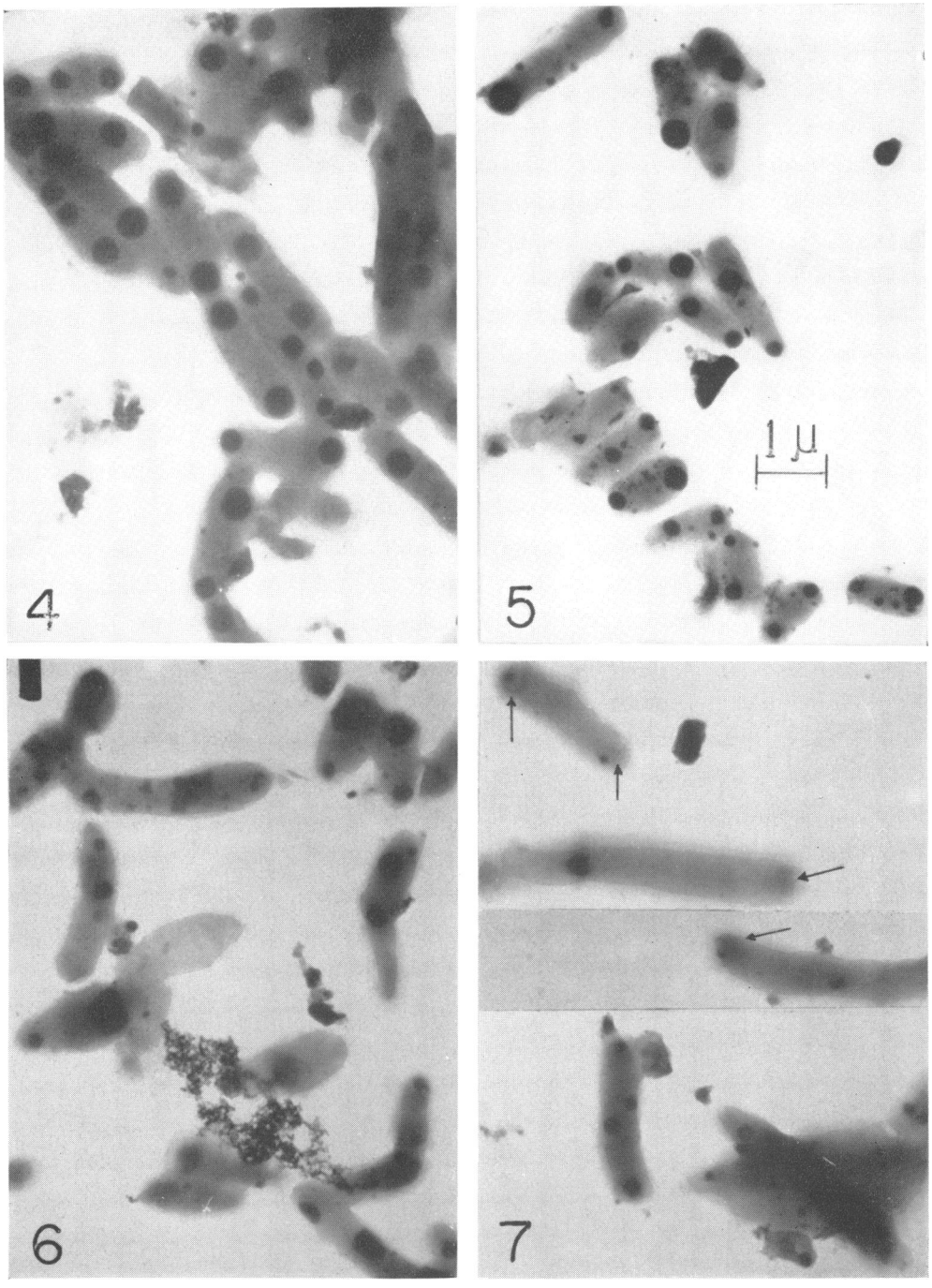
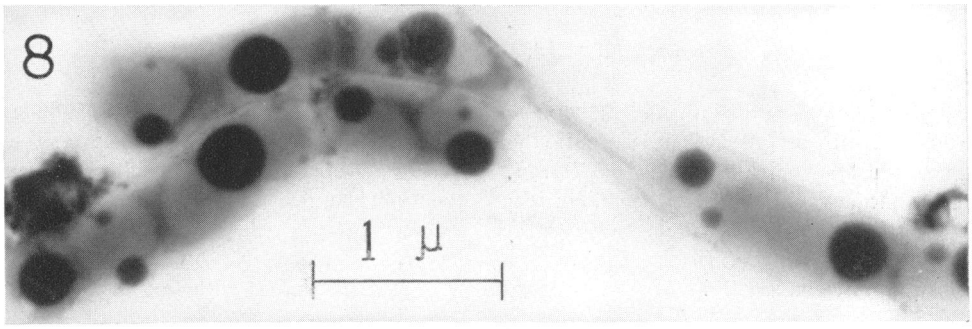


