LETTERS TO THE EDITOR

1. From Dr. R. E. PFALTZGRAFF, of Garkida, Yola, Nigeria, on *A Practical Method of Mailing Small Biopsies:* "Dr. Cochrane has suggested I report this new simple economical way of sending small biopsy specimens. *Method:* The biopsy is fixed by placing in a relatively large amount of whatever fixative solution is chosen, e.g. a small specimen less than 1 cm. in size, is placed in 30 ml. of fixative for 24 hours or more. If passed through several solutions the tissue should remain in the ultimate solution for 24 hours. Cut a finger off a discarded rubber glove, and house the piece of tissue in that, and add 2 to 3 ml. of the fixative solution. Squeeze out all air and fix a rubber band round the neck of the glove finger, in order to seal off the package. Round the constricting rubber band wind 2" to 3" (5 to 7.5 cm.) of "Scotch tape" (equivalent to "sticking plaster" in English usage). The little package is then affixed by sticking plaster to a suitable light card, and inserted with the covering letter into a regular air mail envelope. If the card fits the envelope, there will be less chance of destructive movement inside the envelope during its travels. By keeping the weight of biopsy specimens in their envelopes less than ½ oz. (about 15 gm.), the use of air mail becomes a practical method of sending them about the world to desired laboratory destinations".

2. From R. RHODES-JONES, ESQ., F.I.M.L.T., of the East African Leprosy Research Centre, on the *Technique of Staining Leprosy Bacilli in Smears.* He refers to the article by A. R. DAVISON on this subject in LEPROSY REVIEW **31**, 4. He states that Dr. Davison in that article quoted from a letter from him and that Dr. Davison had failed in that article to notice that slides 1–3 had faded, and it was for that reason Mr. Rhodes-Jones had to restain them. Slides 4 to 6 had *not* faded, so were not restained, though it would seem from Dr. Davison's article that they were. In all the slides the bacilli had stained blue, which masked the pale pink diphtheroids. This is the meaning of the term "masked".

[In this question of fading of stained specimens, we think a possible explanation is the effect of excess of light. In East Africa the intensity of natural radiation of ultraviolet light is 12 times that in Switzerland. To put it another way, places in East Africa *in one month* may have the ultraviolet radiation of a whole year in Switzerland. In a laboratory with plenty of windows, specimens placed even for 1 hour or 2 hours not too far away from a window, and perchance not covered, might well get into trouble even before staining? *Editor.*]

Anonymous or Unclassified Mycobacteria

3. From H. C. DE SOUZA-ARAUJO, M.D., DR. P. H., of Instituto Oswaldo Cruz, Laboratory of Leprology, Rio de Janeiro, on the subject of Mycobacteria:

Ten years ago the NEWS LETTER of the Society of American Bacteriologists (Vol. XVI, N.1, Jan. 1950, p. 6) published that Dr. Frederick Eberson, M.D., Chief, Clinical Pathology, Assistant Chief, Laboratory Service, Kennedy Veterans Administration Hospital, Memphis, Tennessee, U.S.A., was "seeking cultures of chromogenic strains of *Mycobacterium tuberculosis* or similar unclassified acid-fast bacteria" for studies.

By letter of 25th February 1950 to Dr. Eberson I offered to furnish him "more than 30 trains of acid-fast bacteria isolated by myself from leprous patients, directly or with the 'help' of various hematophagi; from leprous patients with pulmonary tuberculosis, under Streptomycin treatment; from rats and mice inoculated with Stefansky bacillus (Strain of the Institut Pasteur of Paris); from ticks (*Amblyomma rotundatum*) captured in cold-blooded animals (*Bufo marinus*, *Bufo crucifer* and *Constrictor constrictor*), in various occasions; and from effluent sewage of OMS tank of two leprosaria, one in this city and another in São Paulo. If you want a set of these cultures and promise me to help in their classification, I will be glad to send you a sample of each one, by air-mail, as soon as you answer this letter", etc.

