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AGENCY FOR INTERNATIONAL DEVELOPMENT  
WASHINGTON, D.C. 20523

DATE: 8/4/88

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18  
FROM: AID/SCI, Victoria Ose *VO*  
SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 5.224  
1st PR rec'd 11/12/88

Attachment

5. 2007  
X

FIRST PROGRESS REPORT OF RESEARCH PROJECT  
"IMMUNODIAGNOSIS AND SEROEPIDEMIOLOGY OF TUBERCULOSIS"

SUBMITTED TO : U.S. Agency for International Development

GRANT NUMBER : 936-5542-G-00-5043-00

TOTAL PROJECT PERIOD : 04/22/1985-10/31/1987

PERIOD COVERED BY THIS REPORT : 04/22/1985-10/31/1985

PRINCIPAL INVESTIGATOR : Sanit Makonkawkeyoon, Ph.D.

Department of Clinical Immunology

Faculty of Associated Medical Sciences

Chiang Mai University, Chiang Mai, Thailand

1. Personnel Management and Activities:

Dr. Sanit Makonkawkeyoon, principal investigator, worked very closely with Archarn Sichen and Archarn Luksana in the attempt to isolate and purify species specific haptens from M. tuberculosis using various biochemical techniques. He also worked along with Archarn Sakchai in the isolation, cultivation, and identification of M. tuberculosis from patients. Mass culture of various species of mycobacteria were prepared for immunization of rabbits to produce hyperimmune sera against various species of mycobacteria. Blood specimens were collected from various groups of patients, such as smear positive for acid fast bacilli (AFB), and culture positive for M. tuberculosis, smear positive AFB but culture negative, smear negative AFB but culture positive, and both smear and culture negative.

Two full-time medical technologists were recruited in this research project. One of them was assigned to assist in the isolation and purification of species specific haptens. The other one was assigned to assist in the work

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of isolation and identification of mycobacterium culture, preparation of mycobacterium antigen, and immunization of rabbits with various species of mycobacteria.

One part-time janitor was hired to look after experimental animals, especially rabbits which were used for hyperimmunization with various types of mycobacterium. Another part-time janitor was employed for taking care of blood specimens and culture transportation from Tuberculosis Control Center Region 10 to the Department of Clinical Immunology, Faculty of Associated Medical Sciences, located about 4-5 Kilometers from the Center.

One part-time secretary has been hired to handle documents and to organize records. One part-time typist was also hired for typing records, research data, progress reports, and manuscripts for publication.

## 2. Purchase of Equipment and Materials:

All listed equipment in this research project, such as ELISA reader, deep freezer, lyophilizer, thin layer chromatography, rotary evaporator, rabbit cages, safty hood and 8-channels micropipette have been ordered, and some have already arrived and are in use. The purchase of scientific materials and reagents from a local agency is quite easy, and only some minor problems have been encountered.

## 3. Progress Report:

### A. OBJECTIVES:

The overall objectives of this research project are :

- (1) To obtain species specific haptens from M.tuberculosis.
- (2) To develop an enzyme linked immunosorbent assay by using the species specific haptens from M.tuberculosis for serodiagnosis of tuberculosis.

- (3) A very simple and practical method of ELISA is developed and used in the field for seroepidemiological study of tuberculosis.

B. ACTIVITIES :

PART I. ISOLATION AND PURIFICATION OF SPECIES SPECIFIC HAPTENS AND DEVELOPMENT OF AN ELISA.

EXPERIMENT NO.1

PURPOSE : LIPOPOLYSACCHARIDE EXTRACTION

M.TUBERCULOSIS H37Rv 13.0 G (WET WEIGHT)

+

200 ML 90% PHENOL-WATER

↓

SONICATION 200 WATT, 3 MIN, 2 TIMES

+

DIST. WATER 100 ML

↓

SONICATION 200 WATT, 3 MIN, 2 TIMES

↓

CENTRIFUGE 900 G

↓

SUPERNATANT

↓

CENTRIFUGE 18,000 G

↓

SUPERNATANT

↓

DIALYSIS AGAINST DIST. WATER 2 DAYS

↓

CONCENTRATED TO 20 ML BY DIALYSING TUBE  
AT 4 DEGREE CELCIUS

↓

ADDED 150 ML COOLED ABSOLUTE ETHANOL

↓

CENTRIFUGE 900 G, 15 MIN

↓

WASH PRECIPITATE WITH COOLED ABSOLUTE ETHANOL 2 TIMES

↓

SOLUBILIZED PRECIPITATE IN 10 ML DIST. WATER

↓

CRUDE PHE-W EXTRACT  
(PHE/W-I)

EXPERIMENT NO.2

PURPOSE : LIPOPOLYSACCHARIDE EXTRACTION

M.tuberculosis H37Rv 31 G

↓

ADD 40 ML 90% PHENOL-WATER

↓

SHAKE IN WATER BATH 68 DEGREE CELCIUS, 20 MIN

↓

SONICATE 200 WATT, 5 MIN, 4 TIMES

↓

CENTRIFUGE 900 G, 30 MIN

↓

SUPERNATANT

↓

CENTRIFUGE 18,000 G, 30 MIN

↓

SUPERNATANT

+

EQUAL VOL. OF COOLED ABSOLUTE ETHANOL

↓

FILTER THROUGH GLASSWOOL COLUMN (2CM DIAMETER x 8 CM LONG)

↓

FILTRATE

+

160 ML OF COOLED ABSOLUTE ETHANOL

↓

LET STAND 4°C, 8 DAYS IN 250 ML FLASK

↓

POUR OUT ETHANOL

↓

EVAPORATE TO DRY

↓

ADD 5 ML DIST. WATER TO DISSOLVE PPT. WHICH ATTACH ON BOTTOM OF THE FLASK

↓

(PHE/W-II)

EXPERIMENT NO.3

PURPOSE : LIPOPOLYSACCHARIDE EXTRACTION

M.tuberculosis H37Rv 28 G

↓

ADD DIST. WATER 60 ML

↓

SONICATE 200 WATT, 5 MIN

↓

ADD 60 ML of 90% PHENOL.

↓

SHAKE 68°C IN WATER BATH 3 HR

↓

LET STAND OVERNIGHT

↓

SONICATE 300 WATT, 3 MIN

↓

WATER LAYER

↓

CENTRIFUGE 18,000 G

↓

SUPERNATANT

↓

ADD 6 VOL. OF COOLED ABSOLUTE ETHANOL.

↓

4°C OVERNIGHT

↓

CENTRIFUGE 1,500 G 20 MIN

↓

WASH PPT. WITH COOLED ABSOLUTE ETHANOL 2 TIMES

↓

ADD DIST. WATER 20 ML.

↓

PRE/W/ETOP

↓

G-25

↓

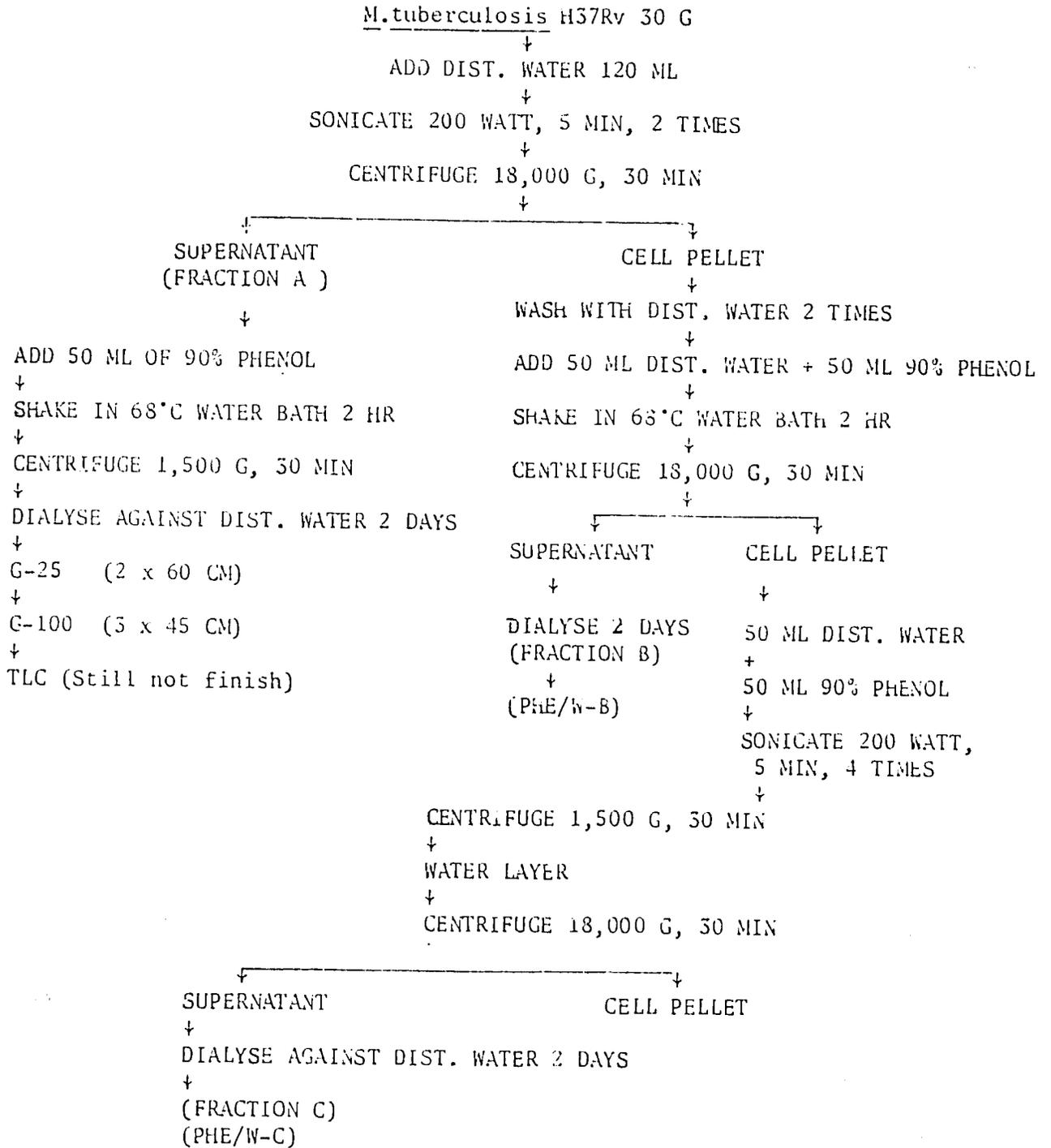
G-100

↓

TLC. (Still not finish)

