

**STANDARDISATION OF ENZYME-LINKED
IMMUNOSORBENT ASSAY (ELISA) FOR THE DIAGNOSIS OF
TUBERCULOUS MENTNGITIS (TBM)**

**DISSERTATION SUBMITTED FOR THE DEGREE OF
DOCTOR OF MEDICINE IN MICROBIOLOGY**

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INTRODUCTION

Tuberculosis is a disease known to mankind at least for the last 6,000 years (1). Some Egyptian mummies showed a deformity of the spine which in all probability is Pott's disease or tuberculosis of the vertebrae. This is probably the earliest evidence of the disease (1). Over subsequent centuries, there are descriptions in old Hindu literature, descriptions by Hippocrates and others. The disease was nearly always fatal in ancient times. The aetiopathology of the disease was gradually unveiled in the late nineteenth century. The presence of tubercles was demonstrated by Laennec and Bayle (2). The specific agent causing tuberculosis was isolated by Robert Koch in 1882. However, as recently as the late nineteenth and first half of twentieth centuries there was still no definitive treatment available. Isolation of the patient and good nursing care was the only regimen available, apart from the surgical treatments like artificial pneumothorax and thoracoplasty (2). In 1944, Schatz, Bugie and Waksman published their landmark paper on the bacteriostatic effect of streptomycin on mycobacteria that revolutionized the treatment of tuberculosis. In 1949 streptomycin came into general use. Other drugs

like p-amino salicylic acid and isonicotinic acid hydrazide became available as antituberculous drugs in the decade that followed. With this, the epidemiology of tuberculosis changed rapidly. In Western countries the incidence of tuberculosis started falling with each successive year (3).

As a definitive treatment for tuberculosis became available other problems gained prominence. Tuberculosis is essentially a chronic disease. In most cases it takes weeks to months for the disease process to manifest itself clinically. Even then, patients may present with vague symptoms like loss of appetite and lethargy. Such vague presentations lead to prolonged decrease in work efficiency and spread of infection before the diagnosis is made. Both consequences have grave socioeconomic implications. There is, therefore, a need for early, definitive diagnosis and institution of the proper regimen.

In chronic cases of pulmonary tuberculosis or tuberculous lymphadenitis there are certain fairly satisfactory methods for definitive diagnosis like X-rays or biopsy of the involved material. Usually such cases are not emergency cases, so that a biopsy report obtained

after a few days does not cause grave harm to the health of the patient. But there are certain forms of the disease which present as emergencies. Usually these are complications of the tuberculous disease process, e.g. severe hemoptysis, intestinal obstruction and intestinal perforation. These emergencies can be treated along general principles of management of such cases with subsequent institution of antituberculous therapy. But there are certain other emergencies in which the tuberculous lesion itself constitutes the actual emergency. These conditions are tuberculous meningitis and miliary tuberculosis involving the central nervous system. In these cases prompt institution of specific treatment is mandatory for survival since tuberculous meningitis is invariably fatal when untreated (4).

The treatment of any form of tuberculosis usually extends for a minimum period of one year. In several cases it may extend for even longer periods. Because of the long duration and a long course of injectable drugs, there is the problem of toxicity and cost of therapy. In view of these antituberculous therapy is not to be decided lightly upon, unless supported by very definite diagnostic evidence.

The incidence of tuberculous meningitis is very high in children and young adults. The paediatric age group is the most vulnerable age group for this condition. Affected children have a grave prognosis. If the treatment is not started early in the course of the disease, morbidity and mortality figures reach very high levels.

Therefore, early definitive diagnosis attains paramount importance. However, the commoner forms of meningitis or meningoencephalitis namely tubercular, pyogenic and viral are very often difficult to distinguish both clinically and by routine examination of the cerebrospinal fluid (CSF). The definitive diagnosis can only be made if tubercle bacilli are demonstrated in the CSF. The rate of finding such bacilli in smears of CSF concentrate is less than 50% in all the reported data (5). In routine practice, the positivity of the smear is much less than 30% (6). The other alternative of isolating and identifying mycobacteria by culturing is no great help in an emergency, as it takes at least 4-6 weeks for tubercle bacilli to grow on a culture medium. Culture positivity in cases of tuberculous meningitis is also quite low (7).

Taking into consideration this problem of early diagnosis of one form of meningitis from the other, the use of a newer technique to detect the presence of mycobacteria in the CSF of a case of meningitis was mooted. Enzyme - linked immunosorbent assay (ELISA) is used to detect nanogram quantities of many different antigens, and it was thought that mycobacterial antigens in the CSF should be amenable to detection by the technique. This technique will give results within 24 hours, and should, therefore, prove to be more useful than many other tests devised to permit a definitive distinction to be made between tuberculous and nontuberculous meningitis.

REVIEW OF LITERATURE

Tuberculosis has a worldwide distribution. At one time it was a disease with very high morbidity and mortality throughout the world. With the advent of chemotherapeutic measures and improved sanitation conditions the morbidity and mortality figures have dropped significantly in the developed countries. At the turn of the century, the mortality figures in most countries were very high, e.g., in 1901 the United Kingdom had a mortality rate of 126 per 1,00,000 population, Germany and Norway 192 and the United States of America 169 (8). These figures have come down very drastically. In 1958 mortality rates in these four countries were 9, 17, 8 and 7 respectively (8). Along with a decrease in mortality rates the prevalence of infection as indicated by tuberculin sensitivity has also fallen to a level of 1-2% at the age of 14 years (9). In countries like India the prevalence rates for infection are very high. At the age of 14 the rate is about 29% whereas at the age of 35 it crosses 60% (10). For this survey only people without BCG scar were taken into consideration. Most developing

and underdeveloped countries do not have accurate data regarding prevalence and incidence rates of tuberculosis. In India the first large - scale organized tuberculin testing was started in 1948 as a prelude to the introduction of BCG vaccination in the country. A national sample survey was carried out in 1955-57 (11). The information collected then was the first authentic report of morbidity status in India. Some salient features of this report were as follows -

- 1) Prevalence rate of active and probably active tuberculosis varied from 13 to 25 per 1,000 population aged 5 years and above.
- 2) The rate of bacteriologically positive cases varied between 2 to 8 per 1,000 population.
- 3) Prevalence in the villages was not appreciably less than in the cities.
- 4) Prevalence rates were on the whole lower for females than males, especially in the age group over 35 years.
- 5) Prevalence rate in men showed continuous increase with age.
- 6) In overcrowded slums with low income group population as residents, the prevalence was very high.