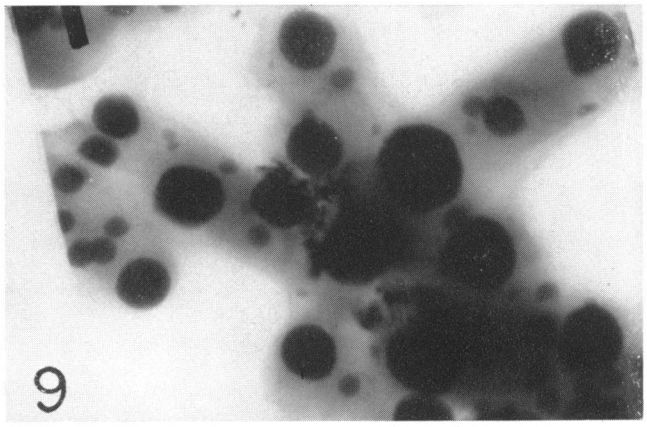
Figure 4. Mycobacterium thamnopheos after incubation for 48 hr in buffer containing orthophosphate, potassium and malate. × 10,000.

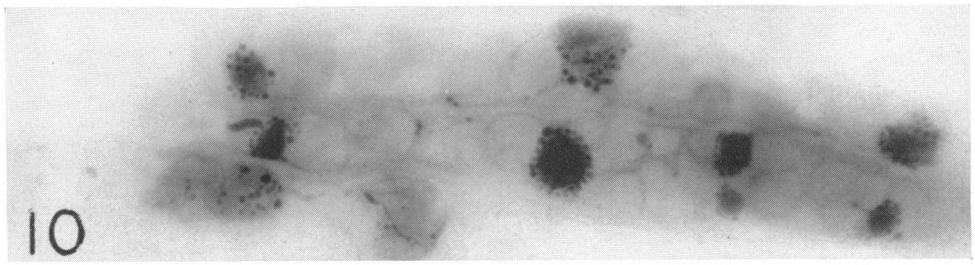
Figure 5. Mycobacterium thamnopheos after incubation for 48 hr in buffer containing orthophosphate, potassium and glycerol. × 10,000.

Figure 6. Mycobacterium thamnopheos after incubation for 48 hr in buffer containing orthophosphate, potassium and glucose. × 10,000.

Figure 7. Mycobacterium thamnopheos after incubation for 48 hr in buffer diluted with distilled water. × 10,000.







