TABLE II. Composition of Various Fresh T-3 Preparations.

T-3 prep. No.	Age, days	I^{131} distribution, % T-3	I^-	Other constituents
1	0	98	2	None
2	1	100	0	Less than 1%
3	3	100	0	*Idem*
4	4	88	4	8%
5	4	98	2	Less than 1%

which had minimal impurities on receipt. Although kept under identical conditions quantitative aspects of development of impurities seemed to be distinctly different. Time intervals employed were within or only slightly beyond the accepted usable life of such radioactive preparations.

Discussion. It was observed that there were marked differences in radiopurity of T-3 preparations obtained from different sources, that markedly impure preparations were obtained occasionally from sources having a record of high purity, and that some pure preparations developed significant proportions of impurities over a short time interval. Only the first of these problems may be directly traceable to source of supply; time and temperature of shipment, particularly where unusual delays are involved, may be responsible for the second and third observations. Nevertheless, these phenomena certainly explain the findings shown in Table I and may account for spurious results occasionally experienced with this type of test. Chromatographic monitoring of each lot of T-3 on receipt may be a sufficiently simple procedure for routine practice. We have found use of a standard lyophilized or pooled serum of additional value as a monitoring device for each run. This is possible with any modification of the T-3 test using only patient's serum or plasma and an adsorbent other than patient's own erythrocytes.

TABLE III. Effect of Preparation Age on Proportions of Various I^{131} Constituents.

T-3 prep. No.	Age, days	I^{131} distribution, % T-3	I^-	Other constituents
1	0	98	2	None
	6	96	4	Less than 1%
	18	91	2	7%
3	3	100	0	Less than 1%
	20	85	3	12%
2	1	100	0	Less than 1%
	6	90	1	9%

Summary. Using a standard thyrodiagnostic test based on *in vitro* partitioning of triiodothyronine-I^{131} between plasma proteins and erythrocytes of whole blood, results were found to differ depending on the commercial source of labeled triiodothyronine. Chromatographic study showed these preparations to be heterogeneous. Impurities were occasionally present in fresh materials and developed in significant proportions during the accepted usable life of the preparation.

1. Hamolsky, M. W., Golodetz, A., Freedberg, A. S., *J. Clin. Endocrin. and Metab.*, 1959, v19, 103.

2. Sterling, K., Tabachnick, M., *ibid.*, 1961, April, in press.

3. Meade, R. C., *ibid.*, 1960, v20, 480.

4. Adams, R., Specht, N., Woodward, I., *ibid.*, 1960, v20, 1366.

5. Mandl, R. H., Block, R. J., *Arch. Biochem. Biophys.*, 1959, v81, 25.

Received February 8, 1961. P.S.E.B.M., 1961, v106.

Fluorescent Antibody Stainability and Other Consequences of the Disruption of Mycobacteria. (26443)

CHARLES C. SHEPARD AND DAVID KIRSH

Communicable Disease Center, Public Health Service, U. S. Department of HEW, Atlanta, Ga.

During the last few years a number of attempts in this laboratory to stain mycobacteria with fluorescent antibody have been unsuccessful, and the lack of publication of successful efforts by others suggests a similar experience elsewhere.

The mycobacteria are characterized by a high lipid content and their lipophilic be-

havior(1) indicates that lipid is located on the surface. Yet water soluble preparations, usually in the form of culture filtrates, are able to react with immune serum in complement fixation and immunodiffusion reactions. It seemed possible that it was only the outer surface of the cell wall that consisted of lipid, and that the inner surface might have a structure resembling other bacteria and would contain protein or carbohydrate antigens.

To test this proposition we have disrupted mycobacteria by vibration with glass beads in the manner used successfully to prepare cell walls of many nonacid-fast bacteria (see review of Salton, 2). It was found that the mycobacteria do stain very well by fluorescent antibody after such treatment. Disruption of the mycobacteria was accompanied by a release of soluble protoplasmic constituents. This soluble fraction contains the largest amount of antigen, as judged by its inhibition of the fluorescent antibody reaction.