The morbidity picture in India compares poorly with Western countries. The morbidity picture that we have today is similar to what Western countries had 50 years ago. With improved measures for diagnosis and treatment of tuberculosis and improved sanitation measures, Western countries have managed to improve their morbidity figures. Taking this into account, the Government of India set up the National Tuberculosis Institute (NTI) at Bangalore. NTI was to propose a comprehensive, realistic and economically feasible tuberculosis control programme for the entire country. The district tuberculosis programme was evolved by NTI in 1962 (12). Pulmonary tuberculosis being the commonest form of the disease as well as an infectious one, measures were to be taken to control its dissemination in the community as early as possible. Diagnostic facilities were installed at various places and drugs were supplied to every case of confirmed tuberculosis through the district tuberculosis programme. All these attempts were aimed at detecting infectious cases early in the course of the disease.

Tuberculosis exists in many different forms. The commonest form of the disease is pulmonary tuberculosis.

Tubercular lymphadenitis is probably the next most common condition. Other comparatively less common forms include abdominal tuberculosis, skeletal tuberculosis, miliary tuberculosis, tuberculous meningitis (TBM) and urogenital tuberculosis.

A patient of tuberculosis usually presents with constitutional symptoms and specific symptoms. The constitutional symptoms are lassitude, loss of weight and fever. The specific symptoms usually depend upon the site of infection, e.g., cough is the commonest symptom in pulmonary tuberculosis. Pain in the chest might be associated with pleurisy. Amenorrhoea may be a presenting feature in genital tuberculosis in a young woman. Vague pain in the back may be because of affected vertebrae.

In the course of the disease the central nervous system is affected in one or more ways. The bacilli reach the brain and meninges commonly via the blood stream. There might be invasion of meninges alone to give rise to TBM. Basal meningitis is seen commonly in such cases. One or more tuberculomas might be formed. The incidence of affection of the brain and the meninges is much higher in children and young adults. About 80% of all cases of TBM

are below 20 years, though no age is exempt (13). The official mortality statistics of India for the last decade show that tuberculosis accounts for about 10% of all the reported deaths. Out of this about 15% are due to tuberculosis of the nervous system. About 60% of the deaths from tuberculosis of the nervous system are of an age less than 15 years. Males are affected 1.5 times more often than females (14, 15).

Clinical features of tuberculous meningitis

The patient may present in any one of the following common forms (16):

- 1) Acute tuberculous meningitis - Along with symptoms of meningeal irritation like restlessness and irritability with headache, symptoms of a more general nature like loss of appetite, fever and unexplained loss of weight are present. Signs of meningeal irritation like neck rigidity, Kernig's sign, Brudzinski's sign are present. Deep reflexes are exaggerated. Lateral rectus palsy might be apparent. With further progression of the disease, clouding of consciousness sets in. Signs of increased intracranial tension are seen.

- 2) Tuberculous arachnoiditis - The characteristic feature is an insidious onset of multiple cranial nerve palsies. Third, 4th and 6th cranial nerves are affected early. Signs of meningeal irritation or increased intracranial tension might be absent. In the later stages of the disease hydrocephalus and increased intracranial tension may result.
- 3) Tuberculous meningoencephalitis - Headache of an insidious onset, effortless vomiting, visual disturbances or seizures simulate a case of any space occupying lesion. Often fever and pain in the neck are not associated with it. Clinical examination may show bilateral papilloedema. Localising signs may be present. With the progression of the disease an acute meningitis with further neurological deficit may set in.
- 4) Mixed variety - Clinical picture may not fit in any particular form. An overlap of signs and symptoms may be present. Meningeal irritation, cranial nerve deficits, increased intracranial tension and focal deficits, all may be present.

Complications and sequelae (17)

Obstruction to the circulation of the cerebrospinal fluid (CSF) due to organization of exudate in the subarachnoid space is a frequent occurrence, especially when treatment is started late, and results in hydrocephalus. The outlook, especially for mental function, is grave in such cases. Paresis of limbs and of cranial nerves are usually transient features and subside after institution of specific therapy. Basal meningitis leading to exudation and subsequent fibrosis may involve cranial nerves. Blindness can commonly result in such cases. Mental retardation is seen frequently in the paediatric age group if treatment is delayed.

Diagnosis of tuberculosis

Tuberculosis is a chronic disease with slow progression of the lesion. The disease process manifests itself in acute form only rarely, e.g., in TBM. The definitive diagnosis of tuberculosis in nonemergency cases is done by laboratory examination of infected material. Sputum, laryngeal swab, gastric aspirate or urine are collected and subjected to microscopic examination of the

smear. Usually for such procedures the samples are concentrated by various methods like centrifugation of urine or sputum concentration by Petroff's method. The smear is stained by Ziehl-Neelsen's method to visualize acid-fast bacilli. The same concentrated material is inoculated on to suitable culture media to isolate mycobacteria. Lowenstein - Jensen medium is commonly employed for this. In certain circumstances guineapigs are used to detect the presence of pathogenic mycobacteria. Guinea pigs are highly susceptible to tubercle bacilli. Concentrated material at neutral pH is injected subcutaneously in the thigh of the guineapig. Two animals are used for each sample. One animal is killed and dissected aseptically at the end of 3 weeks. The animal is observed for a caseous local lesion, enlargement of draining lymph nodes and spleen and a few discrete lesions in the lungs. Smears from caseous material are observed for the presence of acid - fast bacilli. In doubtful cases tissues fixed in formalin are studied for histopathological evidence of tuberculosis. If the guineapig dissected at the end of 3 weeks does not show any evidence of tuberculosis the other guineapig is dissected at the end of 6 weeks and processed in a similar manner (18).