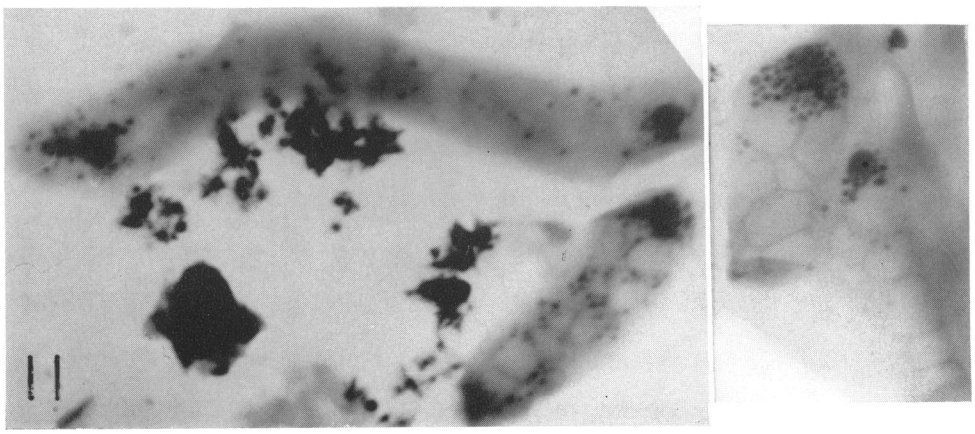


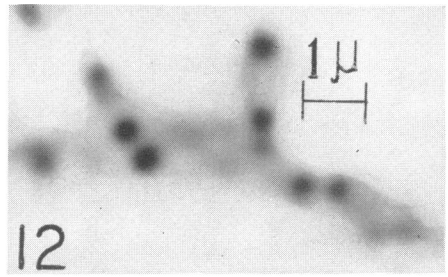
Figure 8. BCG after incubation for 8 days in buffer containing orthophosphate, potassium and malate. × 25,000.

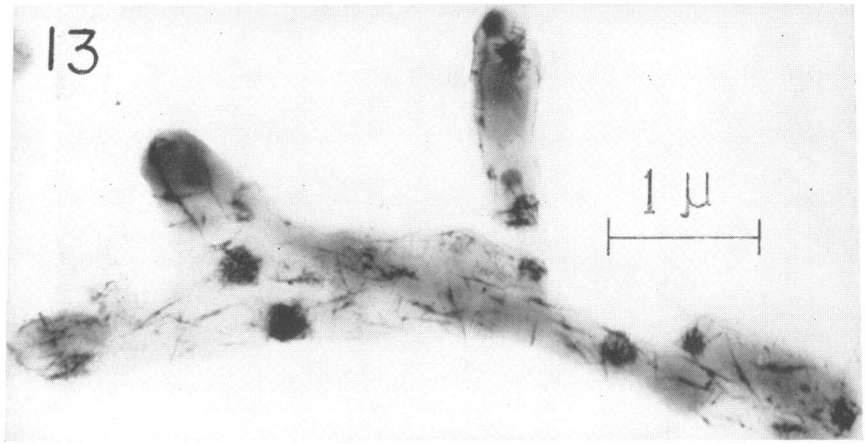
Figure 9. BCG after incubation for 8 days in buffer containing orthophosphate, potassium and glycerol. × 25,000.

Figure 10. BCG after incubation for 8 days in buffer containing orthophosphate, potassium and glucose. × 25,000.

Figure 11. BCG after incubation for 8 days in buffer diluted with distilled water. × 25,000.

In the presence of potassium and orthophosphate with malate or glycerol as substrate the cells contained evenly contoured electron-scattering particles up to 450 mμ in diameter; small electron-scattering particles were also present (figures 8 and 9). In the presence of glucose, the electron-scattering particles were smaller, corresponding to a lower index of metachromasy (table 5). Moreover, the intensely electron-scattering material was in minute spherules apparently within circumscribed particles (diameter ca. 0.25 mμ) of density higher than the surrounding cytoplasm, or in particles ringed with tiny globules of elec-