EXPERIMENT NO.4

PURPOSE : LIPOFOLYSACCHARIDE EXTRACTION



EXPERIMENT NO.5

PURPOSE : POLYSACCHARIDE EXTRACTION

M.tuberculosis H37Rv 38 G

↓

MIXTURE OF 20 ML of 50% SULFOSALICYLIC ACID AND 60 ML of ETHER

↓

SONICATE 200 WATT, 3 MIN

↓

ADD 50 ML of 50% SULFOSALICYLIC ACID

↓

SONICATE 300 WATT, 2 MIN

↓

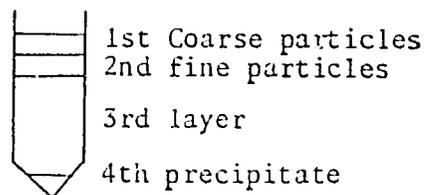
CENTRIFUGE 900 G, 45 MIN

↓

THIRD LAYER

↓

CENTRIFUGE 18,000 G, 30 MIN



SUPERNATANT

↓

EVAPORATE ETHER IN 30 DEGREE CELCIUS WATER BATH

↓

DIALYSE AGAINST DIST. WATER 2 DAYS

↓

CONCENTRATE BY DIALYSING TUBE (CUT OFF 8,000)

↓

CENTRIFUGE 900 G, 15 MIN

↓

SUPERNATANT

↓

POLYSACCHARIDE EXTRACT

↓

SE-I

EXPERIMENT NO.6

PURPOSE : POLYSACCHARIDE EXTRACTION

M.tuberculosis H37Rv 20 G

↓

ADD 30 ML OF 50% SALFOSALICYLIC ACID + 90 ML OF ETHER

↓

STIRRED IN ICE BATH 8 HR

↓

LET STAND 4°C FOR OVERNIGHT

↓

CENTRIFUGE 1,500 G, 30 MIN

↓

THIRD LAYER

↓

CENTRIFUGE 18,000 G, 30 MIN

↓

SUPERNATANT

↓

DIALYSE AGAINST DIST. WATER 2 DAYS

↓

CONCENTRATE TO 5 ML BY DIALYSING TUBE (CUT OFF 8,000)

↓

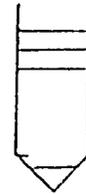
CENTRIFUGE 900 G, 15 MIN

↓

SUPERNATANT

↓

SE-II



1st Coarse Particles  
2nd fine particles

3rd layer

4th precipitate

ELISA ASSAY:

Purpose : Detection of antigenicity and specificity of polysaccharide and lipopolysaccharide crude extract

Method : Fractions of crude extracts were diluted to 1:10, 1:100 and 1:1000 in coating buffer (carbonate/bicarbonate buffer pH 9.6). Two hundreds microlitres of each diluted crude extract was added to each well of ELISA plate and incubated at 25°C for 3 hours. The washing solution consisted of PBS pH 7.2 containing 0.05% Tween 20, 0.02% sodium azide. All reagents were used in 180 microlitre volumes, except antigen coating and washing which used 200 and 400 microliter respectively. Each washing was done 3 times. The incubation times with serum and the rabbit anti-human norseraiish peroxidase conjugate were 1 hr each. The substrate stock solution consisted of ABTS (2,2'-Azinobis (3 ethylbenthiazoline sulfonic acid)) 16 mg/ml in acetate buffer pH 4.5. The substrate working solution was prepared fresh just before use by combining 19 ml. of acetate buffer, 1 ml of substrate stock and 7 microlitre of 50% hydrogen peroxide.



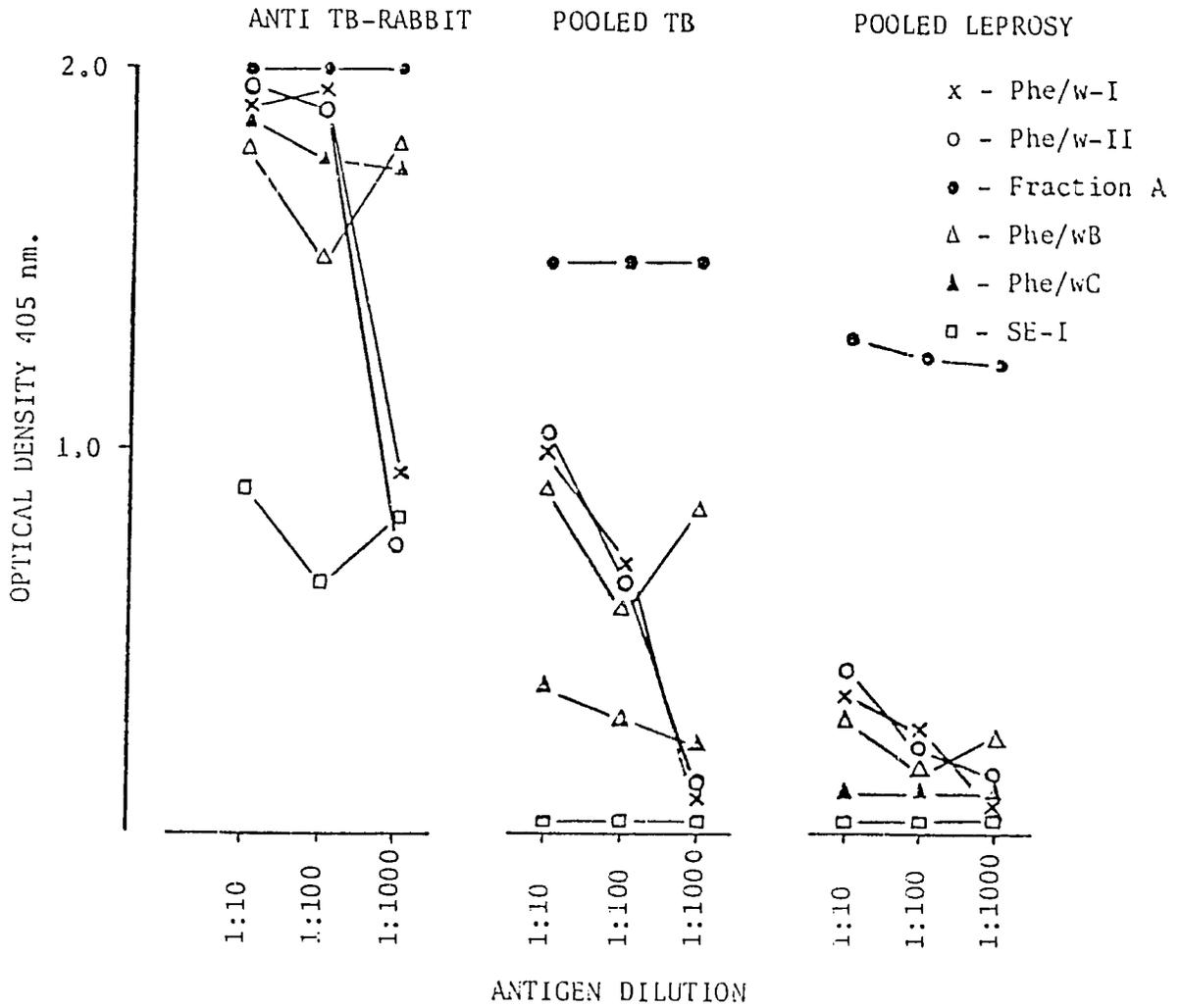


FIGURE 1. ELISA test for crude extrats by Phenol-water and sulpho-ether method react with anti-TB (rabbit), Pooled human TB serum, Pooled human leprosy serum.

EXPERIMENT No.7

Purpose : Test of antibodies preserved by filter paper

Method : One free drop from capillary tube (25 microlitre) of anti-TB blood (rabbit) was dropped on 1.0 x 1.5 cm. filter paper Whatman No.1. The filter paper was dried at room temperature, then kept in a small plastic bag in refrigerator.

Elution method

The filter paper containing blood was cut above blood spot marker, approximately 1 cm. from the end. The piece was put in a tube consisting of 1 ml. of PBS, 0.05% Tween 20 and 0.04%  $\text{NaN}_3$ . The tube was shaken in 56°C water bath for 1 hr. The supernatant was aspirated for ELISA test. Eluates were compared with the same amount of the blood (25 microlitre) added directly into 1.0 ml of the same diluent, then centrifuged and the supernatant fluid kept at -20°C until used.

Table 2. ELISA test for detection of anti TB antibody preserved by filter paper technique.

Dilution of test sample	ELISA VALUE (405 nm)				
	Blood Freeze at -20°C	Filter paper technique storage at 4°C for			
		3 days	10 days	17 days	24 days
1:100	0.999	0.909	0.854	0.896	0.854
1:200	0.487	0.457	0.409	0.397	0.386
1:400	0.236	0.199	0.181	0.169	0.198
1:800	0.142	0.095	0.106	0.119	0.111
1:1600	0.116	0.101	0.112	0.114	0.116
1:3200	0.065	0.053	0.047	0.045	0.056

ELISA CONDITION

Crude TB antigen dilution 1:400 in coating buffer

Coating time 3 hrs. 25°C

Washing solution PBS pH 7.2, 3 times

Incubation time 60 min.

Substrate 30% H<sub>2</sub>O<sub>2</sub> 7 ul/20 ml acetate buffer

Developing color time 15 min.

Serum dilute in PBS/Tween/NaN<sub>3</sub>

Conjugate : anti rabbit Igs dilution 1:10,000 in PBS/Tween

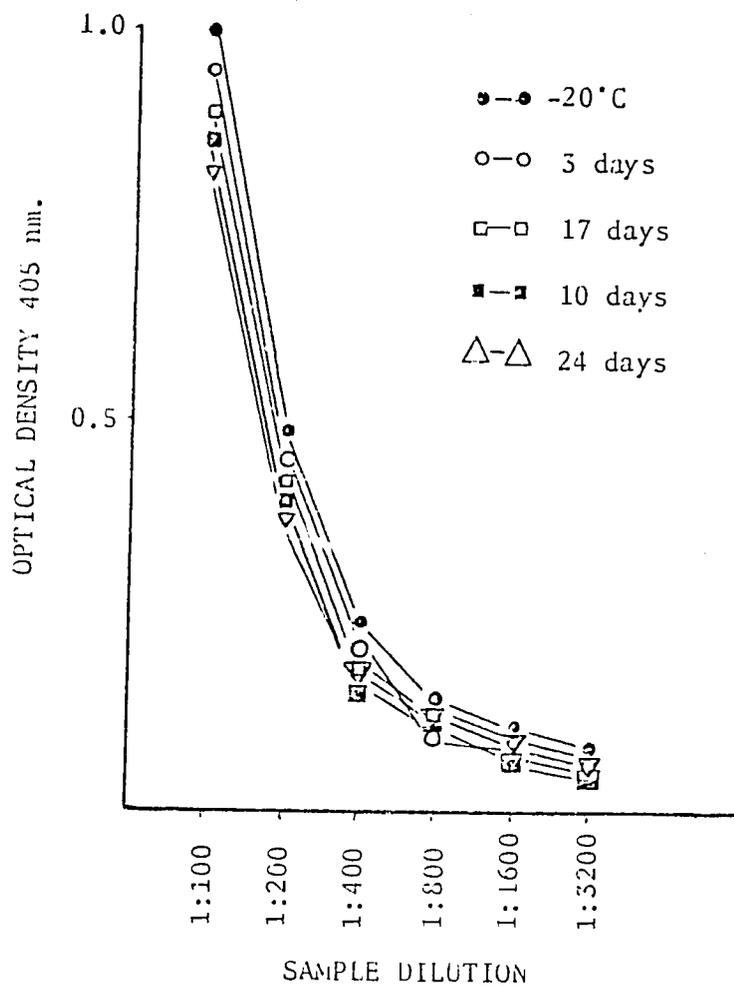


FIGURE 2. ELISA test for blood sample preserve by filter paper technique

Summary :

Lipopolysaccharide and polysaccharide were extracted from M. tuberculosis H37Rv by phenol-water and sulfo-ether methods. The crude extracts were further purified by gel-filtration chromatography (sephadex G-25, G-100) and thin layer chromatography. Most fractions from phenol-water extract possessed antigenicity by ELISA assay when tested with pooled human tuberculous serum and anti-TB from rabbit, whereas extract by sulfo-ether method showed loss of antigenicity.