If a biopsy of an infected lesion can be obtained it is fixed in formalin and examined for any histopathological evidence of tuberculosis.

Tuberculin testing is done routinely in a case of suspected tuberculosis. In India a diameter of induration of 10 mm or more at the end of 48 - 72 hours in response to 1 Tuberculin Unit (T.U.) injected intradermally is taken as an evidence of infection (19). In early years a diameter of 5 mm or more was taken as the threshold level. Exposure to nonpathogenic or saprophytic mycobacteria gives rise to cross reactive sensitisation to purified protein derivative (PPD) which is used for testing the response, but infection with pathogenic tubercle bacilli gives rise to specific higher sensitivity to the antigen injected. Immunisation of the person with BCG vaccine also gives rise to positive tuberculin reaction. The tuberculin test can be negative in a person with active tuberculosis in certain instances. A patient simultaneously infected by viruses like measles usually does not give positive tuberculin reaction. An under-nourished child or a child with advanced tuberculosis is also unlikely to give positive results. In general, tuberculin testing is not

a very reliable method to diagnose a case of tuberculosis.

For the diagnosis of pulmonary tuberculosis, radiographic evidence of the disease is very useful. Early tubercular infiltration, pneumonic or bronchopneumonic lesions, cavitating lesions, disseminated or miliary lesions and lymph node involvement; all can be visualized by radiography. To adopt this method for mass screening, X-rays of a very small size are offered. This procedure, 'Mass Miniature Radiography (MMR)' is used as a large scale screening procedure in the community.

For a case of TBM, radiological examination of the skull is not usually useful. Rarely, hydrocephalus or a space occupying lesion may be visible. The only useful examination for such cases is the examination of CSF. In routine practice, the diagnosis of TBM is done as follows -

Diagnosis of tuberculous meningitis

TBM being an emergency condition early diagnosis and prompt treatment are the keypoints in the recovery of the patient. Todate, the diagnosis is based mainly

on the clinical presentation aided by routine examination of CSF.

Family history of an active case of tuberculosis or exposure in other forms as from neighbours or schoolmates can give a clue to a lesion in the lungs, and an attack of meningitis in a case of tuberculosis points to a tubercular aetiology of the meningitis. A history of failure to thrive and increased irritability in an infant can also contribute to the diagnosis of military tuberculosis and TBM.

The tuberculin test is positive in most cases of TBM. In very advanced cases of TBM or military tuberculosis, especially in grossly undernourished patients it may be negative.

Laboratory examination of the CSF

Routine examination of the CSF is undertaken before administration of drugs. In cases of basal meningitis the pressure is usually elevated. The fluid is usually clear but a cobweb or pellicle might appear after keeping the sample for 1-2 hours at 4°C.

If the fluid is examined immediately, a classical case of TBM will show the following picture (20):

- 1) Cells : Cell count in the CSF is increased. More than 50% are mononuclear cells. Cell counts are rarely above 1000/cmm., more frequently they are in 50-200/cmm. range and may be as low as only a few lymphocytes. In acute severe disease, the CSF may contain predominantly polymorphonuclear leukocytes, thus resembling pyogenic meningitis.
- 2) Protein : Protein is almost always increased except in cases of a near complete block to the flow of CSF.
- 3) Glucose : Glucose is usually decreased as compared to simultaneously determined blood glucose.
- 4) Chlorides : Chloride levels are found decreased but they are not given diagnostic importance in recent years (21).
- 5) Smears : Smears of the sediment after using concentration methods are positive for acid-fast bacilli in about 10-22% of cases (22). The cobweb or the pellicle on the top of the fluid specimen, if used to make smear gives good results.

6) Culture : A centrifuged deposit of the CSF used for culture gives better results than the smear alone.

In about 38-88% of cases the culture is positive (22,23).

From the above battery of tests the only tests that are useful for urgent diagnosis are cell count, smear report and biochemical investigations of CSF. Tuberculin test is read after 48-72 hours and it might help in deciding the direction of treatment but culture methods are not useful because pathogenic mycobacteria take anywhere between 3 to 6 weeks to form visible colonies.

Differential diagnosis of TBM

In the course of the disease a patient of TBM presents either with acute onset of symptoms of meningitis or with an insidiously progressing meningitis. The clinical picture and the differential diagnosis change depending on the mode of onset.

In cases presenting with acute or subacute onset of symptoms of meningitis, pyogenic meningitis and viral encephalitis need to be differentiated from TBM. For this, clinical presentation of the patient and laboratory

examination of the CSF are the only two parameters which help in the diagnosis. The values of the various constituents of CSF which help in the differential diagnosis, overlap to an extent of 30-40% in cases of TBM, pyogenic meningitis and viral encephalitis (24). This is mainly because of the cellular exudate, as in the acute cases the exudate consists mainly of neutrophils, irrespective of aetiology.

The chronic onset TBM is mainly to be differentiated from syphilitic, cryptococcal, candidal and leptospiral meningitis, toxoplasma encephalitis or cysticercosis (22). In most of these including TBM the clinical picture is very vague and diagnostic physical findings are rare (23). Routine laboratory examination of CSF is also not of significant help (26). As a result, empirical treatment is started in many instances. An apparent response to treatment may prove the aetiology of meningitis retrospectively.