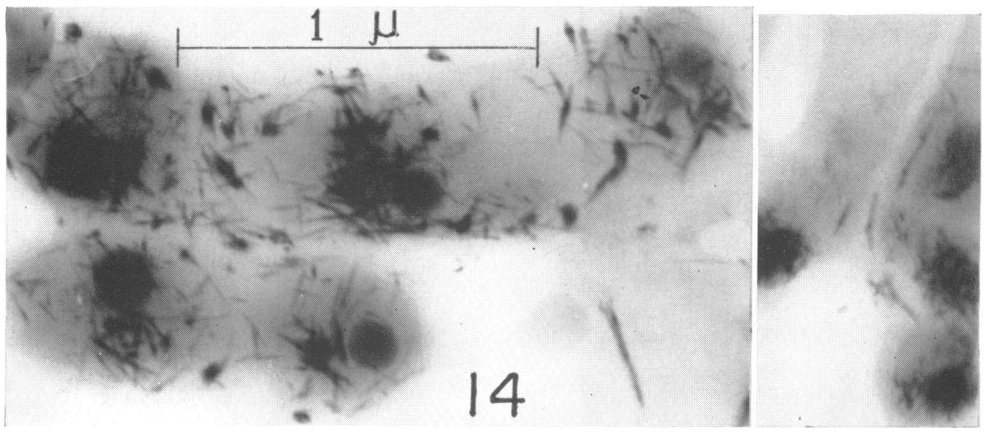


Figure 12. Mycobacterium thamnopheos unstained save for incubation for 20 hr in 3 per cent glycerol broth containing 0.05 per cent potassium tellurite. Light picture. × 8,300.