The result of ELISA demonstrated that the antibodies to mycobacterial antigen are preserved quite well by filter paper technique through 24 days.

EXPERIMENT No.8

PURPOSE . Oligosaccharide Extraction and Purification

M.tuberculosis strain H37Rv cell, washed with water 3 times

↓

Autoclave

↓

5 G of cells

↓

50 ml 70% Ethanol

↓ stirring 70°C, 20 min.

Centrifuge 5,000 G 30 min

↓

↓

Supernatant I                      Cell wall pellet

↓

Reextraction

↓

← --- Supernatant II                      Cell wall pellet

↓

Extract with 50% ethanol 50 ml

↓

70°C, 20 min.

↓ 2 time

← --- Supernatant III                      Cell wall pellet  
(Discard)

Combine supernatant I, II and III  
concentrate to 100 ml at 37°C

↓

50 ml  $\text{CHCl}_3/\text{CH}_3\text{OH}$  (2:1) 1 hour

↓

Water layer                       $\text{CHCl}_3/\text{CH}_3\text{OH}$

↓

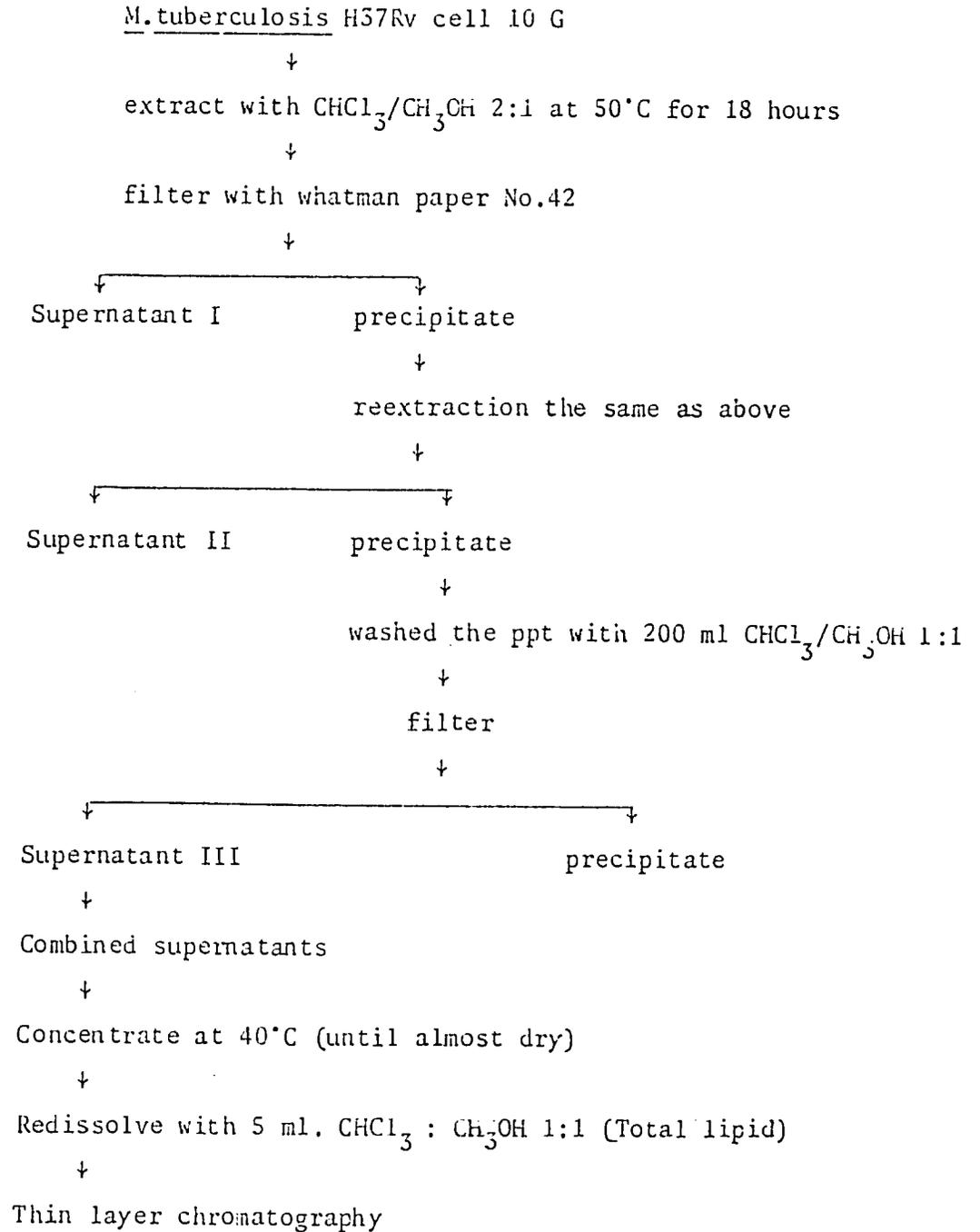
50 ml  $\text{CHCl}_3/\text{CH}_3\text{OH}$  (2:1) 1 hr.

↓

Water layer (wait for chromatography on Dowex)

EXPERIMENT No.9

PURPOSE : Glycopeptidolipids/Lipooligosaccharide Extraction and Purification



Thin layer Chromatography System for Total Lipid and Lipid fractions

Sorbent Silica gel type 60

Supporter glass plate 20 cm x 20 cm

Sorbent layer 300 u - 500 u

Developing solvent Chloroform : Methanol : Water 65 : 25:4

Colour Reagent 0.1% orcinol in 40%  $H_2SO_4$   
(Spray reagent)

Time of Colorized 110°C 20 min.

The result of thin layer chromatography of total lipid extraction by chloroform and Methanol (Expt. 9) was shown in figure 5, while table 3 showed the R<sub>f</sub> values of various spots of extract A

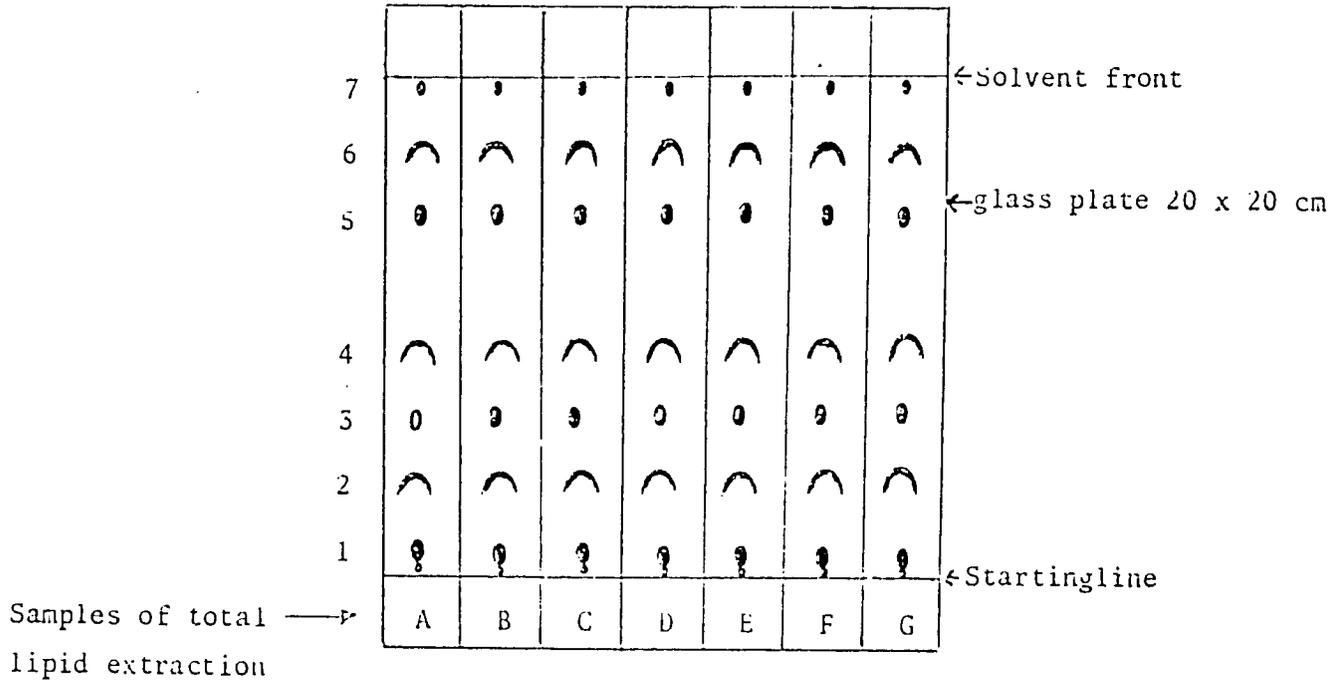


Fig. 3 TLC of total lipid in Chloroform : Methanol : water 65:25:4 and colourize with 0.1% orcinol in 40% H<sub>2</sub>SO<sub>4</sub>

Table 3. R<sub>f</sub> values of various spots of total lipid from plate area A

Spot No.	Solvent front (cm)	distance from starting line (cm)	R <sub>f</sub>
1	16.7	1.5	.09
2	16.7	2.5	.15
3	16.7	5.3	.32
4	16.7	7.4	.44
5	16.7	13.0	.78
6	16.7	14.1	.84
7	16.7	16.5	.98

Each spot from identical TLC plates was scraped off and extracted with chloroform : methanol : water 65:25:4. The extract solution was tested for antigenicity to anti-tuberculosis sera by ELISA test. The results and condition of ELISA are shown in table 4.