Dr. Eberson answered me by air-mail letter of March 6, 1950, telling: "Dear Dr. Araujo: Your response in reply to my note in the S.A.B. News Letter is most gracious. I appreciate your generous offer and shall be pleased to have a set of the cultures for inclusion in a study of chromogenic 'variants' of *Mycobacterium tuberculosis*. If it is not too much trouble, can you let me have certain data pertaining to the history of these cultures, particularly, the human strains . . . etc". He added: "P.S. It has occurred to me to ask if you have available any serums from patients or animals, snakes, etc. corresponding to the cultures of chromogenic organisms or other T.B. strains. I propose to do serologic studies as an aid in attempting to classify these cultures", etc.

On 31st March 1950 I sent to Dr. Eberson, by air-mail registered, 51 tubes with cultures and 13 tubes with sera of patients, accompanied with a full explanatory list, which will be transcribed in the following page. In the same day I sent him also one confirmatory letter. On 28th April I wrote again to Dr. Eberson confirming the above remittance and sending him, in separate, another sub-strain "Chaves" recovered from experimental lesion produced in rhesus monkey and the serum of patient Emilia, corresponding to strain n.14 of the list.

Only in May 8, 1950 Dr. Frederick Eberson wrote me: "In confirmation of your letter dated April 28th, referring to the shipment of cultures, I received on Saturday May 6th, 51 tubes of cultures and 13 specimens of serum. *The package arrived in excellent condition.* Upon removing the wrappers and sorting the cultures, I

found 13 which have no visible surface growth and 6 which were grossly contaminated with a black or greyish white mold. Transplants are being made on all the strains and I *anticipate very interesting findings*. Thank you again for your very generous contributions and your courtesy", etc. Dr. Eberson says that he had received the culture and the serum sent to him on 28th April.

I do not understand why was spent 38 days to be open my package. The tubes being flat so long time (38 days) explains the wetting of the cotton-cover with condensed water of the medium and germination of the spores of *Aspergillus niger* existing in the same. My cultures of mycobacteria preserved in standing position in general last 2 to 3 years without mold contamination.

In his letter of November 30th, 1950, Dr. Eberson informed me: ". . . It may interest you to know that the culture N.24, *Mycobact. lutzi*, appears to be a Nocardia strain with inhibitory properties for tubercle bacilli", etc.

On 19th December 1950 I sent to Dr. Eberson 17 tubes of cultures, of which nine new strains or sub-strains and the other to replace those arrived contaminated. The new strains or sub-strains were included in the general list below.

List of Cultures of Mycobacteria furnished in 1950 to Dr. Frederick Eberson, Chief Pathologist of the Kennedy Veterans Administration Hospital, Memphis 15, Tennessee, U.S.A.

No. of Tubes; Origin of the Cultures; Other data.

1—Strain "José", 1941. Isolated in October and November from closed skin leprous lesions of a 7-year boy. Case L2, son of leprous parents, from Piauhy.
2—Strain "Alcebiades", 1942. Isolated from *Amblyomma cajennense* experimentally infected in skin lesion of A.P., aet.32, L3–N1 case, Paraná.
3—Strain "Ramtun", 1942. Isolated from *Boophilus microplus* experimentally infected in skin lesion of J.R., aet.45, L3–N1 case, from Paraná.
4—Strain "Rudan", 1942. Isolated from *Boophilus microplus* experimentally infected in skin lesion of P.R., aet.26, L3–N1 leprosy case, from Paraná.
5—Strain "J. Carlos", 1943. Isolated from *Triatoma infestans* experimentally infected in skin lesion of J.C., aet.23, L3 leprosy case, from S. Paulo.
6—Strain "Chaves", 1949. Isolated from skin lesion of right thigh of J.C., aet.30, L2–N1 leprosy case, aviator, 7th biopsy.
7—Sub-strain "Chaves", 1950. Recovered from experimental lesion in mouse.
8—Sub-strain "Chaves", 1950. Recovered experimental lesion in the same part
9—Sub-strain "Chaves", 1950. Recovered from experimental lesion produced in his wife Maria D., aet.18, she being a tuberculoid incipient case.
10—Strain "Hecke", 1949. Isolated from leprous skin lesion of Agronomist Hecke, aet.26. Case L2–N1, biopsy of right buttock. From Paraná State.
11—Sub-strain "Hecke", 1949. 3rd recovery from experimental lesion in mouse.
12—Sub-strain "Hecke", 1949. Recovered from experimental lesion in Chaves.
13—Sub-strain "Hecke", 1949. Recovered from infection of guinea-pig: 6 days.
14—Strain "Emilia", 1950. Isolated from leprous skin lesion of E.P., aet.53.
15—Strain "A. Alho", chromog., 1948. Isolated from sputum of this leper with pulmonary TB, under treatment with Streptomycin. Gave another strain.
16—Strain "JP Souza", chromog., 1948. Isolated from sputum of this leper with pulmonary TB, under treatment with Streptomycin. Gave another "R" strain.
17—Strain "Santos", chromog., 1948. Isolated from sputum of this leper with pulmonary TB, under treatment with Streptomycin. Gave another "R" strain.
18—Strain "Stefansky I", 1948. Isolated from experimental infection of rat.