Vibration with glass beads had also been used by Ribi *et al.*(3) to prepare suspensions of cell walls, as judged by electron microscopy. They observed a pronounced aggregation of cell walls and unbroken organisms with consequent low yield of suspendable cell walls, and later turned to the pressure cell technic(4), where the aggregation was less severe. Clumping has been observed in the present study, but it did not hinder the observation of the markedly increased staining by fluorescent antibody. The efficiency of mycobacterial disruption was controlled by measurements of the release of soluble protoplasmic constituents.

Materials and methods. Mycobacterial cultures were maintained on Loewenstein-Jensen medium. Suspensions for study were prepared from growth in Tween-albumin medium (TB broth base of Difco), washed in water 1 to 3 times, and resuspended in water to a concentration that when diluted 10 times gave an optical density of 0.40 in the Coleman spectrophotometer with a 19 mm cuvette at 600 mμ ($D_{600} = 4.0$). The vibration was performed in the Mickle apparatus with an amplitude of 7 mm, with 4 ml of bacterial suspension and 4 g of glass beads (No. 12, Cataphote Corp., Toledo, Ohio). The sediments and supernates were collected after centrifugation at 1000 g in an angle head for 30 minutes. Bacteria were counted in microdrops by the "rapid method"(5,6), the final dilution (10^{-3}) being made in formol-milk containing 0.1% gelatin. Dry weights were performed after drying for 24 hours at 105°. The accuracy of the dry weight determinations was restricted by the availability of material but it was attempted to weigh at least 4 mg of solids in duplicate; the duplicates agreed within ± 10%. Protein was measured by the Lowry method(7). Carbohydrates were estimated by the anthrone procedure(8). An estimate (called D_{265}^{PCA}) of the amount of bacterial protoplasm was formed by measuring optical density at 265 mμ of extracts with hot perchloric acid. Four-tenths ml of bacterial preparation was mixed with 3.2 ml H_2O + 0.4 ml 60% perchloric acid and heated to 90° for 15 minutes. Centrifugation at 1000 g for 30 minutes was used to clarify the extract. The presence of Tween in the extracted material gives a turbid extract, which can be clarified by ether extraction overnight. Optical density was read in the Beckman spectrophotometer in 1 cm cells at 220, 230, 240, 260, 265, 280, 300, and 320 mμ, and the curve constructed to ensure its typical form. The minimum was located at 230 or 240 mμ, and the ratio between optical density at the minimum and at the maximum (265 mμ) was less than 0.7 in all instances except in the case of sediments of well disrupted organisms, which had very low optical densities. This procedure does not differentiate between RNA, DNA, and smaller compounds, but is a convenient and sensitive indicator of bacterial protoplasm, as distinguished from cell wall material.

Antimycobacterial sera were prepared usually by intravenous injection of rabbits with about 0.4 mg (dry weight) of bacilli at weekly intervals for 4 weeks, with bleedings before injection and 2 weeks after 4th injection. Complement fixation tests showed antibody responses usually to titers of 1:256 to 1:2048 from levels usually less than 1:10 before immunization. Antibody response was also revealed by fluorescent antibody results.

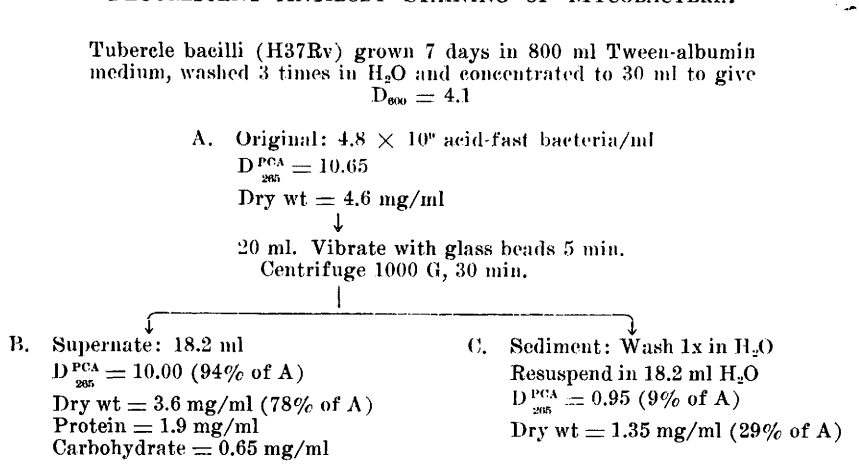

FIG. 1. Procedure for separation of intact bacilli (A) into soluble fraction (B), and disrupted organisms (C).