Figure 13. Mycobacterium thamnopheos, same cell group as in figure 12. Electron picture. × 20,000.

Figure 14. Mycobacterium thamnopheos prepared as for figures 12 and 13. Electron picture. × 48,000.

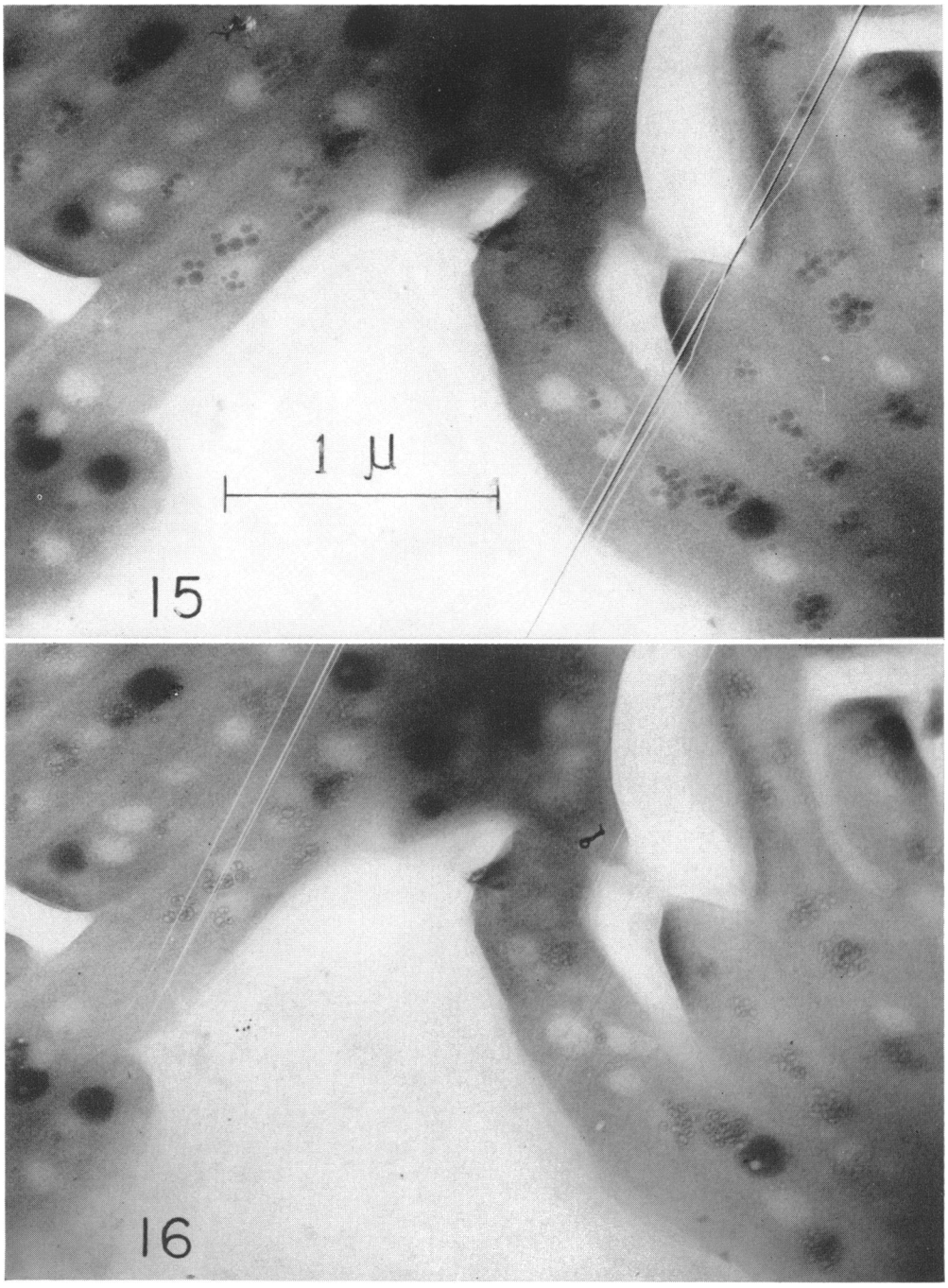
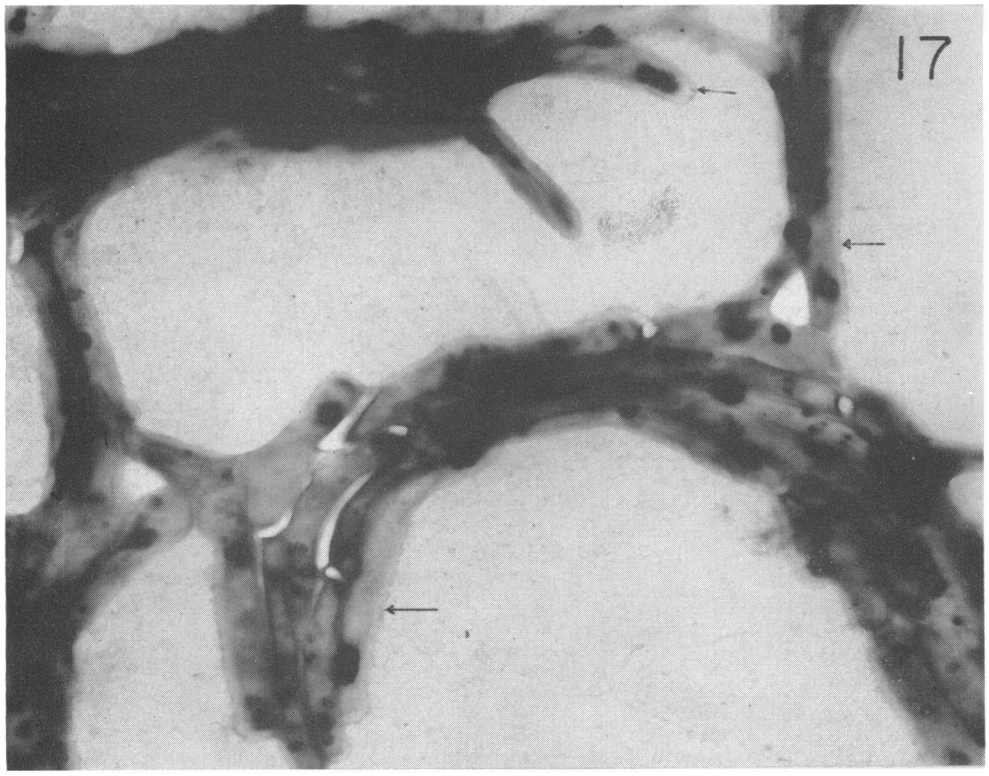