Table 4. ELISA test for specificity of TLC fraction of Total lipid from experiment 9.

spot from TLC	O.D.against Tuberculosis serum	O.D.against Leprosy serum
1	1.189	0.666
2	0.324	0.254
3	-0.219	-0.385
4	0.256	0.190
5	0.129	0.354
6	0.138	0.240
7	0.316	0.189

CONDITION OF ELISA

Antigen : fraction in 0.1% sodium deoxycholate (undilute)

Serum test : patient's TB serum, Leprosy serum, both diluted 1:100  
in 1% BSA/PBS

Conjugate : human Ig S-P dilution 1:5,000 in 1% BSA/PBS

Substrate : 16 mg ABTS in acetate buffer 20 ml/7 ul 30% H<sub>2</sub>O<sub>2</sub>

Coating time : overnight, 25°C

Incubate time : 1 hour

Developing time : 20 min.

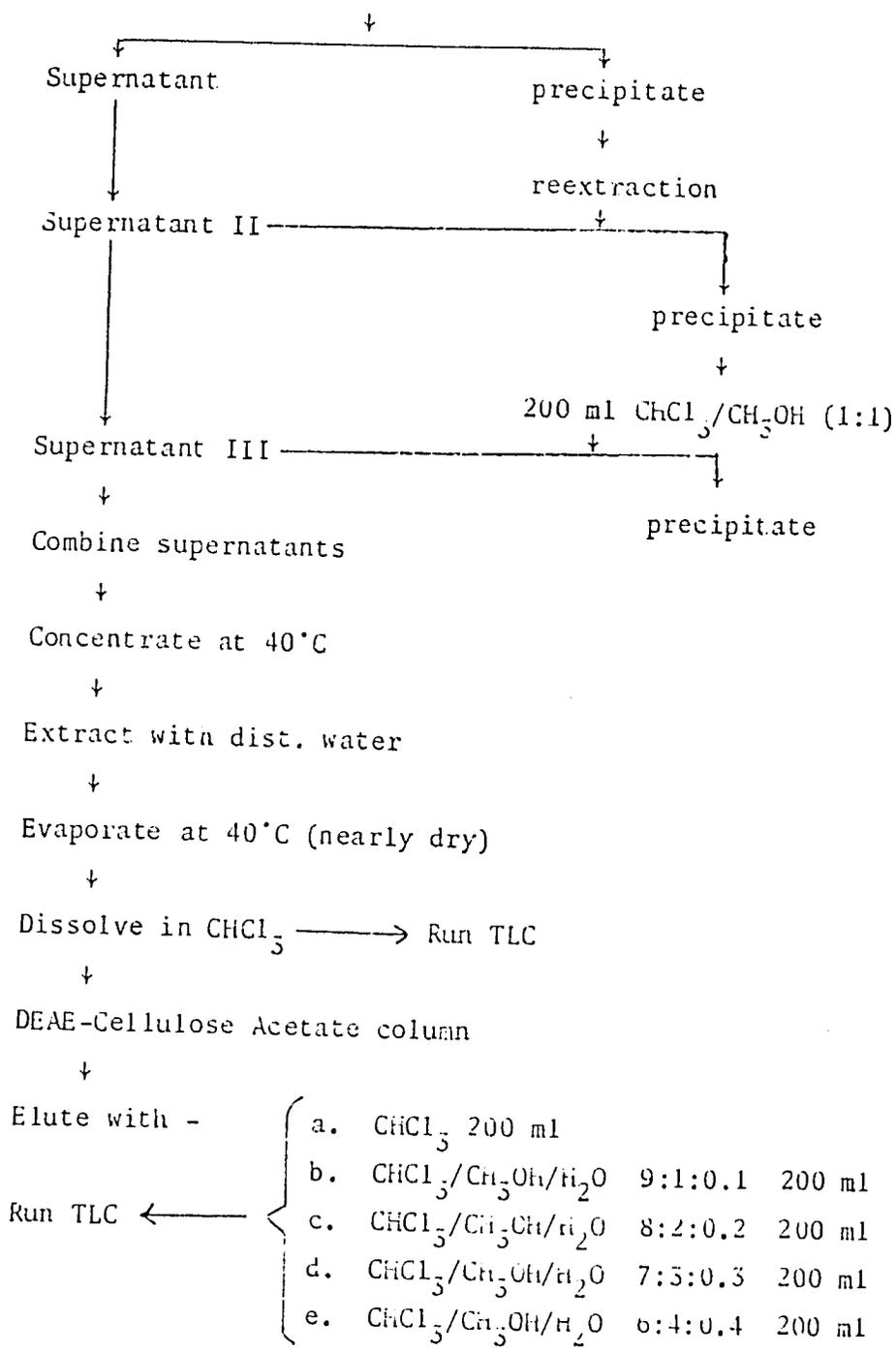
EXPERIMENT No.10

PURPOSE : Glycopeptidolipids/Lipooligosaccharide Extraction and Purification.

M.tuberculosis H37Rv cell 10 G

↓  
400 ml (CHCl<sub>3</sub>/CH<sub>3</sub>OH (2:1) 50°C 18 hours

↓  
filter with Whatman No.42



The result of thin layer chromatography of total lipid extraction by chloroform and methanol and water (Expt.10) were shown in fig.4 and table 5.

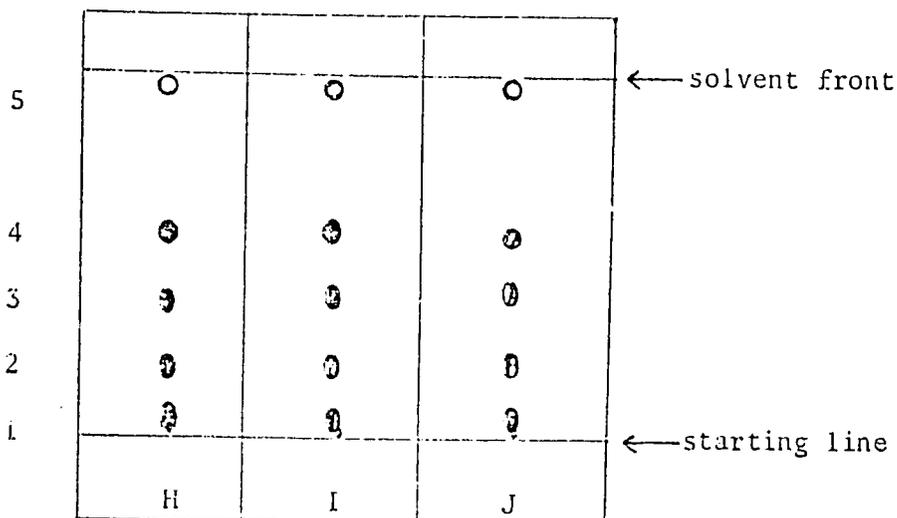


Fig 4. TLC of purify total lipid by  $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}$  65:25:4 and colorize with 0.1% orcinol in 40%  $\text{H}_2\text{SO}_4$

Table 5. Rf values of purify total lipid extraction

spot No.	Solvent front (cm)	Distance from starting line (cm)	Rf
1	16.5	0.5	.03
2	16.5	4.8	.29
3	16.5	6.5	.39
4	16.5	11.5	.70
5	16.5	16.2	.98

The results of ELISA test for specificity of TLC fraction of purify: total lipid from experiment 10. were shown in table 6.

Table 6. ELISA test for specificity of TLC fraction from total lipid.

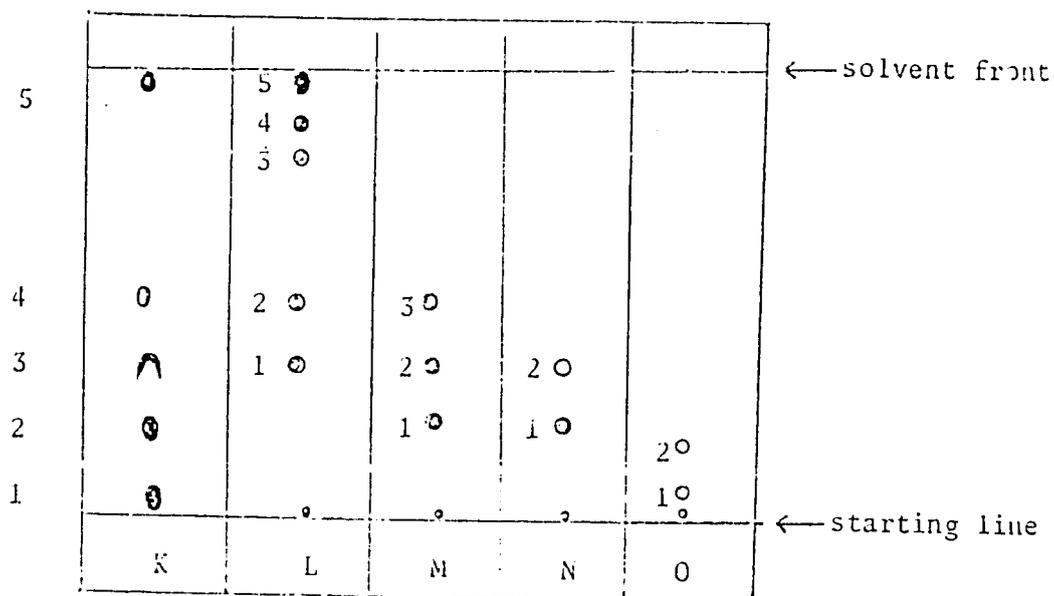
TLC fraction from purify total lipid	Tuberculosis serum	Leprosy serum
1	1.140	0.796
2	0.274	0.188
3	0.920	0.421
4	0.437	0.251
5	1.011	0.558

ELISA condition

Diluent for TLC fract <sup>n</sup>	= 0.1% sodium deoxyholate
Ag coating time	= overnight, 25°C
Washing solution	= PBS
Diluent for serum	= 1% BSA in PBS
Dilution of serum	= 1:100
Reaction time in serum step	= 1 hour
Diluent for conjugate	= 1% BSA in PBS
Dilution of anti human Ig-P	= 1:5,000
Reaction time in conjugate step	= 1 hour
Chromogen/substrate	= 16 mg (ABTS) + 7 ul 30% H <sub>2</sub> O <sub>2</sub> in 20 ml acetate buffer pH 4.5
Color developing time	= 20 min

The Eluents of purified lipid, after passing through a DEAE cellulose acetate column, were collected and each stepwise fraction concentrated and applied on TLC. The result of each stepwise eluent in TLC are shown in figure 5. Table 7 showed Rf values of each spot from various eluents.

Fig.5 the TLC of each eluent of purified lipid from DEAE cellulose acetate column.