19—Strain "Stref. II", 1948. Isolated from white rat. Pasteur Inst. strain.
20—Sub-strain "Stef. II", 1949. Recovered from black mouse infected with same.
21—Strain "Stef. III", 1949. Isolated from black mouse infected with leproma.
22—Sub-strain "Stef. III", 1949. Recovered from black mouse infected with same.
23—Strain "Stef. IV", 1949. Isolated from black mouse infected with leproma.
24—Strain *Mycobacterium lutzi*, 1947. Isolated from *Amblyomma rotundatum* parasiting of *Bufo marinus*, from Rio City. New species.
25—Strain *Mycob. sp.* 1949. Isolated from same tick above of same toad.
26—Strain *Mycob. sp.* 1949. Isolated from same tick from same toad. Nat. museum.
27—Strain *Mycob. sp.* 1950. Isolated from same tick from same toad. State Rio.
28—Strain *Mycob. sp.* 1950. Isolated from same tick from same toad. State Rio.
29—Strain *Mycob. sp.* 1949. Isolated from same tick from *Bufo crucifer* of Rio.
30—Strain *Mycob. sp.* 1949. Isolated from same tick captured in *Constrictor constrictor* of the garden of the Nat. Museum, of Rio de Janeiro.
31—Strain "Geraldo V.", 1948. Isolated from pus of cervical lymphnode of this leper with ganglioar TB. Died 2 days later.
32—Strain "A. Alho II", 1948. Isolated from sputum of this leper with pulmonary TB, separated from strain 15, chomogenic.
33—Strain "JP Souaz II", 1948. Isolated from sputum of this leper with pulmonary TB, separated from strain 16, chromogenic.
34—Strain "M. Rodrigues", 1948. Isolated from sputum of this leper with pulmonary TB, like the other, under treatment with Strepromycin.
35—Strain "A. Simoes", 1948. Isolated from sputum of this leper with pulmonary TB, under treatment with Streptomycin.
36—Strain "Raymundo T.", 1948. Isolated from sputum of this leper with pulmonary TB, under treatment with Streptomycin.
37—Strain "Dinorah", 1948. Isolated from sputum of this leper woman suffering with pulmonary TB, under treatment with Streptomycin.
38—Strain "G. Silva", 1948. Isolated from sputum of this leper with pulmonary TB, under treatment with Streptomycin.
39—Strain "A. Lopes", 1948. Isolated from sputum of this leper with pulmonary TB, under treatment with Streptomycin.
40—Strain "Waldivia C.", 1948. Isolated from sputum of this woman leper suffering from pulmonary TB, under treatment with Streptomycin.
41—Strain "Minervina F.", 1948. Isolated from sputum of this woman leper suffering from pulmonary TB, under treatment with Streptomycin.
42—Strain "Geraldo R.", 1948. Isolated from suptum of this leper with pulmonary TB, under treatment with Streptomycin.
43—Strain "Alcides G.", 1948. Isolated from sputum of this leper with pulmonary TB, under treatment with Streptomycin.
44—Strain "Clovis S.", 1948. Isolated from sputum of this leper with pulmonary TB, under treatment with Strepromycin. Gave another chromog. strain.
45—Strain "Adauto P.", 1948. Isolated from sputum of this pulmonary tubercle patient. No leprosy. TB alone.
46—Strain "FF. Goulart", 1948. Isolated from sputum of this leper with pulmonary TB, under treatment with Streptomycin.
47—Strain "C. Amorim", 1948. Isolated from sputum of this leper with ulmonary TB, under treatment with Streptomycin.
48—Strain *Mycob. sp.* 1948. 1948. Isolated from effluent water of sewage biological OMS tank from Leper Hospital Curupaity, Rio de Janeiro.
49—Strain recovered from tuberculosis lesion of guinea-pig infected with the above 48 strain.
50—Strain *Mycob. sp.* 1946. Isolated from effluent water of sewage biological OMS tank of Padre Bento Leper Hospital of Sao Paulo. 1st strain.
51—Strain *Mycob. sp.* 1946. Isolated from the same effluent water above. 2nd strain.