Figure 15. BCG. Incubated 5 days at 37 C on collodion film overlying egg medium. Unfixed except for desiccation. × 38,000.

Figure 16. BCG. Same field as in figure 15. × 38,000.



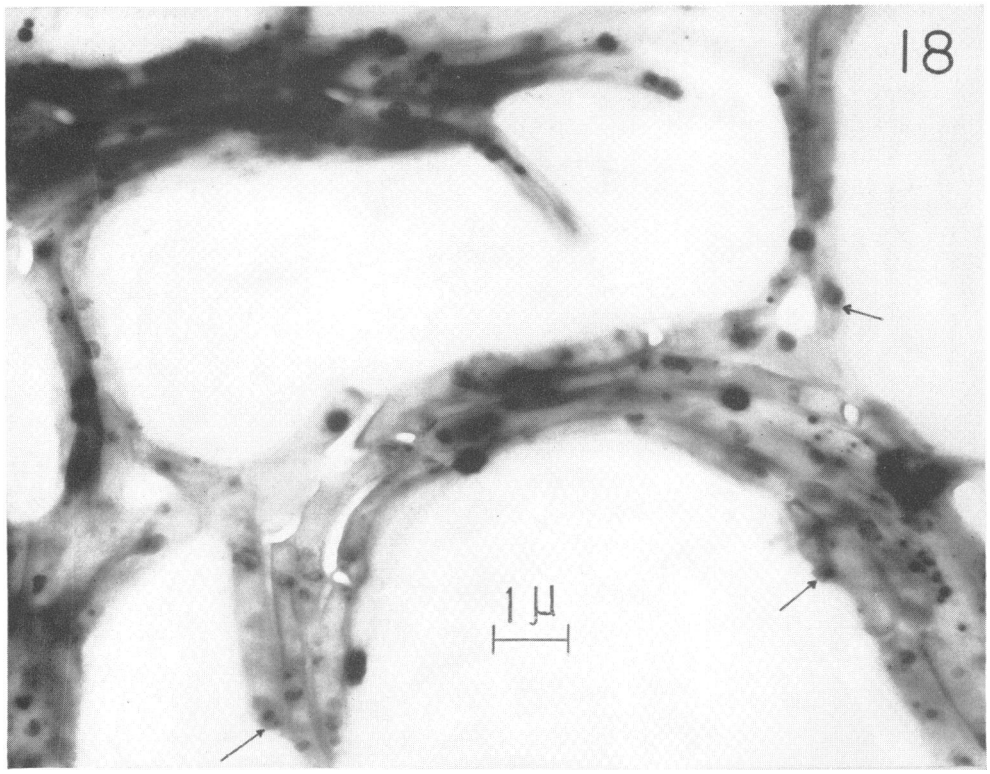


Figure 17. H37Rv. Incubated 4 days at 37 C on collodion overlying 5 per cent glycerol infusion agar. Microcolony fixed for 2 min in vapor of 2 per cent solution of OsO_4 and preserved in desiccator until studied in electron microscope. RCA plate #6377. × 10,000.

Figure 18. H37Rv. Same field as in figure 17, but printed more lightly.

tron-scattering material (figure 10). In the preparation from dilute buffer without substrate the electron-scattering material was largely in minute spherules and was reduced in amount (figure 11).

The reduction of potassium tellurite. Further light on intracellular reduction was obtained from light- and electron-microscopic pictures of cells of *M. thamnopheos* incubated with tellurite. *M. thamnopheos* grown on 0.5 per cent glycerol agar for 24 hr was suspended in 3 per cent glycerol broth containing 0.05 per cent potassium tellurite, and incubated at 37 C. After incubation for 20 hr the suspension was washed 3 times by centrifugation in distilled water and mounted on formvar on copper mesh screens. After taking electron micrographs with weak beam intensity, the mesh was placed on a glass slide and immersed in cedar oil for observation with the light microscope. The cells were unstained save for the tellurium deposited by reduction of the potassium tellurite.

In figure 12 the light-microscopic and in figure 13 the electron-microscopic picture of the same cell group are shown. In the light picture the tellurium deposits appear to be localized in discrete reduction sites such as are seen when tetrazolium salts are reduced to their colored formazans. There is also some opacity outside the circumscribed reduction sites, but no crystals of metallic tellurium are resolved. In the electron picture the same circumscribed reduction sites are seen to be centers of deposit of tellurium, much of which is in fine needle-shaped crystals. Moreover, crystals of tellurium appear throughout the cytoplasm, even in areas where no circumscribed reduction sites are discernable. Figure 14 shows another cell group in which the felt-work of crystals about the reducing sites is more clearly evident. (Morton and Anderson, 1941).

Fine structure of strains BCG and H37Rv. Before attempting interpretation of the above data in terms of organization and function within the cells of mycobacteria, electron pictures of BCG and H37Rv, processed in the attempt to throw light on fine structure, may be considered.

Figure 15 shows a group of cells of BCG in an electron microscope picture taken with low intensity of electron beam. Figure 16 shows the same field after exposure to an intense electron beam. The large electron-scattering particles have been volatilized to some extent and the small ones to a greater extent by the intense electron bombardment. The arrangements of the small electron-scattering particles in rosette-like groups (Takeya *et al.*, 1954) is striking, and may suggest some structural differentiation of and within reducing sites whose nature has thus far not been revealed.

