Culture Possibilities of *Mycobacteria* at 5°C

72 strains of 'Bacilles paratuberculeux' (so-called atypical *Mycobacteria*) have been inoculated. These strains came from very different sources, both regarding material from which the initial isolation was made and countries of origin (Table I).

Table I

Number of strains	Origin	Temperature and mean periods of growth (as already known)
6	Japan (Prof. UYEDA)	20–37° (15 days) 45° (35 days)
8	USSR (Dr. OGLOBINA)	30–37° (15–20 days) 6 of them grow at 45° (35 days)
5	France (Miss NOUFFLARD)	20–37° (15–20 days) 3 of them grow at 45° (35 days)
20	Holland (Prof. MANTEN)	20–37° (15–20 days) 11 of them grow at 45° (35 days)
8	Canada (Prof. MANKIEWITZ)	20–37° (15 to 20 days) 5 of them grow at 45° (35 days)
5	Malmö (Sweden) (Dr. JUHLIN)	20–37° (15–20 days) 45° (35 days)
6	Australia (Dr. KOVACS)	20–37° (15–20 days) 45° (35 days)
9	Iran (Dr. TABATABAÏ)	20–37° (30 days)
4	Belgium (Dr. VERSTRAETE)	20–37° (30 days)
1	Austria (Prof. Diemhofer) (*M. vaccae*)	20–37° (15–20 days)

All strains have been isolated from various pathological and non-pathological products: expectorations, gastric washes, lymphatic ganglia, residual water. In no case was there a *M. tuberculosis* var. *hominis*.

All have been inoculated with Löwenstein-Jensen and Dubois media, and kept at 5°C (±1°C). For results see Table II. These results show first of all the necessity of inoculating bacteria on different media if studies of temperature influence on growth are made. Should this precaution not be taken, results tend to be inaccurate.

Development of cultures at 5°C appears much later than growth at previously known temperatures (Table I). For 71 of the strains, growth started at 5°C after 30 days, while an abundant growth was only apparent after 50 or 60 days. A single strain, *M. vaccae* (Bönicke), gave abundant growth within 20 days.

Coloration by Gram and Ziehl methods for strains grown at 5°C shows no difference from the coloration for the same strains grown at higher temperatures. Serial reinoculations are also possible at 5°C.

Discussions on possible variations in biological characteristics for strains grown at 5°C will appear in later publications.

Table II

	Cultures	
	Positive at 5°C	Negative at 5°C
Dubos medium	59 (82%)	13 (18%)
Löwenstein-Jensen medium	51 (71%)	21 (29%)

Résumé. Les auteurs ont constaté que sur 72 souches de Myobactéries atypiques 82% d'entre elles pouvaient développer à 5°C. Les cultures à cette température commencent à être visibles vers le trentième jour et sont abondantes vers le cinquantième ou le soixantième jour.

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The Application of Fluorescent-Antibody Test to Cysts of *Entamoeba invadens*

The fluorescent-antibody test has been used extensively by GOLDMAN[1] to study the antigens of *Entamoeba*. Although in an initial study he had successfully applied the test to both the cysts and trophozoites of *Entamoeba histolytica*[2], most of his work is based on trophozoites as antigens.

This is a preliminary report into the study of the antigenic relationship of *Entamoeba* using cysts as antigens. The cysts, although not as readily available in cultures, have certain advantages over trophozoites. They can be stored for long periods at low temperatures and can be washed almost entirely free of extraneous antigens and bacteria without losing their viability.

The strain of *E. invadens* (BC) used in this study was obtained through the courtesy of Dr. E. W. MCCONNACHIE, Cambridge (England). This strain was originally monoxemic, growing with *Escherichia coli*. At present it is contaminated with at least one more bacterium. The strain encysts in large numbers when grown in Difco's Endamoeba medium[3], 5–6 days after inoculation.

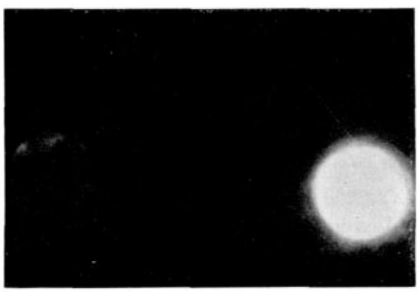
To prepare the antigens, the cysts were washed three times in sterile distilled water by centrifugation and stored at 5°C overnight. This process was repeated daily

[1] M. GOLDMAN, Exp. Parasit. *10*, 366 (1960).
[2] M. GOLDMAN, Am. J. Hyg. *58*, 319 (1953).
[3] L. R. CLEVELAND and J. COLLIER, Am. J. Hyg. *12*, 606 (1930).

for 3 days until a pure suspension of cysts, completely free of trophozoites, was obtained. The cysts were transferred to isotonic saline before injection into rabbits for immunization. The immunization schedule consisted of 4 injections given intravenously at 7 day intervals. The total number of cysts injected was approximately 1,000,000 cells per animal.