NB. At the occasion the Author obtained 4 other pure strains from sewage water of OMS purifying tank, all with the characteristic of TB bacillus.

Second remittance:

52—Strain *Mycob. sp.* 1950. Isolated from *Amblyomma rotundatum* parasite of snake *Drimachon bifossatus*. No. 1.
53—Strain *Mycob. sp.* 1950. Isolated from same tick above from same snake.
54—Strain *Mycob. sp.* 1950. As above. No. 3.

These three strains are similar to *Mycobaterium lutzi*.

55—Sttain "Chaves II", new strain, 1950. Isolated from residual skin lesion of the left knee of that patient. 14th biopsy. Identical to "Chaves I."
56—Sub-strain "Hecke", 1950. Recovered from experimental lesion produced in the leprous patient Chaves.

57—Sub-strain "Hecke", 1950. Recovered from experimental lesion produced in the leprous patient N.14 of Recife, Pernambuco.
58—Sub-strain "Emilea", 1950. Recovered from white rat experimental lesion.
59—Sub-strain "Emilia", 1950. Recovered from experimental lesion produced in the face of rhesus N.2. (*Maccaca mulatta.*)
60—Sub-strain "Emilia", 1950. Recovered from experimental lesion produced in leprous patient N.21, of Recife, Pernambuco.

1. *Technique of the Cultures.* The above cultures were obtained from skin lesions, subcutaneous lymph, lymphnodes or sputa of leprosy patients, and from triturates of ticks and triatomas, and biopsies of experimental lesions produced in men, murines, and monkeys. After trituration and suspension in saline solution, the materials were treated by 10% solution of NaOH (Petroff method) or 5% solution of H2SO4 (Loewenstein Method), kept in the incubator at 37·C during 30 minutes, then washed by 2 or 3 centrifugations and the sediments sown in series of 9 tubes of Loewenstein medium and 1 tube of 5% glycerin broth as control. Incubation at 37C° for 30 days, because we know that pathogenic mycobacteria germinate from 14 to 29 days. Those growing in a few days are, in general, symbiotic bacteria. All 15 advanced leprous cases, suffering from pulmonary tuberculosis, confirmed by X-ray, No. 32 to 47 (excepted No. 45 who had no leprosy), after having taken the maximum dosage of Streptomycin then used gave, from their sputa, pure cultures of *Mycobacterium tuberculosis*, and died within a few days or months later.

2. *Staining Properties.* All the above 60 cultures were permanently acid-alcohol fast, staining by classic Ziehl-Neelson method, and granulated, coccothrix form stained by Fontes method. Dr. L. M. de Andrade, using auramine, proved that 49 out of the first 51 samples sent to Dr. Eberson, were fluoroscent, being 2–plus 34, 1–plus 15 and 2 negative (Technic of Emil Bogen: American Rev. of Tuberculosis, 44 (3), Sept. 1941, p. 367). Such results were confirmed, in other transplants of the same cultures, by Prof. Joao Christovao Cardoso, now President, Conselho Nacional de Pesquisas.

3. *Dubos Test for Virulence.* Testing the 51 cultures first sent to Dr. Eberson, Dr. L. M. de Andrade, then chief of the Laboratory of Mycobacteria of the Instituto Oswaldo Cruz, proved that 25 gave 2–plus, 7 1–plus, 18 negative and 1 unsufficient material. (Technic of Dubos and Middlebrook: Amer. Rev. of Tuberculosis, 58, Dec. 1948, p. 698). Professor Pierre Hauduroy, Director, International Centre of Cultures, Lausanne, Suisse, confirmed such results in a few strains sent to him. Some leprosy-strains being too pigmented, gave doubtful results (Dr. Andrade). All the 15 strains, from sputa of leprosy patients with TB gave 2–plus fluoroscence and 2–plus Dubos test.