Most of the fluorescent antibody stains were performed by the indirect procedure with the anti-rabbit conjugates prepared from chicken or goat immune sera. Direct stains were also effective. The serum and conjugates were diluted in VBA (2.5% bovine plasma albumin in veronal buffered saline; final pH 7.5) before being applied to the slide. A fresh pipette was used for each dilution. Incubation was at 37° for 30 minutes in each case.

Labelling was performed by adding fluorescein isothiocyanate (chromatographically pure isomer I) to 20 times its weight of the globulin fraction, present as a chilled and stirring 2% solution in 0.025 M Na_2CO_3 + 0.025M $NaHCO_3$ + 0.15 M NaCl. After 6-18 hours the conjugates were dialyzed thoroughly and absorbed 3 or 4 times with tissue powders (prepared usually from monkey liver). The illumination for microscopy, provided by a Reichert assembly carrying an HBO-200 Osram lamp, was filtered through a half-thickness Corning 5840 filter. A Wratten 2B filter was in the ocular. With this filter system the blue-gray autofluorescence of unstained bacilli, distinctly visible in clumps of organisms, could be easily distinguished from the yellow-green fluorescence of the bacilli after they had been stained with fluorescent antibody. The smears were usually prepared fresh for each day's work. They were air-dried, fixed with acetone for 10 minutes at room temperature, then allowed to dry at room temperature. They could be preserved at −20° or lower if, before use, they were allowed to come to room temperature in a closed box to prevent condensation. The fluorescent antibody procedures are those described earlier by others, especially by Coons and his colleagues; a recent handbook(9) describes the general technics in detail.

Complement fixation tests were done by the modified Kolmer procedure employed in this laboratory for diagnosis of viral and rickettsial diseases.

Results. 1. *Control of disruptive treatment.* That the mycobacteria were thoroughly opened up by the agitative treatment is shown in Fig. 1. Most of the protoplasm was released into the soluble fraction (B) (94% as judged by D^{PCA}_{265} determinations). This represented 70-80% of the dry weight of the intact organism. The sediment (disrupted bacilli, C) probably consisted chiefly of cell walls since its D^{PCA}_{265} value was only 9% of A, which would represent only 0.4 mg/ml. The spectrophotometer curves for the same 3 materials are shown in Fig. 2. The minimum for C at 240 was 87% of the maximum at 265, indicating that the D^{PCA}_{265} value overstates the protoplasmic content. It is not clear whether the protoplasm was present in this fraction as intact bacilli entrapped in the clumps, or as adhering protoplasm that did not leave the disrupted bacilli. As stated, dry weight values are imprecise at present because of inadequate bacterial mass.

Preparation of all the mycobacterial spe-

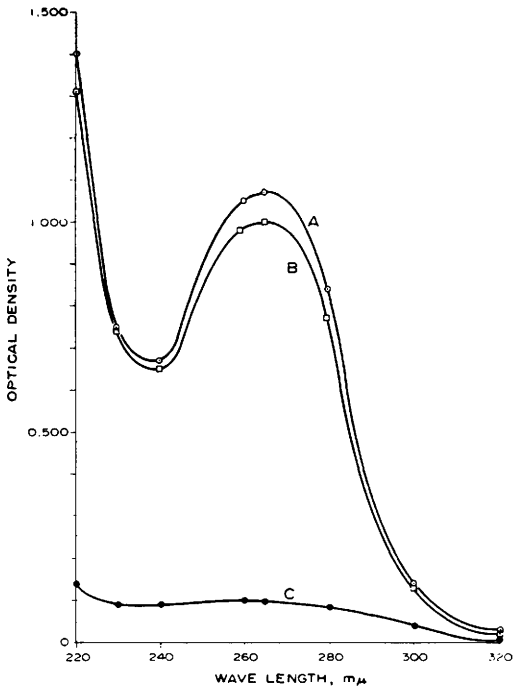

FIG. 2. Spectrophotometric curves of perchloric acid extracts of materials of Fig. 1. (A = intact bacilli, B = soluble fraction, C = disrupted bacilli.)