The only definitive way to ascertain the presence of mycobacteria in CSF is to visualize them in smears or cultures or, alternatively, to look for specific evidence

of their presence. This can be (1) specific antibody directed against mycobacterial antigens or (2) antigens of mycobacteria, or (3) disintegration products of mycobacteria.

In addition to this, many other nonspecific tests have been tried in which attempts were made to establish diagnostic findings for TBM. Some of these are:

- 1) Quantitation of different classes of immunoglobulins in the CSF (27) and their comparison with serum levels.

Results of such studies show wide variation in the amounts of immunoglobulin classes. The difference in the concentrations in various diseases were not significantly different.

- 2) Estimation of free sialic acid in CSF (28) - Sialic acid content of normal and tubercular CSF was comparable but in cases of pyogenic meningitis it was significantly higher. This might help in differentiating pyogenic meningitis from other conditions, but other causes of meningeal and cerebral inflammation were not taken into account in this study. Therefore the interpretation of results cannot be done with certainty.

- 3) Isocitric dehydrogenase levels in the CSF (29). The enzyme levels were increased in TBM and in benign and malignant brain tumors. In congenital hydrocephalus it was not increased. This paper does not mention any other inflammatory condition of the brain and meninges.
- 4) Serum fucose levels (30) - Serum fucose levels and erythrocyte sedimentation rate (ESR) were studied in cases of pulmonary tuberculosis. Serum fucose was proved to be a better indicator than ESR before starting the treatment. The study claims usefulness of the estimation in the diagnosis of tuberculosis, but it can only be used as an ancillary indicator in the diagnosis.
- 5) ⁸²Bromide test - ⁸²Br. was used in the differential diagnosis of 'lymphocytic' meningitis in 1960 by Crook et al. (31). In cases of meningitis the normal blood brain barrier is disturbed and allows diffusion of many substances into the CSF. The authors have used this phenomenon to judge the difference in a normal and an abnormal partition coefficient. The coefficient was expressed as a

ratio of concentration in serum to the concentration in the CSF. They reported a normal coefficient of 2.6 ± 0.4 ($M \pm S.D.$). They also found a partition coefficient of 1.0 or less in cases of TBM. Bharucha et al. (32) studied proved cases of TBM and suggested a modification. In cases of active TBM ratios were found to lie in between 1.61 - 1.99. A ratio below 1.6 indicated bad prognosis and ratios of 1.99 and above were found in cases of tuberculomas, miliary tuberculosis or inactive cases of TBM.

These nonspecific tests have not been studied in details so as to ascertain their usefulness. Basically, being nonspecific tests, the chances of false positive results in clinically similar conditions appear to be high. Therefore it is necessary to have tests which will detect specific evidence of the mycobacteria in biological fluids.

Tests for detection of anti-mycobacterial antibodies

Reggiardo and Middlebrook (33) have used glycolipids extracted from M.bovis - BCG to conduct serological studies in human populations. In 1981 Reggiardo and Vasquez (34) published their data on comparison of

haemagglutination test and ELISA performed on sera of patients with active pulmonary disease before and during chemotherapy. They have used glycolipid extracts from M.bovis-BCG as the antigen. In their hands haemagglutination test and ELISA were equally sensitive.

In 1975 Nicholls (35) had proposed revival of whole cell agglutination test for serodiagnosis of tuberculosis. The author had used phenolkilled M. tuberculosis H₃₇Ra (Human avirulent strain) cells as antigen. According to him a titre of 1:125 and above was indicative of active tuberculosis. But Mitchison and others (36) have refuted that claim. According to them no differentiation was possible between active pulmonary tuberculosis, healthy controls and patients with nontuberculous conditions.

Radioimmunoassay (RIA), one of the most widely used immunoassays, has been used to detect the presence of mycobacterial antibodies. A report published by Winters and Cox (37) suggests that antibody detection by RIA - as they have done - is unlikely to prove very useful in the diagnosis of the disease.

Thoen and others (38) used ELISA to detect antibodies in tuberculous animals. They have used protein

- A conjugated with horse - radish peroxidase. Since protein - A binds with the Fc portion of immunoglobulins especially immunoglobulin G (IgG) (39), the antibody in the form of immunoglobulins was detected. The authors had used sera from animals which were bacteriologically proved to have tuberculosis.

Test for the detection of mycobacterial antigens

Not many papers are found in the literature where attempts are made to detect mycobacterial antigens in various biological fluids. Strauss and others (40) have standardized a competitive RIA using labelled PPD. The assay system could detect 5 ng of mycobacterial antigens. One week old sputum cultures were used as a source of antigen. The sputum was not used directly to check the presence of antigens. This method lengthens the time to arrive at a definitive diagnosis. Also the expensive material and radioisotopes needed for RIA restrict its usefulness as a readily available diagnostic procedure.

Thoen and his colleagues (41) have used ELISA for the detection of mycobacterial antigens. They have used methodology essentially similar to that for detecting the

antibodies. They were able to detect mycobacterial antigens in tissue homogenates of infected cattle. The results obtained by these workers suggest a possibility that ELISA can be used to detect mycobacterial antigens not only from tissue homogenates but also from other biological fluids.

Tests to detect specific components of mycobacterial cells

Mycobacterial cell wall has a high mycolic acid content. Hence specific mycolic acids or their cleavage products which can be detected by gas-liquid chromatography are used as diagnostic markers. Larsson and others (42) have used selected ion-monitoring for the detection of tuberculostearic and C_{32} -mycoseric acids in mycobacteria. Like Strauss and others (40), they have used 3-day old sputum cultures from patients with pulmonary tuberculosis and have looked for specific products by gas chromatography-mass spectrometry (GC - MS). The sensitivity of detection of mycolic acids is 1-2 pg. But availability of an expensive instrument is a major obstacle for the procedure. Guerrant and others (43) have used a similar method to detect mycolic acid cleavage products but 10-15 mg of the sample needs to be processed for this method. A large

amount of sample means a longer waiting period as the growth rate of mycobacteria is very slow.