- K = purify lipid
- L = eluent of purify lipid from  $\text{CHCl}_3 : \text{CH}_3\text{OH} : \text{H}_2\text{O}$  9:1:0.1
- M = eluent of purify lipid from  $\text{CHCl}_3 : \text{CH}_3\text{OH} : \text{H}_2\text{O}$  8:2:0.2
- N = eluent of purify lipid from  $\text{CHCl}_3 : \text{CH}_3\text{OH} : \text{H}_2\text{O}$  7:3:0.3
- O = eluent of purify lipid from  $\text{CHCl}_3 : \text{CH}_3\text{OH} : \text{H}_2\text{O}$  6:4:0.4

Table 7. Rf values of various spots on TLC

	1	2	3	4	5	solvent front
K	.04	.28	.38	.40	.93	16.0
L	.37	.44	.76	.79	.99	15.7
M	.28	.38	.45			15.5
N	.26	.34				15.5
O	.04	.26				15.5

Each spot from TLC was scraped off then extracted with  $\text{CHCl}_3$  :  $\text{CH}_3\text{OH}$  :  $\text{H}_2\text{O}$  = 15:25:4. The extracted solution was concentrated and tested for antigenic specificity to M. tuberculosis by ELISA method.

The results of ELISA test for each spot against T.B. antiserum and Leprosy antiserum gave very low O.D. values, therefore, all were not suitable for being used as antigens or haptens.



The results of TCA precipitation of cell wall polysaccharides from M. tuberculosis and then passing through sephadex G 75/25 column are shown in figure 6 and table 8.

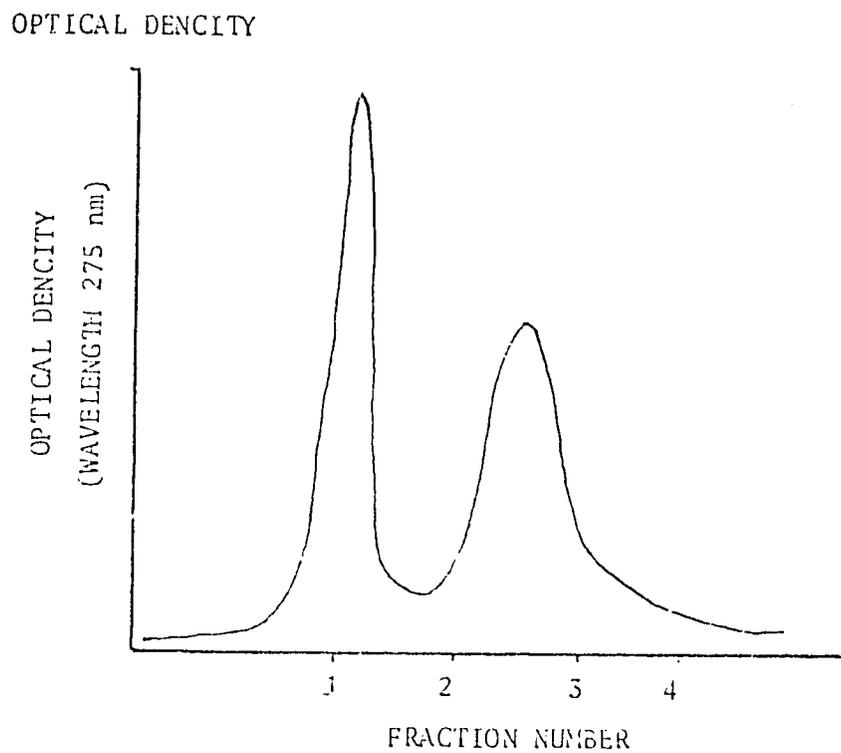


Figure 6. Separation of cell wall polysaccharide by trichloroacetic acid and passing through sephadex G 75/25 column.

Table 8. ELISA test of fractions from M. tuberculosis extracted by trichloroacetic acid and passed through sephadex G 75/25 Column.

Fraction No.	Antigen control	human T.B. serum	human leprosy serum	rabbit anti T.B. serum
1	0.044	0.150	0.166	0.154
2	0.068	0.245	0.310	0.648
3	0.059	0.555	0.410	0.945
4	0.066	0.128	0.156	0.501
5	0.068	0.137	0.168	0.475
6	0.057	0.169	0.180	0.297
7	0.064	0.175	0.192	0.250
8	0.069	0.140	0.147	0.205

Antigen : fraction from G 75/25 - undilute

Serum test : pooled T.B. serum, leprosy serum, rabbit anti T.B. serum dilution 1:6,400 in PBS/Tween/ $\text{NaN}_3$

Conjugate : human Igs-P, rabbit Ig-P dilution 1:5,000 in PBS/Tween

Substrate : ABTS in acetate buffer 20 ml + 7 ul 50%  $\text{H}_2\text{O}_2$

Coating time : overnight

Incubation time : 1 hr.

Developing color time : 30 min.

PART II. ISOLATION AND IDENTIFICATION OF *M.tuberculosis* AND PRODUCTION OF  
HYPERIMMUNE SERA TO VARIOUS SPECIES OF MYCOBACTERIA.

1. Collection of Specimen

Two hundred blood samples were collected from TB patients whose sputum smear was positive for acid-fast bacilli at the Tuberculosis Center Region 10, Chiang Mai, Thailand. Ten millilitres of blood were collected by veinipuncture. Serum was separated and kept in aliquots at -20°C and -70°C until used for antibody assay. Additionally, small volumes of blood sample were collected by filter paper technique. Small portions of blood collected via veinipuncture were filled into 2 heparinized microhematocrit tubes and one free drop of blood was absorbed on one end of 1 x 4 cm, using Whatman No.1 filter paper strip. After drying at room temperature for 1 hour, 4 absorbed filter paper strips from one blood sample were kept in plastic bag at 4°C

2. Cultivation and Identification of *M.tuberculosis*.

Sputum was collected from TB patients at Tuberculosis Center Region 10. Bacilli in sputum were first examined in smear stained by Ziehl-Neelsen technique and Auramine O staining technique. Sputum was treated by 5% oxalic acid for 25 min. for decontamination and inoculated triplicate on Lowenstein-Jensen slant. Cultures were incubated at 37°C and observed for growth at weeks 1 and 4. Cultures showing typical colonies and no sign of contamination were subcultured on Lowenstein-Jensen slant and Lowenstein-Jensen deep for further biochemical identification. After 2 weeks of incubation, cultures which showed maximum growth were tested for Niacin accumulation, Nitrate reduction and semiquantitative and heat-stable Catalase production. Cultures showing confused biochemical reactions were subcultured and tested once again.

One hundred and fifty two cultures were done for identification of M.tuberculosis. Fourteen cultures were contaminated during subculture. Forty eight cultures were completely identified and all are M.tuberculosis. This group of cultures showed positive reaction with Niacin accumulation and Nitrate reduction test. Semiquantitative catalase test was less than 45 min. and heat-stable catalase test (pH 7, 68°C) were negative. The remaining ninety cultures also showed positive reaction for Niacin accumulation and Nitrate reduction. Test for heat-stable catalase was negative and semiquantitative catalase test is being done.

### 3. Mass Culture for Antigen Preparation

Mycobacterium tuberculosis H37Rv and 18 atypical mycobacteria were cultured for mass antigen preparation. Mass culture of only M.tuberculosis H37Rv was used for species specific antigen isolation and purification. The bacilli were cultured at 37°C for 2-6 weeks in Sauton's liquid media. Cultures were done in 500 ml culture flask that contained approximately 250 ml culture media. Cultures showing maximal growth were autoclaved and harvested by centrifugation at 8,000 G for 30 min. One culture flask gave approximately 2-4 gm of bacilli (Wet weight). The bacilli were washed 2 times with normal saline solution (NSS) and resuspended in NSS to make a suspension containing 60 mg bacilli/ml (Wet weight). This suspension was sonicated for 5 minutes, 4 times in an ice bath by using a Braun-Sonic 1510 with an effect of 100 W. The sonicated material was divided into 2 portions; one was mixed with an equal volume of incomplete Freund's adjuvant and stored at -20°C until used for immunization. Another portion was centrifuged at 8,000 G for 30 min and supernatant was collected and kept at -20°C until used for ELISA assay.

Eighteen atypical mycobacterial antigens of the following bacilli were prepared :

<u>M. phlei</u> TMC # 1548	<u>M. chelonae</u>
<u>M. fortuitum</u> TMC # 1529, ATCC # 6841	<u>M. triviale</u>
<u>M. smegmatic</u> TMC # 1546	<u>M. scrofulaceum</u>
<u>M. avium</u> serotype 2, # 724 (NIH)	<u>M. vaccae</u> TMC # 1526
<u>M. avium</u> TMC # 706	<u>M. intracellulare</u> TMC # 1403
<u>M. gordonae</u> TMC # 1324	<u>M. bovis</u> (BCG)
<u>M. marinum</u> TMC # 1218	<u>M. bovis</u> (BCG) TMC # 1030
<u>M. kansasii</u> TMC # 1204	<u>M. gastri</u> NIHJ # 1616, ATCC # 15754
<u>M. terrae</u>	<u>M. nonchromogenicum</u> NIHJ # 1622, ATCC # 15930

#### 4. Production of Antisera

Antisera against mycobacterial antigens were produced in rabbits. Ten antigens were first used for immunization including M. gordonae, M. smegmatis, M. phlei, M. marinum, M. bovis (BCG) TMC # 1030, M. avium TMC # 706, M. kansasii, M. terrae, M. triviale and M. intracellulare. New Zealand white rabbits were used for immunization with each antigen emulsion. The dose was 0.5 ml in each hind leg given as a single deep intramuscular injection. Rabbits were further immunized with 0.1 ml of antigen emulsion given intracutaneous injection in the neck region every 14 days, i.e., on days 14, 28, 42 and at monthly intervals thereafter. Bleeding was done every 7 days, i.e. on days 0, 7, 14, 21 and so forth. Sera were separated and stored at -20°C for further antibody assay.

Antibodies in immunized sera were assayed by two techniques, indirect ELISA and immunodiffusion in two dimension (Ouchterlony). In ELISA assay, Nunc-Immuno Plates were coated with 200 ul of a 1/5200 dilution of mycobacterial antigens. After overnight incubation at 4°C, excess antigens were

washed out. A 180 ul of 1/640, 1/1280, 1/2560 and 1/5120 dilution of rabbit sera collected on each bleeding and a 1/10000 dilution of peroxidase-conjugated anti-rabbit Igs (Cappel) were added, incubated at room temperature for 1 hr. and washed out respectively. ABTS/H<sub>2</sub>O<sub>2</sub> used as chromoger/substrate was added to react for 50 min at room temperature. Absorbance at 405 nm was read in an ELISA reader (Titertek Multiskan)

Figures 7 to 16 showed the antibody response to immunized mycobacterial antigens detected by ELISA technique. Antibody against immunized mycobacterial antigens could be detected in sera bleeding on days 14, 21 and 28. However, the antibody response to M.terrae and M.intracellulare antigens was relatively lower than other antigens.