Figure 17 shows part of a microcolony of virulent human tubercle bacilli strain H37Rv after fixation for 2 min in the vapor of 2 per cent OsO_4 solution, and figure 18 the same field lightly printed. Many of the electron-scattering areas in figure 17 are resolved in the lighter print in part into electron-scattering granules within circumscribed areas of greater density than the surrounding cytoplasm (arrows). Attention is directed also to the osmophilic surface film (arrows) evident in cells of figure 17; this we believe may be the ester described by Middlebrook *et al.* (1947).

DISCUSSION

We may now consider the questions that were raised in the introduction. With respect to the relationship of reducing sites to electron-scattering metachromatic granules the following observations are relevant:

Circumscribed areas of intense oxidation-reduction, revealed by reduction of tetrazolium salts or tellurite to microscopically visible deposits, exceeded in absolute numbers the microscopically visible granules revealed by metachromatic staining.

The proportion of microscopically visible reducing sites remained approximately constant at about 1 reducing site per μ of rod length, whereas the proportion of microscopically visible metachromatic granules varied with conditions of growth and age of culture.

In metabolic experiments with surviving washed cells in buffer the presence of a suitable oxidizable substrate, orthophosphate and potassium ion led to the considerable building up of metachromatic granules; surviving cells in dilute buffer only were depleted with respect to metachromatic deposits. The reducing sites did not undergo parallel change under these circumstances. In cells depleted of metachromatic material the residual metaphosphate often occurred as minute granules within or around

circumscribed areas of greater density than the surrounding cytoplasm.

In the presence of potassium tellurite the reducing sites appeared in the light microscope as black circumscribed areas; no tellurium crystals were resolved. In the electron microscope pictures these reducing sites were seen to be darkened by a felt-work of fine crystals; tellurium crystals were also found throughout the cytoplasm. These observations are compatible with the following interpretation:

Energy-yielding metabolic reactions result in electron transfer to suitable hydrogen acceptors (e. g., tellurite) throughout the cytoplasm; in the circumscribed sites of intense reduction, which we interpret as bacterial mitochondria, these electron transfers may result in microscopically recognizable accumulations of reduction products (i. e., tellurium, formazan). Some of the energy of oxidation-reduction reactions may be conserved in the form of phosphoric acid anhydrides, microscopically recognizable as metachromatic metaphosphate granules within or about the reducing sites. These metaphosphate deposits are added to or drawn upon as varying metabolic conditions require. Kornberg *et al.* (1956) have recently purified an enzyme from *Escherichia coli* which synthesizes long-chain, acid-insoluble metaphosphates. These metaphosphates are metachromatic. The reaction requires Mg^{++} and is completely inhibited by fluoride; it is not inhibited by arsenate or 2,4-dinitrophenol.

Are bacterial mitochondria structural homologues of the mitochondria of higher plants and animals? The mitochondria of higher forms are enclosed in a double membrane, the inner layer of which is infolded to form *cristae mitochondriales*. Although there is evidence that the mitochondria of mycobacteria exhibit osmotic phenomena strongly suggestive of a limiting membrane (Mudd, 1956), no evidence has come to light suggesting a double membrane or *cristae mitochondriales* in bacterial mitochondria. In thin sections of bacteria in large, dense granules which may be interpreted tentatively as mitochondria rendered strongly electron scattering by metaphosphate (Niklowitz and Drews, 1955, Bassermann, 1956), little internal structure has been discerned. In ultrathin sections of bacteria which presumably have accumulated little or no metaphosphate, inconspicuous circumscribed areas of increased density are sometimes discernible in the cytoplasm. These we believe to be the same organelles as have repeatedly been shown in electron pictures of *E. coli* lysed by coliphage (Mudd, 1951*a*; Hartman *et al.*, 1953) and interpreted as mitochondria. Recognizable internal structure in these granules either intact or in section has not been described. The high degree of coordination required for such biochemical functions as succinoxidase action or oxidative phosphorylation certainly presupposes structural organization at least at the molecular level, but we must conclude that up to the present such organization has not been satisfactorily described in morphological terms. Tsuda (1956) has demonstrated cytoplasmic granules with double membranes and cristae mitochondriales in the fungus *Penicillium chrysogenum*.