After going through all the available tests for detecting mycobacterial infections, it was realized that only an assay which is highly sensitive will be helpful in early diagnosis, since slow growing organisms like mycobacteria will not be present in the body in sufficiently large numbers so as to release their antigens or cell wall components in large amounts. Hence, less sensitive immunological techniques (see later) like counter immunoelectrophoresis or passive haemagglutination were not considered very useful as the detection limits of these techniques usually lie in microgram quantities. ELISA which can be as sensitive as RIA was chosen as the best available method on this basis.

Immunological tests have been used in diagnostic medicine for nearly a hundred years. In early days only flocculation tests and complement fixation tests were available, e.g., Widal test for typhoid antibodies and Wassermann reaction for antibodies to Treponema pallidum. Later on inert particles coated with soluble antigens were used in agglutination tests to detect antibodies.

Bentonite or latex particles when coated with the antigens did react with specific antibodies but they were found to be difficult to coat, preserve and standardise (44). Red blood cells, on the other hand, have been found to be extremely convenient passive carriers of antigen.

Middlebrook and Dubois in 1948 (45) and Boyden in 1951 (46) have shown that mycobacterial extracts coat the red cells well. Boyden has shown that tanned red cells are better carriers than fresh cells (46). Using passive haemagglutination, antibody titres of biological fluids can be detected. If instead of an antigen, an antibody is used to coat the red cells, the antigen content of the fluid can be detected. This technique is called reverse passive haemagglutination. Alternatively, haemagglutination inhibition assays can also be used to detect antigens.

Soluble antigens can form insoluble immunoprecipitates with specific antibodies in a gel matrix. Ouchterlony in 1958 (47) introduced the method of double diffusion in agar gel in which antigen and antibody diffused into the gel from two wells side by side. This made it possible to visualise immunoprecipitates in agar gel and titrate antigens or antibodies. In this method a lot of antigen and antibody was lost without coming in contact with each

other as they did not diffuse in each other's direction alone. Cossart (48) used immune-electro-osmophoresis, i.e., movement of antigen and antibody in an electrical field. In this method waste diffusion was minimised and the duration of the test was drastically reduced. The precipitates could be seen at the end of 2 hours instead of 24-48 hours.

In the second half of this century, assays that separated antigen - antibody complexes from unreacted material and used various substances as labels for these complexes came into use. This made it possible to quantitate antigens or antibodies instead of merely titrating them as in the older tests. The haemagglutination assay may be called one of the earliest immunoassays. Different forms of labels used constituted different forms of immunoassays, e.g., radiolabelled material constituted radioimmunoassay (49), enzyme labelling constituted enzyme immunoassays (EIAS) or enzymelinked immunosorbent assays (ELISA)(50), a fluorescent group gave rise to fluoroimmunoassay (51). Other uncommonly used immunoassays are spin immunoassay (52) in which a stable free radical is used and a viroimmunoassay (53) in which protein - bacteriophage conjugates are used.

ELISA was first introduced by Engvall and Perlmann in 1971 (54). They had used sheep anti-rabbit immunoglobulin coupled to alkaline phosphatase for the detection of rabbit-immunoglobulin. In their assay system, the sensitivity was similar to radioimmunosorbent assays.

In ELISA one reactant (usually the antibody) is linked to an enzyme. The antibody reacts with specific antigens to form immune complexes. After getting rid of the excess of reacting materials the bound enzyme in the immune complexes is estimated by a suitable method. The indicator colour of the resultant reaction is either read on a spectrophotometer or, at times, read visually.

Several different types of EIAs can be used. The variants are (50) :

- 1) Competitive EIA for antigen - Labelled antigen competes with unlabelled antigen for binding to a limited quantity of antibody. The antibody bound antigen is separated from the free antigen by the use of solid phase antibody or a second antibody with a specificity for the first. The enzyme activity in either the bound

or free fraction is determined. This procedure is analogous to classical RIA method.

- 2) 'Immunoenzymometric' assay for antigen - Antigen reacts with excess labelled antibody and after incubation excess solid phase antigen is added. The solid phase antigen reacts with the excess of labelled antibody. Two phases of antigen can be separated and enzyme activity measured. This assay is analogous to the immunoradiometric assay.
- 3) 'Sandwich' EIA for antigen - An antigen with at least two reactive sites reacts on one side with excess solid phase antibody and on the other side with labelled antibody.
- 4) EIA for antibody - Antibody binds to an excess of solid phase antigen and after washing reacts with added labelled antibody specific for the first antibody.
- 5) Homogenous EIA for haptens - This type of EIA does not require separation of free and bound label because the assay depends on inhibition of the enzyme label by antibody binding. The hapten - antibody complex inactivates the labelled enzyme, hence it is not available for estimation.

Alkaline phosphatase was the first enzyme used for labelling antibody. Today, along with it many other enzymes have been tried. Some of the commonly used enzymes are listed below.

Enzymes used as labels in EIAs (50)

Enzyme	Source
1) Malate dehydrogenase	Pig heart mitochondria
2) Glucose-6-phosphate dehydrogenase	<u>Leuconostoc mesenteroides</u>
3) Glucose oxidase	Fungi
4) Peroxidase	Horse - radish
5) Acetylcholin-esterase	
6) Alkaline phosphatase	Calf intestinal mucosa or <u>E.Coli</u>
7) Glucoamylase	<u>Rhizopus nizens</u>
8) Lysozyme	Egg white
9) β -galactosidase	<u>E.Coli</u>

Coupling

Coupling of the enzyme and the protein without loss of active sites of the two is a crucial step in EIAs.

Coupling can either be done by incorporating an intermediary between the two components or by activating one of the components initially to react with the second.