In Ouchterlony technique, antibody response to only five mycobacterial antigens including M.gordonae, M.marinum, M.bovis (BCG), M.kansasii and M.phlei could be detected in sera bleeding on days 42 and 49. The sera showed antibody titers between undiluted to 1/4 dilution. The Ouchterlony technique is still used to monitor antibody titer in all sera bleeding on the days thereafter. Full bleeding will be done when antibody titer is 1/4 to 1/8. 3

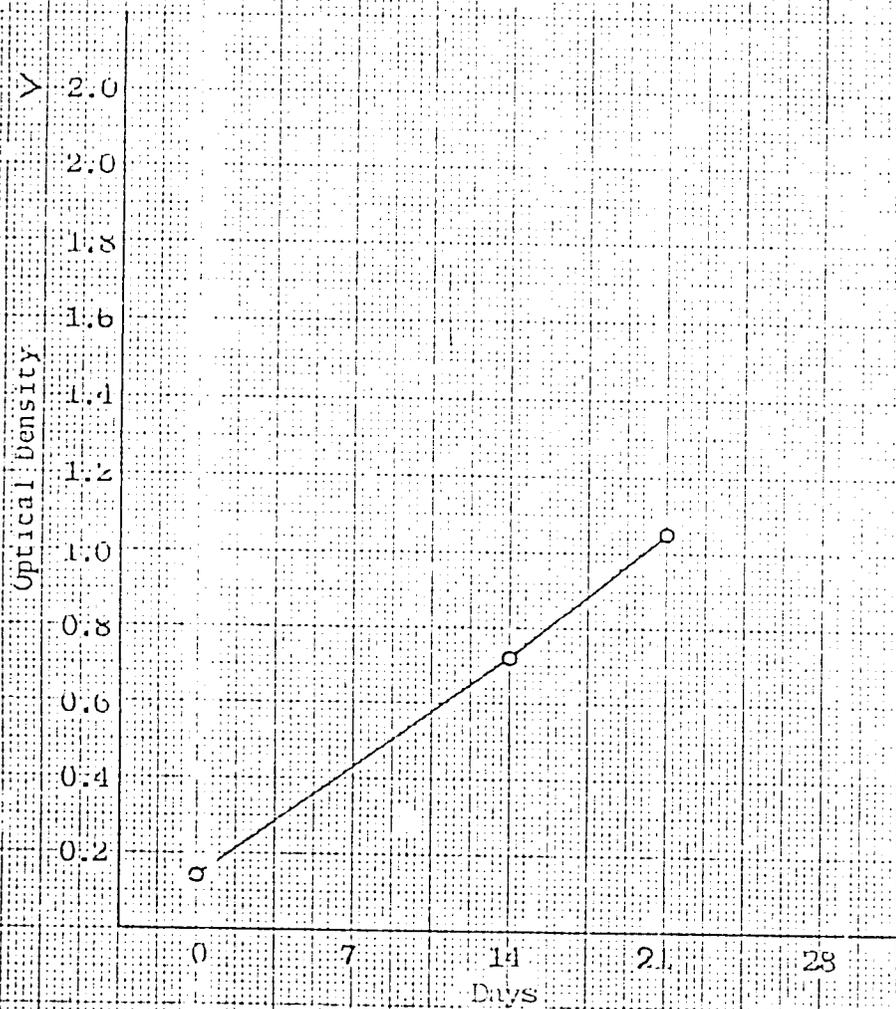


Fig.7 Rabbit antibody response to M. gordonae by ELISA assay at the serum dilution 1:640 and the corresponding antigen 1:3200

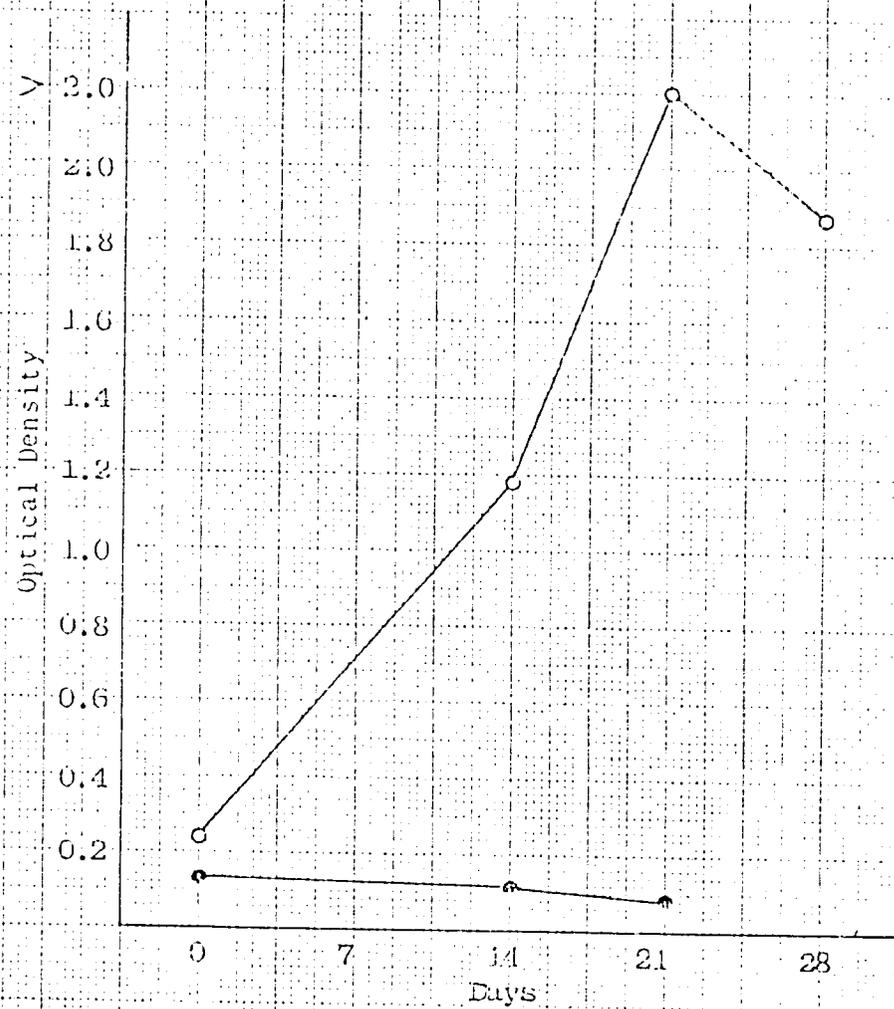


Fig.8 rabbit antibody response to M. smegmatis by ELISA assay at the serum dilution 1:640 and the corresponding antigen 1:3200.

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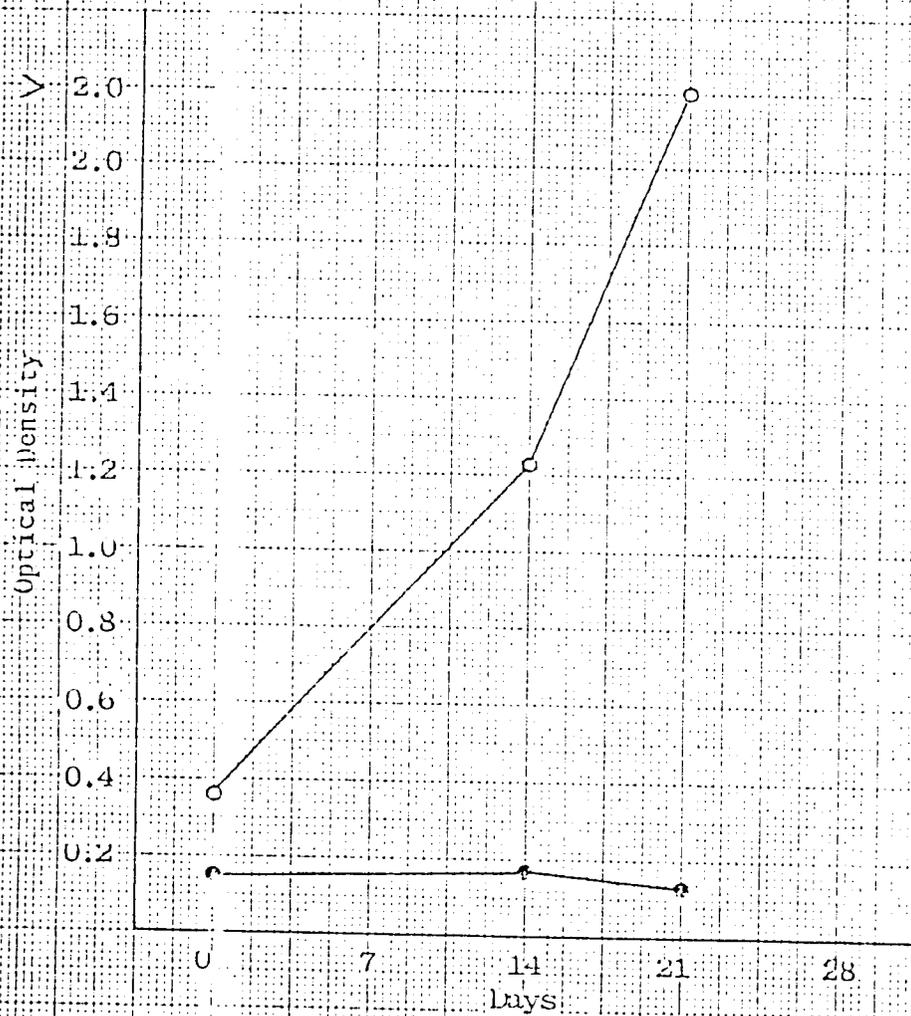


Fig.9 Rabbit antibody response to M.phlei by ELISA assay at the serum dilution 1:640 and the corresponding antigen 1:5200