cies listed in Tables I and II have been monitored with bacterial counts, protein and D_{265}^{PCA} determinations. No important differences among species were noted. Incomplete break-up of the bacteria was revealed by low D_{265}^{PCA} for the supernate relative to the starting material and by lower protein values. The amplitude of vibration needs to be a full 7 mm to ensure efficient break-up in 5 minutes.

The disruption is associated with a variable amount of clumping of the remaining bacteria and cell walls, sometimes with attachment to glass surfaces. This leads to an overestimate of degree of bacterial disruption if judged by decrease in turbidity. It may also lead to poor recovery.

Acid-fast stains were not helpful in assessment of the efficiency of disruption. The smears were difficult to interpret quantitatively because of clumping and poor staining. There was a decrease in both acid-fast and nonacid-fast bacilli that roughly paralleled the degree of disruption indicated by D_{265}^{PCA} and protein determination. Koch in 1897(10) described a loss of acid-fastness of tubercle bacilli when they were ground in the dried state in an agate mortar, and he used acid-fast stains to control the efficiency of grinding of tubercle bacilli during preparation of "TO" and "TR", which were the respective supernate and sediment of a subsequent centrifugation, and would be analogous to fractions B and C in Fig. 1. It seems likely that the process employed here releases more protoplasm into a soluble state, presumably because denaturation of protoplasmic constituents is avoided, and that loss of protoplasm results in loss of stainability with the methylene blue counterstain.

2. *Increase in stainability with fluorescent antibody that accompanies bacterial disruption.* Comparisons of stainability of several species of mycobacteria before and after disruption are given in Table I. No staining of bacterial bodies was observed with the first 5 mycobacteria, although there was some staining of the background between the organisms. Staining of the background is not very useful in fluorescent antibody studies because it lacks morphology and its specificity is difficult to control. The last 3 cultures, which are the rapidly growing species, could be well stained while still intact.

Addition of Tween 80 to a concentration of 0.05% or 0.5% (w/v) to the dilutions of antimycobacterial sera did not affect staining.

3. *Relative amounts of antigen available for antibody inhibition in the 3 fractions.* The antimycobacterial sera were diluted in the bacterial preparations to see if the staining could be inhibited. This approach is particularly convenient with fluorescent antibody methods because the indicating antigen is fixed to the slide and is not confused with the inhibiting (absorbing) antigen, which is carried away in the normal washing procedure(13). Representative examples of results for the various mycobacteria are given in Table II. The soluble fraction was the most effective inhibitor in each instance. The preparations of disrupted bacilli were not much more effective inhibitors than those of intact bacilli. The results of Table I sug-

TABLE I. Fluorescent Antibody Staining of Mycobacteria before and after Disruption by Vibration with Glass Beads. Indirect stain with antimycobacterial serum prepared in rabbits against the homologous culture. Numbers refer to brightness of staining (0 = no staining, 4 = brilliant staining) with antimycobacterial serum diluted 1:10.

Mycobacterial culture and abbreviation	Fluorescent antibody staining: Unbroken organisms (A) Bacilli	Unbroken organisms (A) Background	Disrupted organisms (C) Bacilli	Disrupted organisms (C) Background
Tb (*M. tuberculosis*, strain H37Rv)	0	0	4	4
BCG (Rosenthal vaccine strain)	0	2	3	3
Ka (*M. kansasii*(11), strain Barbee)	0	2	4	4
Ba ("Battey strain"(11), strain Wolfe)	0	2	3	3
NT and QT (hamster organism, Binford(12))	0	4	4	4
Fo (*M. fortuitum*)	3	3	3	3
Ph (*M. phlei*)	4	4	4	4
Sm (*M. smegmatis*)	3	3	4	4

gest that the inhibitory power of the latter 2 fractions reflects their content of the extrabacillary antigen that produces background staining, rather than the amount of antigen located on the organisms or their cell walls and available to fluorescent antibody.