Glutaraldehyde has been used for cross-linking of proteins (55). Glutaraldehyde acts as a bifunctional group and hence can be used very conveniently in coupling reactions. It can either be used in a 'one-step' (55) or a 'two-step' (56) linking method. In one-step linkage, the enzyme and the protein are mixed together in the presence of glutaraldehyde. The conjugates formed are heterogenous and have a high molecular weight. Two-step linkage can only be used for horse-radish peroxidase (HRP). HRP is unusual in that it normally reacts with only one glutaraldehyde molecule so that self-coupling does not occur. In this method, peroxidase is first treated with glutaraldehyde, the excess of which is removed and the 'activated' peroxidase is allowed to react with the free amino groups of the protein to be coupled. Loss of activity is much less in this method than by the one-step method.

The other way of linking the enzyme and the protein is again used for HRP. HRP has several oligosaccharide

groups and their oxidation to aldehyde groups that can react with amino groups is the basis of this method (57). The HRP is treated with 1-fluoro-2,4 - dinitrobenzene to block its free amino groups and prevent self-linking. The saccharides are oxidised with sodium periodate, the 'activated' peroxidase is then allowed to react with the free aminogroups of the second protein and the Schiff bases formed are reduced to sodium borohydride to give stable linkages.

Dimaleimide linkage is used by Kato et al. (58) to link rabbit - anti IgG with β -D-galactosidase. In this method, dimaleimide derivatives cross link proteins by reacting with the sulphydryl groups.

Use of different solid phases

In most types of EIAs the labelled antigen is separated from unlabelled antigen by using various types of solid phases. Initially Engvall of Perlmann (54) had used polystyrene tubes as a solid phase. The amount of protein needed to coat these tubes was very high. Later, these plates were replaced by microtitre plates. The capacity of each well is about 0.3 ml. Many small wells on a single

plate offered the advantages of using less quantity of protein to coat the well and to run many samples at a time. These plates are used very commonly for qualitative and quantitative EIAs. Polyacrylamide or agarose beads can be used as a solid phase (59). The time consuming process of centrifugation to separate these beads from the soluble or liquid phase is shortened if antigen or antibody is covalently linked to magnetic polyacrylamide-agarose beads and a magnetic rack is used to separate the beads from liquid phase (59). Kato and others (60) have used IgG - coated silicone pieces as a solid phase. Activated thiol - sepharose was used by Umeda and others (61). Lehtonen and others (62) have compared results of polystyrene, nylon and cyanogen bromide - activated paper as solid phases.

Indicator systems

To detect the presence and quantity of enzyme label present in the assay system, many substrates or substrate - indicator systems are employed. After enzymatic hydrolysis certain substrates produce a coloured product. In the case of alkaline phosphatase, sodium para-nitrophenyl phosphate on enzymatic hydrolysis produces free para-nitrophenol which has an intense yellow colour

which can be read spectrophotometrically. In certain other instances the products of substrate hydrolysis by themselves are colourless. In such a case an indicator which uses one of the reaction products to produce a coloured compound is used. The colour formation is directly dependent upon the amount of enzymatic hydrolysis. HRP uses hydrogen peroxide as a substrate to give rise to nascent oxygen which is used by colourless chromogens, the oxidised product of which is a coloured compound. A number of substances are used as chromogens for peroxidase such as o-dianisidine, o-toluidine, o-phenylene - diamine, 2,2'-azino-di - (3 - ethyl) benzothiazoline - 6-sulphonic acid (ABTS) or 5-amino-salicylic acid. There are various advantages and disadvantages associated with each of them, most of which are discussed by Rook (63) at length.

Applications of EIAs

EIAs have been used extensively to detect antigens, haptens and antibodies in biological fluids. In most cases the assay system can detect the antigens or the antibodies in nanogram quantities. The list of various substances that have been detected by EIAs is given below.

Substance detectedReference No.**A) Infectious diseases-antibodies and antigens**

1) Antibody to salmonella Spp.	64
2) Antibody to <u>V.cholerae</u>	65
3) Antibody to rubella virus	66
4) Antibody to Hepatitis B e antigen (HBeAg)	67
5) Antibody to rotavirus	68
6) Antibody to influenza virus	69
7) Antibody to <u>S.pneumoniae</u>	70
8) Antibody to Echinococcus spp.	71
9) Antibody to plasmodium spp.	72
10) Antibody to Toxoplasma spp.	73
11) Antibody to <u>E.histolytica</u> strain HK9	74
12) HBe Ag	67
13) <u>M.bovis</u> antigens	41

B) Endocrine substances

1) Insulin	61
2) Oestrogen, luteinising hormone, human chorionic gonadotropin	75
3) Progesterone	76
4) Thyrotropin	77
5) Cortisol	78

<u>Substance detected</u>	<u>Reference No.</u>
C) <u>Miscellaneous</u>	
1) α_1 -fetoprotein	79
2) Haptoglobin	80
3) Anti-DNA antibody	81

The above list is only a sample of the many diagnostic uses for which the EIA has been employed in efforts to develop simple and yet sensitive assays for routine laboratory use.

MATERIALS AND METHODS

I. Preparation of reagents for ELISA

1. M.tuberculosis H₃₇Rv irradiated cells.
2. M.tuberculosis H₃₇Rv sonicate.
3. Alkaline phosphatase-antibody conjugate.

1. Preparation of M.tuberculosis - H₃₇Rv irradiated cells

M.tuberculosis - H₃₇Rv strain was used as a standard human pathogenic strain. The culture was obtained from National Collection of Type Cultures (NCTC, London, England). M.tuberculosis H₃₇Rv (NCTC 7416) lyophilized culture was revived in Youmans' medium (Modified Proskauer and Beck synthetic medium) (See Appendix) (82) by inoculating the total contents of the ampoule in 10 ml of medium. After 3-4 weeks of incubation at 37°C. a surface pellicle growth had formed. On transfer into a 250 ml flask containing 100 ml medium, a thick pellicle was obtained at the end of four weeks of incubation at 37°C. The flasks were then shaken to disturb the pellicle which settled at the bottom. As much medium as possible was removed by decantation and the cells were irradiated in a Co⁶⁰ irradiation chamber

(Installed by B.A.R.C., Bombay) with a total dose of 2.4 megarads. The cells were then suspended in phosphate buffered saline (PBS) (See Appendix) and spun at 40,000 g max for 30 minutes. Three such washes were given to remove traces of Youmans' medium. Finally, the cells were suspended in PBS.