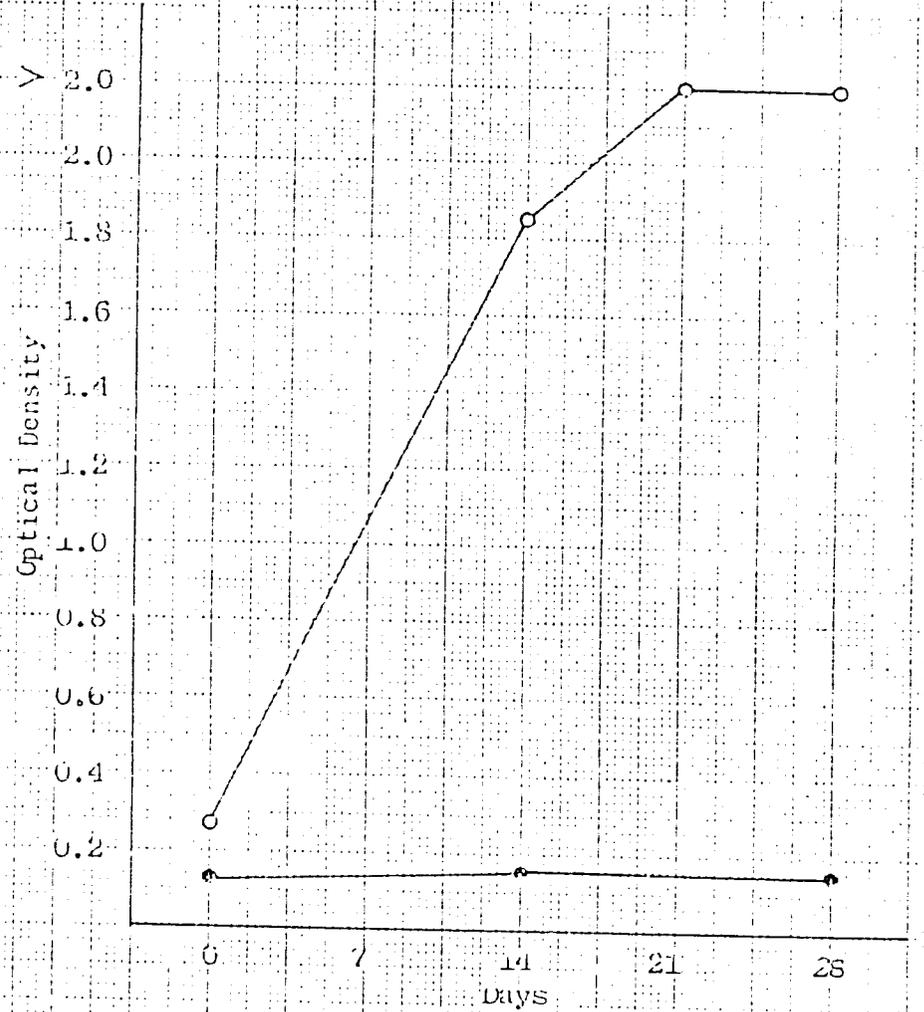


Fig.10 Rabbit antibody response to M.marinum by ELISA assay at the serum dilution 1:640 and the corresponding antigen 1:5200 .

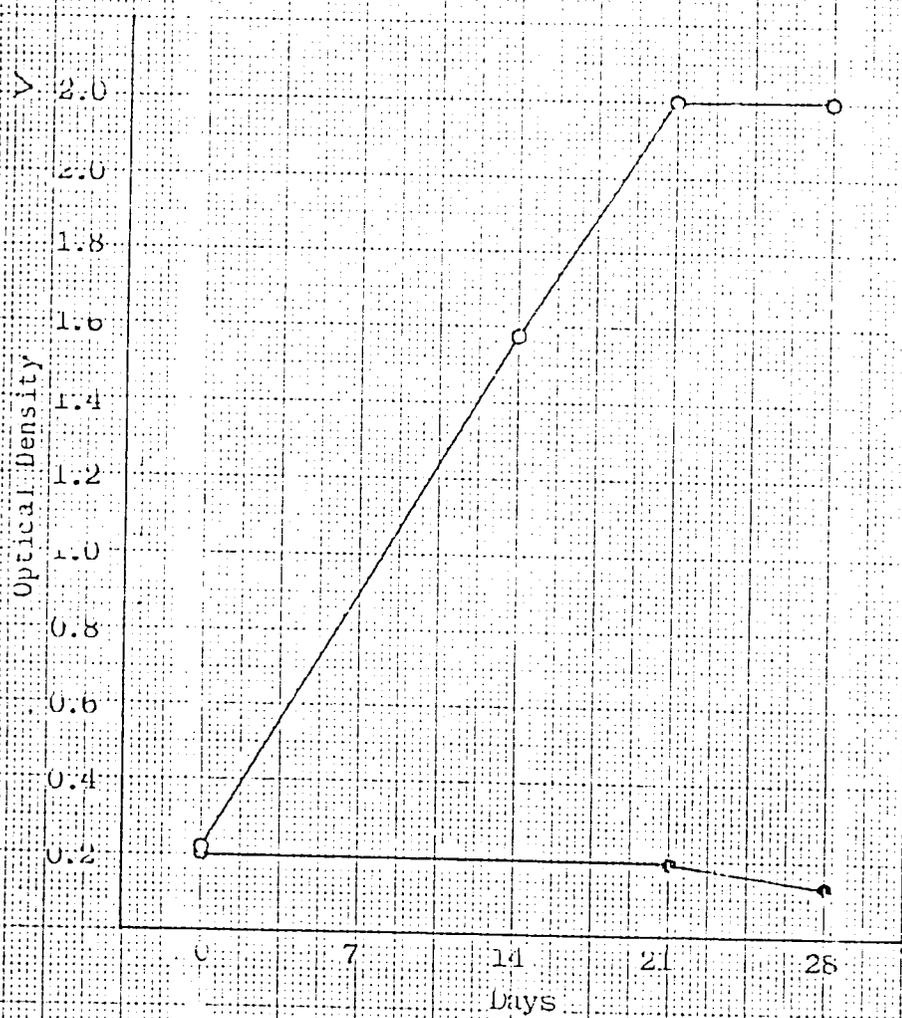


Fig. 11 Rabbit antibody response to *M. bovis* (BGG) by ELISA assay at the serum dilution 1:640 and the corresponding antigen 1:3200

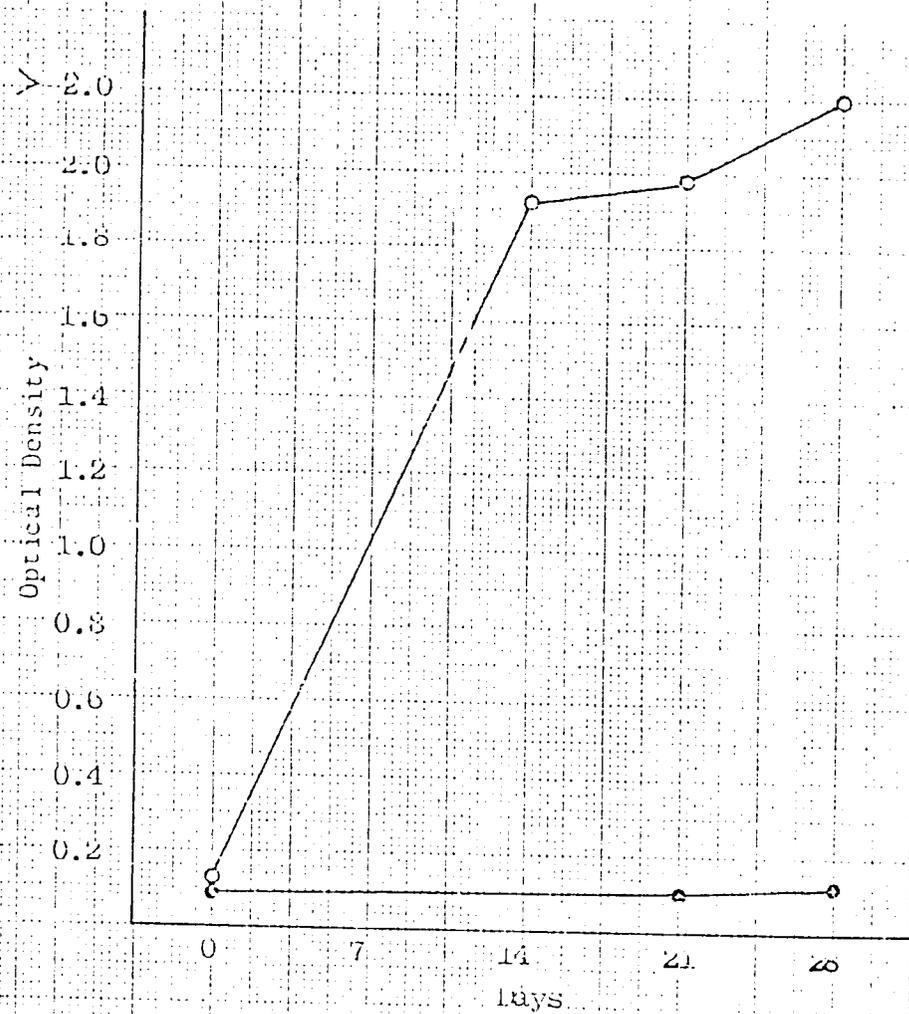


Fig. 12 Rabbit antibody response to *M. avium* by ELISA assay at the serum dilution 1:640 and the corresponding antigen 1:3200

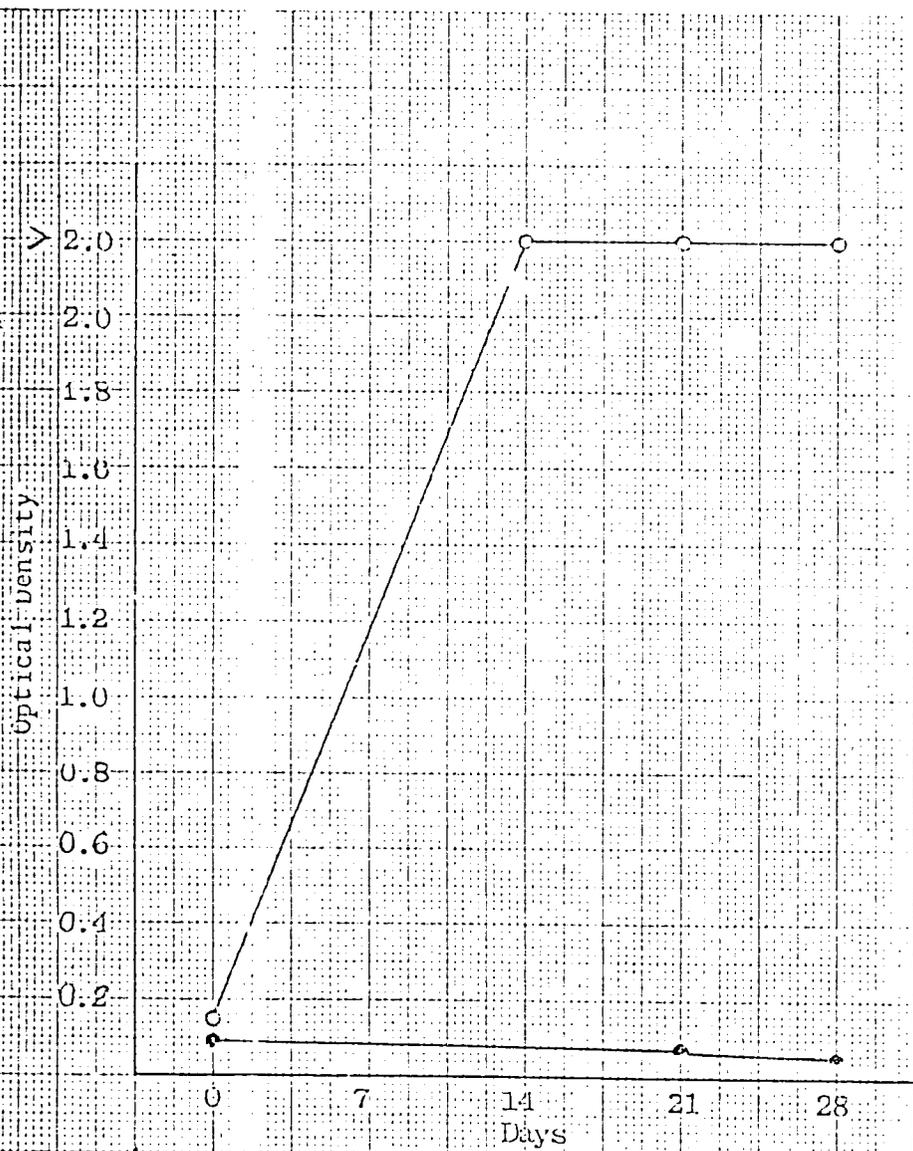


Fig.15 Rabbit antibody response to M.kansasii by ELISA assay at the serum dilution 1:640 and the corresponding antigen 1:3200