4. *Serological relationships between mycobacterial species.* A study of antigenic relationships among the mycobacterial species is being carried out with the rabbit sera and fluorescent antibody stains of the disrupted organisms. The results appear to follow the pattern described by Wilson(14). Cross-reacting antibody is usually present in high titer, and must be removed by absorption with the heterologous antigen to reveal specific homologous antibody. The serological differentiation of *M. tuberculosis* (strain H37Rv), and *M. kansasii* (strain Barbee) is illustrated in Table III.

5. *Considerations of specificity of staining of mycobacteria with fluorescent antibody.* a) In low dilutions normal rabbit sera caused distinct staining of the broken-up organisms, but after immunization there was marked increase in titer. With direct procedures there was 2+ staining of some of the mycobacterial species studied with serum diluted 1:4, but after immunization there was always increase in staining, so that there was 4+ staining at the low dilutions and 2+ staining in dilutions of 1:16 or so. With the indirect procedure pre-immunization sera frequently gave 4+ staining in dilution of 1:10, and after immunization 4+ staining was observed with the rabbit sera diluted 1:100 to 1:1000. Staining of the mycobacteria was also observed with chicken and goat anti-rabbit conjugates in low dilutions, but it was not observed with the dilutions employed in the tests, *i.e.*, 1:6 to 1:8. The lack of staining of the mycobacteria with the anti-rabbit conjugate only was controlled in each test. b) That the staining was not an

TABLE II. Relative Inhibitory Power of the 3 Types of Bacterial Preparations. Homologous antisera were diluted 1:10 in the 3 types of homologous antigen. Sera were then further diluted in VBA and applied to smears of disrupted bacilli (C), as the intermediate layer of an indirect fluorescent antibody stain. Symbols not defined in Table I are: Bl = *M. balnei* (strain X), and VBA = 2.5% bovine plasma albumin in veronal buffered saline.

Mycobacterium		VBA 10^1	VBA 10^2	VBA 10^3	Intact bacilli (A) 10^1	Intact bacilli (A) 10^2	Intact bacilli (A) 10^3	Soluble portion (B) 10^1	Soluble portion (B) 10^2	Soluble portion (B) 10^3	Disrupted bacilli (C) 10^1	Disrupted bacilli (C) 10^2	Disrupted bacilli (C) 10^3
1.	Tb	4	3	1	3	2	0	2	0	0	3	1	0
2.	Ka	4	3	1	3	2	0	0	0	0	2	1	0
3.	Ba	4	3	1	2	1	0	2	1	0	2	1	0
4.	NT	4	3	0	1	0	0	0	0	0	1	0	0
5.	QT	3	3	1	2	1	0	0	0	0	2	1	0
6.	Bl	4	3	2	3	3	0	2	0	0	4	2	0
7.	Fo	3	2	0	1	0	0	0	0	0	2	0	0

TABLE III. Serological Comparison of 2 Mycobacterial Species by Antibody Absorption. See Table I for definition of symbols.

Serum	Dil'n	VBA* Tb†	VBA* Ka†	Tb* Tb	Tb* Ka	Ka* Tb	Ka* Ka
1. Anti-Tb	10^1	4	4	1	0	4	0
	10^2	3	3	±	0	2	0
	10^3	2	1	0	0	1	0
	10^4	±	0	0	0	0	0
2. Anti-Ka	10^1	4	4	2	2	3	±
	10^2	3	3	0	2	1	0
	10^3	1	1	0	2	0	0
	10^4	0	0	0	0	0	0

* Antisera were diluted in VBA, or in the soluble fraction (B) of Tb (*M. tuberculosis*), or of Ka (*M. kansasii*) before being used to stain mycobacterial antigens by indirect method.