4. *Phage Typing.* From the above list I selected 20 strains and sub-strains and sent on 20th December, 1950, to Professor Giuseppe

Penso, Director, Istituto Superiore di Sanitã, of Rome for study, as he asked for when he was in Rio in August 1950. Such cultures were passed to the hands of Dr. Vittorio Ortali, who, on 26th December 1950 wrote to me: "The 20 strains of Mycobacteria to be typed with the available phages were received". By letter of May 29, 1951 said Dr. Ortali: "Now we are working with strains of Mycobacteria isolated from leprosy. I have received some new phages from Canada, and I hope to find some one active". Later on (Sept. 24, 1951) Dr. Ortali informed me: "I tried yours strains with all my phages, and I did not get any reaction. A technician who works with me now tries to find new phages active on some strains of Mycobacteria, among which there are also your strains. If you can send me other strains I will be very grateful. If some phage exist, it would be easier to find in leprosy material . . . ."

As it is very, very difficult to get an acid-fast culture from leprotic skin, and I got twice identical and pathogenic cultures from patients "Chaves" and "Emilia", then I consulted to Dr. Eberson if I could classify the same as *Mycobacterium leprae hominis*. Dr. Eberson answered me by letter of January 23, 1951, as follows: ". . . In regard to your 'Chaves' and 'Emilia' strains, do you not agree that identity of these warrants the name of *Mycobacterium leprae hominis* similar to others belonging to the International Collection? However, the naming of such cultures need not carry the implication that they are in fact etiologic agents in the disease mentioned. I have not had the opportunity to work with the new cultures 52, 53, 54, aside from making necessary transplants. Presumably these strains will conform to varieties known to exist in cold-blooded species of animals. The research problem is, as you see, one of such magnitude that I wish it were possible to devote more attention to the study. . . . In connection with the study of certain chromogens, another important phase of biological interest has been occupying my attention, that of the antibiotic properties. I hope to have some of this ready for publication in the near future", etc.

In answer to my letter of February 28, 1952, Dr. Eberson wrote to me on 6th of March 1952: ". . . Unfortunately my studies with all our acid-fast chromogenic Mycobacteria, including your strains from leprous lesions, have been interrupted during the past few months. In the near future our bacteriological research laboratories will be fully staffed and I hope to continue the studies on an intensive sacle. As I wrote you some time ago, perhaps, it has seemed to me important to classify the entire group of chomogenic acid-fast mycobacteria. This is a gigantic undertaking, aside the many problems introduced by antibiotic therapy, variation and mutation of strains, and so on", etc. This was the last letter I received from Dr. Frederick Eberson.

In 1959 the Veterans Administration and the National Tubercu-

losis Association sent out a questionnaire and requested co-operation of the specialists to resume and intensify the research upon which is now called "unclassified" mycobacteria. I offered immediately to collaborate with both institutions, informing them about my previous co-operation with VA. By letter of March 29, 1960 Dr. Ernest H. Runyon, Ph.D., Microbiology Research Chief, Veterans Administration Hospital, Salt Lake City, Utah, U.S.A., informed me: "About 10 years ago I received from Dr. Eberson transfers of some cultures which were from South America—presumably the ones you sent to him. We have been concerned with strains of mycobacteria which are known to be closely associated with human diseases. Since information concerning the relationship of the South American strains to disease was lacking we did not retain these cultures. . . ." By letter of June 23, 1960 informes Dr. Runyon: "Dr. Floyd Feldmann has kindly sent me the transcription of your correspondence with Dr. Eberson concerning mycobacterial cultures. You are to be congratulated on having isolated so many interesting organisms", etc. In this letter he asked me for transplants of five of my mycobacteria cultures. I sent him ten, to start new co-operation and promised him, for September, new strains from cold-blooded animals.

The anonymous mycobacteria were the theme of the XVth Conference of the International Union against Tuberculosis, held in Istanbul, September, 1959, and will be also of the XVIth Conference to be held in Toronto, in 1961.

The subject is of great importance and merits international co-operation for its study.

Instituto Oswaldo Cruz, Rio de Janeiro,
September 8, 1960 H. C. DE SOUZA-ARAUJO