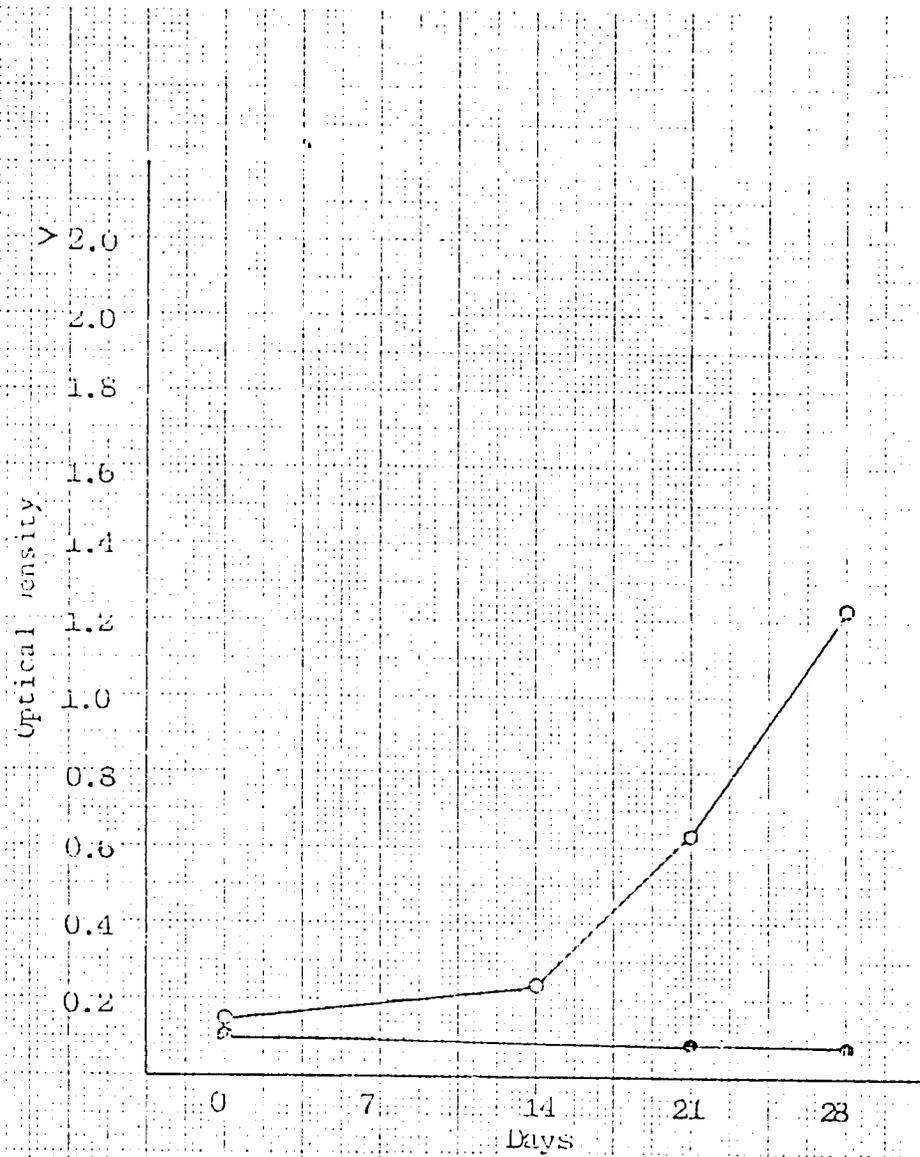


Fig.14 Rabbit antibody response to M.terrae by ELISA assay at the serum dilution 1:640 and the corresponding antigen 1:3200

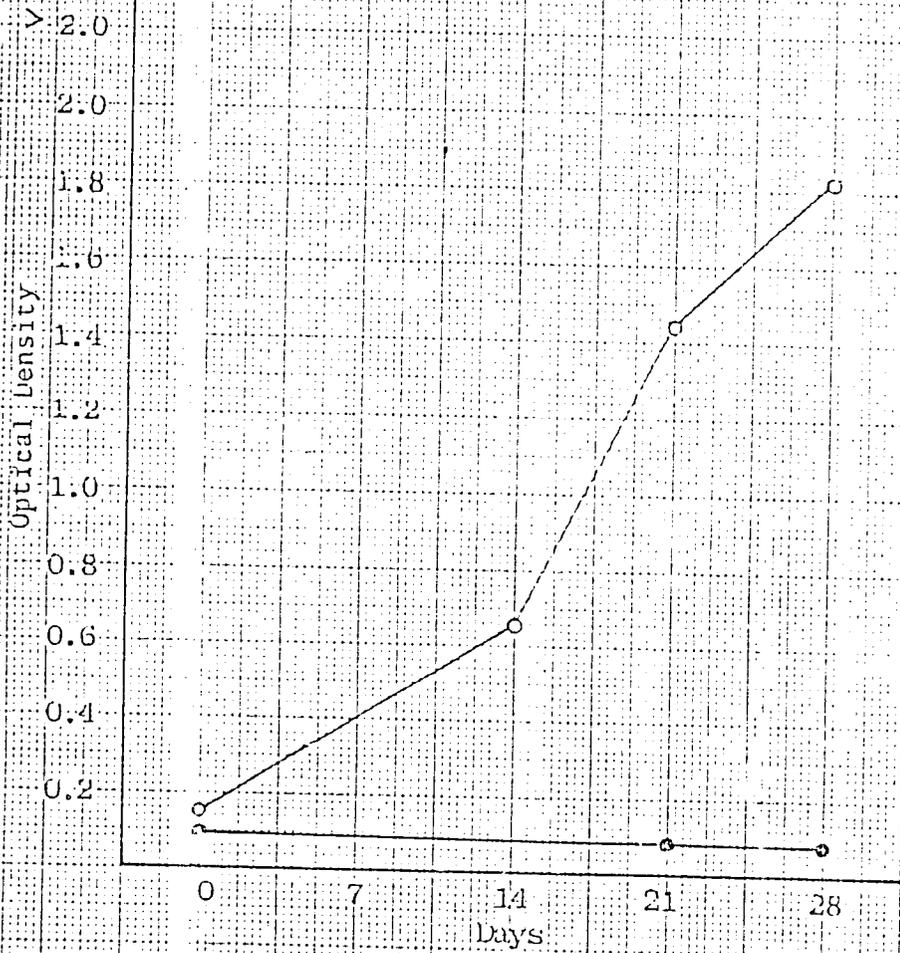


Fig.15 Rabbit antibody response to M.triviale by ELISA assay at the serum dilution 1:640 and the corresponding antigen 1:5200.

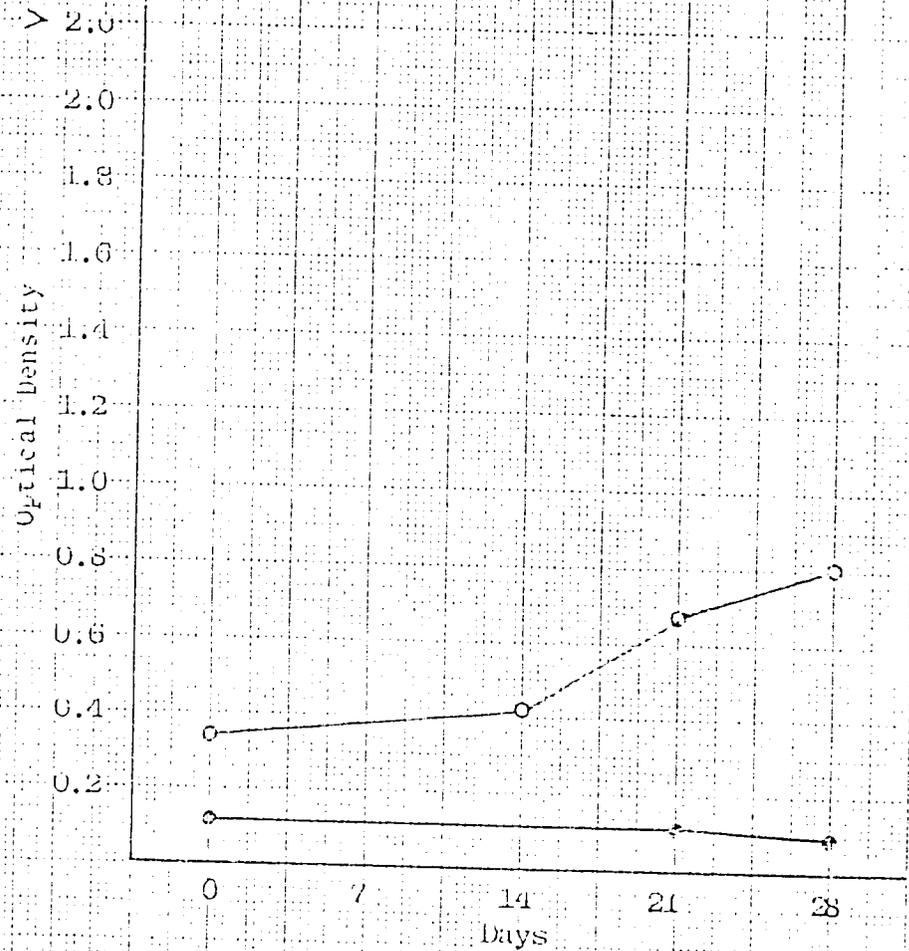


Fig.16 Rabbit antibody response to M.intracellulare by ELISA assay at the serum dilution 1:640 and the corresponding antigen 1:5200.