† Smears were made from disrupted bacilli of *M. tuberculosis* or *M. kansasii*.

artefact produced by clumping of mycobacteria was obvious on comparison of stained and unstained smears, since staining gave the clumps a brilliant yellow-green color and rendered distinctly visible unclumped single bacilli, which were numerous in some preparations (Fig. 3 and 4). c) That the staining was due to fluorescent antibody and not some nonspecific fixation of labelled non-antibody protein, for example, by surface active forces, was attested by the increases in titer that occurred on immunization of rabbits, and by the specificity of the inhibition of staining that could be achieved by dilution of the serum in soluble preparations of antigen homologous to the mycobacterial species in the smear. d) The fluorescent antibody results, however, did not avoid the cross-reactions between mycobacterial species observed with other serological technics (*e.g.* 14). It seems probable that cross-reactions between mycobacterial species, and between mycobacteria and non-acid fast species, will make difficult the application of fluorescent antibody methods to laboratory diagnosis of mycobacterial diseases.

The soluble fractions (B) from *M. tuberculosis, M. kansasii, M. balnei,* and *M. phlei* were tested against the sera prepared against the same organisms in complement fixation tests. Block titrations were performed against homologous and heterologous sera. A condensation of the results with crosses among the 4 species is shown in Table IV.

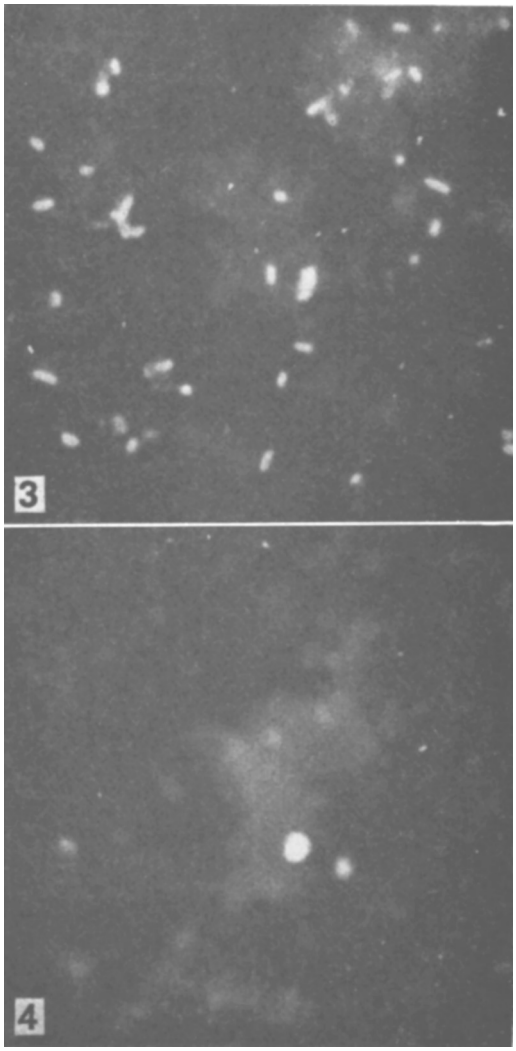

FIG. 3. Disrupted *Mycobacterium balnei* stained with fluorescent antibody. The bacilli, stained the typical yellow-green of fluorescein, were embedded in blue-gray autofluorescent amorphous material. × 885.

FIG. 4. Control unstained smear of the same disrupted *M. balnei* preparation. Only the blue-gray autofluorescent amorphous material was visible. In preparations of intact organisms, staining was confined to background, and bacilli were not discernible. × 885.

There was a helpful degree of specificity in each case.

6. *Antibody response to the 3 types of antigen preparations.* Only incomplete data are available. Rabbits immunized with each of the 3 preparations have responded satisfactorily with antibody, as shown by fluorescent antibody staining with an increase in

TABLE IV. Complement Fixation Titers of Rabbit Antisera Tested against Soluble Fraction (B) of Each of 4 Species, Tb (*M. tuberculosis*), Ka (*M. kansasii*), Bl (*M. balnei*), and Ph (*M. phlei*).