In a recent review Bradfield maintains that the major parts of the oxidation-reduction systems of bacteria are located in very small granules 100–200 A in diameter, widely distributed throughout the entire cytoplasm: "Only if it could be shown that the granules are regularly combined together in substantial and coherent clumps, either enclosed in a membrane or arranged in some fairly constant and characteristic pattern, would the word mitochondrion be justified; to date there appears to be no evidence of this kind whatever" (Bradfield, 1956).

We certainly do not dispute the evidence reviewed by Mudd (1954, 1956) that oxidative-reductive functions may be exhibited by submicroscopic particles in cell-free preparations derived from various bacteria. However, oxidative-reductive activities and even oxidative phosphorylation are also now being ascribed to submicroscopic particulates derived from mammalian mitochondria (e. g., Cooper and Lehninger, 1956).

We believe that the electron micrographs in this study suggest that reduction of tellurite and probably also the deposition of metaphosphate may occur throughout the cytoplasm in part away from the mitochondrial granules. However, the fact remains that reduction of sufficient intensity to produce microscopically visible deposits of formazans, the Nadi reagent, Janus green B, or tellurium is exhibited at circumscribed local sites in the intact bacterium. These sites may persist as definite granules during and after lysis by phage and exhibit

their cytochemical reactions both within and outside of the bacterial cells; they are found in numbers in electron micrographs of the debris of phage-lysed cells (Hartman, *et al.*, 1953; Penso, 1953). These same sites are also stained by the lipid stain Sudan black B.

We were also well aware that overstaining with tetrazolium salts might result in some deposit of the less insoluble formazans, notably that of triphenyltetrazolium, outside the chief sites of reduction; this fact was published by Mudd *et al.* (1951*b*) some time before Weibull (1953) reported it. This difficulty is much less troublesome with neo- and blue tetrazolium or with a tetrazolium used by Drews (1955), whose formazan is distinguished by its lipoid-insolubility; this difficulty can be guarded against, and certainly does not vitiate the extensive evidence furnished by study of stained preparations.

We have reviewed recent evidence by Millman and Youmans, Brodie, Gray and Hartman, showing that the Krebs cycle and oxidative phosphorylation may be exhibited by cell-free particulates of an order of magnitude larger than the 100–200 A particles (Mudd, 1956). To these citations may be added that of Georgi *et al.* (1955). It is these oxidative-reductive sites of microscopic dimensions that we believe to be the functional equivalents of the mitochondria of higher forms. We are not aware of any evidence, however, to indicate that bacterial reducing sites are structural homologues of the mitochondria of higher forms.

Should organelles possessing many of the biochemical functions of the mitochondria of higher forms, but without demonstrated morphological homology with such mitochondria, be termed mitochondrial equivalents, chondrioids, bacterial mitochondria or by some other term? We do believe that the presently recognized attributes of the larger centers of active oxidation-reduction in bacteria are at least compatible with a revised definition of *bacterial mitochondria* (Mudd, 1956).

SUMMARY

Circumscribed sites of oxidation-reduction in mycobacteria were rendered visible in the light microscope by suitable indicator dyes. These visible sites occurred in the ratio of approximately 1 reducing site per μ of rod length, irrespective of the metabolic conditions. Metaphosphate deposits formed in association with many but not all of the reduction sites, and possibly elsewhere in the cytoplasm. These metaphosphate deposits were dependent upon metabolism; they could be caused to increase or decrease by appropriate experimental conditions.

The reduction of potassium tellurite to metallic tellurium was discernible in light microscopic preparations as black deposits corresponding to the reducing sites; in electron microscopic preparations the tellurium crystals were seen to be in greater concentration about the reducing sites, but also to occur throughout the cytoplasm.

We interpret these results and others cited as indicating that energy-yielding reactions occur throughout the cytoplasm but in particular in circumscribed organelles functionally equivalent to the mitochondria of higher forms.

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