The sera taken 7 days after the last immunizing injection were pooled and used for conjugation. The globulin fraction of the serum was conjugated to Fluorescein isothiocyanate on celite[4] according to the method described by RINDERKNECHT[5]. The unconjugated dye was separated by passing it through a column of Sephadex G25[6]. The serum was absorbed twice against rabbit liver powder before use.

The tests were performed using cysts prepared by the following methods: (a) Unfixed cysts suspended in phosphate buffered saline of pH 7.2, 0.01 *M* (PBS). (b) Cysts fixed in 10% formalin at room temperature for $^1/_2$ h. (c) Cysts fixed in 95% methanol at room temperature for $^1/_2$ h. (d) Cysts fixed in 95% ethanol at room temperature for $^1/_2$ h. (e) Cysts fixed in acetone at room temperature for $^1/_2$ h.



The cyst on the right has been treated with conjugated antiserum and the cyst on the left with conjugated normal serum. Both the cysts were from unfixed preparations.

The fixed and unfixed cells were treated with 1:5 dilution of specific antiserum for $^1/_2$ h at room temperature, washed in PBS by centrifugation and examined as a wet preparation with a Zeiss fluorescent microscope.

The controls consisted of cells identically prepared but treated with normal conjugated rabbit serum. In addition, cells were pre-treated with unconjugated antiserum and then with conjugated antiserum to test for any inhibition of the reaction.

The test slides showed intense fluorescence when both the unfixed and chemically fixed cells were used. None of the fixatives tried destroyed the specificity of the reaction. Non-specific staining was negligible in unfixed cells and only slight in the chemically fixed cells. Formalin fixation seemed to be the best as it least affected the specificity of the reaction. However, acetone gave the brightest fluorescence. Pre-treatment with normal antiserum inhibited the reaction. It was possible to remove the fluorescent effect of the antiserum by repeated absorption against a heavy suspension of cysts. This indicated that it was a true antigen-antibody reaction.

Work is at present in progress to study the antigenic relationship of cysts of different species of *Entamoeba* using this technique.

Zusammenfassung. Die Methode fluoreszierender Antikörper wurde zur Darstellung von Blasen bei *Entamoeba invadens* benützt. Sowohl mit nativen, wie auch mit fixierten Zellen konnte eine befriedigende Reaktion erzielt werden. Formalin erwies sich als günstigstes Fixationsmittel.

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Department of Parasitology, Faculty of Medicine, University of Singapore (Malaysia), November 2, 1964.

[4] California Corporation for Biochemical Research, Los Angeles (Calif. USA).
[5] H. RINDERKNECHT, Exper. *16*, 430 (1960).
[6] Pharmacia, Uppsala (Sweden).

Eine Variation der Bichromat-Osmiumsäurefixation für das Nervensystem

Die von DALTON[1] eingeführte Bichromat-Osmiumsäurefixation bedarf einer Verfeinerung, um Nachteile bei den Fixationsresultaten, sowie verschiedene Fixationsartefakte zu vermeiden, welche sich besonders an Zellen des Nervensystems finden. Wir stellten uns die Aufgabe, durch empirische Versuche die Bichromat-Osmiumsäurefixation zu verbessern, damit eine gleichmässige und gute Fixation erfolgt. Es wurde in Versuchen festgestellt, dass sich einige Salze zur Veränderung der Tonizität besonders eignen, ähnlich dem Prinzip von ZETTERQUIST[2] bei der Osmiumsäurefixation. Im Vergleich mit den bisherigen Fixationen an den Zellen des Nervensystems hat die von uns erprobte Fixationsart bessere Resultate gezeigt: die Weitstellung des Ergastoplasmas bei der Osmiumsäurefixation nach PALADE[3] sowie die Aufblähung von Zellkern und Mitochondrien bei der ursprünglichen Daltonschen Bichromat-Osmiumsäurefixation konnte vermieden werden. Die Membransysteme sind wie bei der Daltonschen Fixation sehr gut dargestellt.

Bei den Untersuchungen wurde Nervengewebe von Albinoratten, Katzen und Meerschweinchen entnommen. Bei den gezeigten Abbildungen handelt es sich um eine sympathische Nervenzelle aus dem Ganglion cervicale superius der Ratte (Figur 1) und um die Neuron aus dem

[1] A. J. DALTON, Anat. Rec. *121*, 281 (1955).
[2] H. ZETTERQUIST, *The Ultrastructural Organisation of the Columnar Absorbing Cells of the Mouse Jejunum* (Dept. Anat., Karolinska Institutet; Aktiebolaget Godvil, Stockholm 1956).
[3] G. E. PALADE, J. exp. Med. *95*, 285 (1952).