Serum	Antigen Tb*	Ka*	Bl*	Ph*
Anti-Tb	>256†	64	64	16
" -Ka	64	>256	128	0
" -Bl	>256	32	>256	128
" -Ph	64	64	128	>256

* Antigen dilutions (2 units): Tb 80, Ka 320, Bl 320, Ph 80.

† Numbers refer to reciprocal of highest titer giving 3+ to 4+ fixation against 2 units of antigen.

titer of roughly 100-fold. The rabbits also showed strong antibody responses as demonstrated by agar diffusion and complement fixation. Studies of the efficacy of the preparations as vaccines are in progress.

Discussion. The intact mycobacteria of the more slowly growing species did not stain with fluorescent antibody and this is evidence that the outer surface does not contain antigen. This unusual condition presumably arises from the unique lipophilic surface of these organisms. When the rabbits were injected with the intact bacilli, they formed antibody that was not apparently different from that produced by injection of the soluble fraction or disrupted bacilli; probably the intact bacilli are broken up in the rabbit before it responds by formation of antibody.

Absorption by the soluble fraction of antibody capable of reacting with the disrupted bacilli might be interpreted to mean that there were no antigens present in the cell wall that were not also present in the protoplasm. There was not sufficient evidence of anatomical purity to establish such a conclusion, however, since protoplasm antigen might have persisted on the cell wall, or perhaps translocated there, and the soluble fractions (B) might have contained suspended fragments of cell wall.

Summary. 1) Mycobacteria were broken open by vibration with glass beads. Ninety to 95% of the protoplasm, and 70-80% of the bacterial mass was released into a soluble state and could be separated by centrifugation from the sediment (disrupted bacilli), which consisted largely of cell walls. 2) Intact bacilli of the 5 more slowly growing species studied were not stainable with fluorescent antibody, but disrupted bacilli stained brightly. This was interpreted as evidence that the outer surfaces of intact bacilli of these species are free of antigen. 3) Intact bacilli of 3 of the more rapidly growing species stained well. 4) The soluble fraction contained the greatest amount of antigen as judged by ability to inhibit staining of the disrupted bacilli. It could be used to absorb (inhibit) cross-reacting antibodies to render immune sera more specific in their staining of disrupted organisms. 5) The soluble fraction was a potent antigen in agar diffusion tests. In complement fixation tests it was also an effective antigen with helpful specificity.

1. Mudd, E. B. H., Mudd, S., *J. Exp. Med.*, 1927, v46, 147.

2. Salton, M. R. J., in *The Bacteria. A Treatise on Structure and Function.* I. C. Gunsalus and R. Y. Stanier, Eds., Academic Press, New York, 1960, p97.

3. Ribi, E., Larson, C. L., List, R., Wicht, W., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 263.

4. Ribi, E., Perrine, T., List, R., Brown, W., Goode, G., *ibid.*, 1959, v100, 647.

5. Shepard, C. C., *J. Exp. Med.*, 1960, v112, 445.

6. ———, *Am. J. Hyg.*, 1960, v71, 147.

7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.*, 1951, v193, 265.

8. Seifter, S., Dayton, S., Novic, B., Muntwyler, E., *Arch. Biochem.*, 1950, v25, 191.

9. Cherry, W. B., Goldman, M., Carski, T. R., *Fluorescent antibody techniques in the diagnosis of communicable diseases.* U. S. Govt. Printing Office, 1960.

10. Koch, R., *Deutsche Med. Wschr.*, 1897, v23, 209.

11. Runyon, E. H., *Med. Clin. N. Am.*, 1959, v43, 273.

12 Binford, C. H., *Lab. Invest.*, 1959, v8, 901.

13. Goldwasser, R. A., Shepard, C. C., *J. Immunol.*, 1959, v82, 373.

14. Wilson, G. S., *J. Path. Bact.*, 1925, v28, 69.

Received September 12, 1960. P.S.E.B.M., 1961, v106.