2. Preparation of H₃₇Rv sonicate

Ten ml of packed cells of M.tuberculosis H₃₇Rv prepared as above were suspended in 25 ml PBS and were sonicated in a 'Sonifier' (Branson Sonic Power Co., U.S.A.) at 20 KHz for 15 minutes. The sonicate was centrifuged at 40,000 g max. for 30 minutes. The supernatant was further filtered through a 0.45 µm membrane filter (Millipore Corporation, Massachusetts, U.S.A.). The protein content of this filtrate, designated as 'sonicate', was estimated by the method of Lowry et al. (83) (See Appendix).

3. Preparation of alkaline phosphatase - antibody conjugate

A) Preparation of antibody

a) Preparation of the antigen from H₃₇Rv sonicate

The sonicate was fractionated by Sephadex G-200 column chromatography as described by Deshpande et al.

(84). Briefly, the sonicate was concentrated by vacuum dialysis; the concentrate containing 30 mg. of protein was loaded on a Sephadex G-200 column (Pharmacia Fine Chemicals, Uppsala, Sweden 2.15 cm x 100 cm) and 3.0 ml fractions were eluted with PBS and the absorbance recorded at 280 nm on a PMQ II spectrophotometer (Carl Zeiss, Germany). One major peak appeared in the void volume. Two minor peaks were encountered subsequently (Figure 1). The eluate under the third peak showed one major fast-moving low molecular weight protein (about 20,000 daltons) band and 3 to 4 minor bands preceding it on polyacrylamide gel electrophoresis (Figure 2). This material was designated as Fraction 1 and was used as the antigen for immunization after concentration by vacuum dialysis.

This antigenic preparation was employed because the antigens present in this fraction constitute the water soluble antigens found in culture filtrates, PPD and aqueous extracts of mycobacteria. Since these antigens are easily extractable from mycobacteria, it is logical to expect them to be present in CSF in cases of TBH.

**Figure 1 : Fractionation of H_{37} Rv sonicate
on G-200 column.**

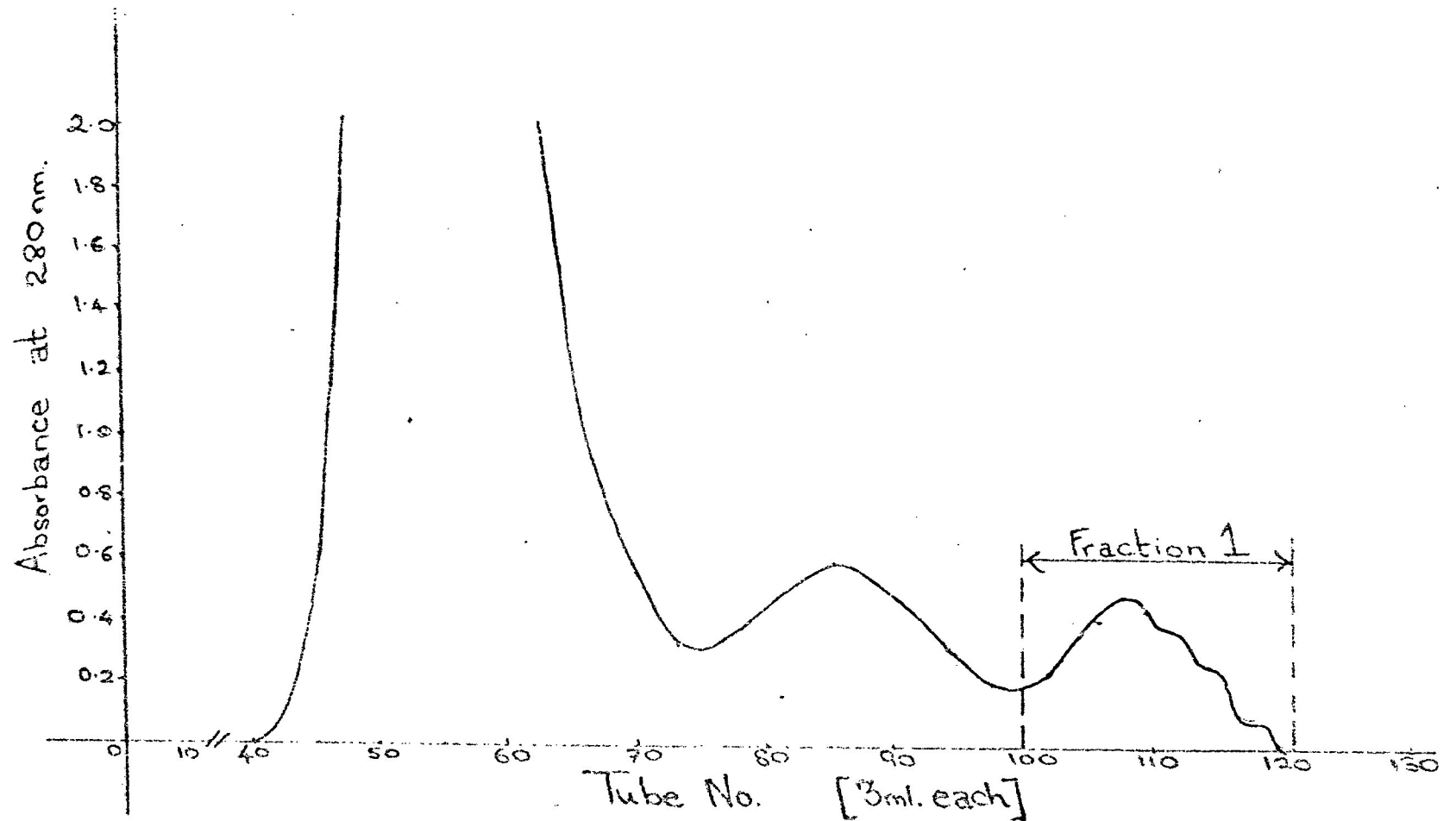
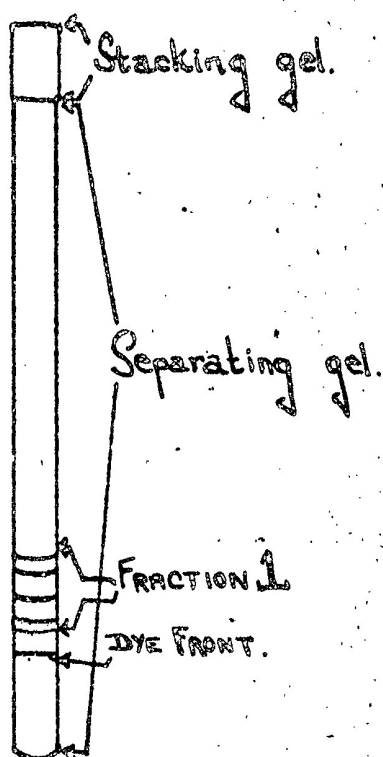


Figure 2 : Fraction 1 from G-200 column on
polyacrylamide gel electrophoresis.



b) Preparation of antibody to Fraction 1

Male rabbits weighing 1.2-1.5 kg were obtained from Haffkine Bio-Pharmaceutical Corporation Ltd., Bombay.

0.5 ml of Fraction 1 containing 3.0 mg/ml of protein was mixed with an equal volume of Freund's incomplete adjuvant (Difco Laboratories, Detroit, Michigan, U.S.A.) and emulsified. 0.25 ml of the emulsion was injected intradermally at 4 different sites on the back of the animal. The hair from the back was clipped prior to immunization. Three more immunizations were done similarly at two-week intervals. On the 7th day after the 4th dose, the rabbits were bled through the ear vein, serum separated and antibody titre checked by agar gel precipitation technique (47) as described in Appendix. After this, immunization was done at monthly intervals. The rabbits were bled from the heart when the antibody titre was 1:8 or more. For this, the subternal route was employed. Sterile 18 gauge needles of 1½ inch length attached to 50 ml syringes were used. About 30-40 ml of blood could be withdrawn from each animal at a time.

Separation of immunoglobulins from the serum

One volume of saturated ammonium sulphate (AS) (See Appendix) was added to two volumes of serum dropwise to attain a final concentration of AS of 33%. The mixture was allowed to stand at room temperature for 30 minutes and then centrifuged at 8,000 g max. for 30 minutes. The sediment was washed twice with 33% AS, centrifuging and discarding the supernatant each time. The sediment was then dissolved in PBS. With every step of AS precipitation the resuspending volume of the buffer was decreased very gradually so as to get a concentrated globulin solution. After dissolving the sediment in PBS it was reprecipitated twice as above and then finally dissolved in PBS again. The solution was dialysed extensively for 48 hours against 4 changes of PBS on a magnetic stirrer at 4°C to remove traces of AS. Precipitates formed during dialysis were removed by centrifugation at 1,00,000 g max. for 60 minutes. Estimation of the protein content was done by the method of Lowry *et al.* (83) as described in the appendix. The antibody titre of the purified fraction was determined by the agar gel-diffusion method as described in the appendix.

C) Coupling of the enzyme and the immunoglobulin (antibody)

The enzyme alkaline phosphatase type VII-S (Sigma Chemical Company, U.S.A.; specific activity 1250 units/mg of protein) was supplied as a suspension in 3.2 M ammonium sulphate solution. The source of the enzyme was bovine intestine. The coupling of the enzyme and the antibody was done by the 'one-step' glutaraldehyde method described by Engvall and Perlmann (54). Essentially, the method was as follows:

0.7 ml of the suspension containing 4.0 mg (5000 units) of enzyme was transferred to a centrifuge tube and spun at 40,000 g max. for one hour. The supernatant was discarded. The immunoglobulin solution prepared as described earlier was adjusted to a concentration of 2 mg/ml in PBS. 0.85 ml of this solution was added to the enzyme pellet. The pellet was dissolved in the solution and the contents of the centrifuge tube were transferred to a dialysis tube. The dialysis was done overnight at 4°C against PBS (pH 7.2) to remove ammonium sulphate. The dialysis bag was emptied in a measuring cylinder and the volume of the solution made to 1.0 ml with PBS. 10 µl of 20% glutaraldehyde solution (Sigma, stock 25% aqueous

solution, diluted to 20% by PBS) were added. The contents were allowed to stand at room temperature for 2 hours after thorough mixing. The mixture was dialysed against PBS at 4°C overnight with 2 changes of 2 litres each. Solid sucrose was added to the mixture to a final concentration of 10% and the mixture was loaded on to a Sepharose - 4 B column (Pharmacia; 1.7 cm x 48.0 cm) previously equilibrated with 0.05 M Tris HCl buffer pH 8.0 (See Appendix). 2.4 ml fractions were eluted with the same buffer. The absorbance of the fractions was read at 280 nm. on the spectrophotometer in quartz cuvettes of 1 cm. path length. A graph of fraction number Vs. absorbance was plotted and the first peak of protein was pooled after checking the enzymatic activity of each fraction. The protein content of the pooled fraction was estimated as described in the appendix. Bovine serum albumin (BSA, Centron, Bombay) was then added to a final concentration of 1%, the conjugate divided into aliquots and stored at -20°C. Freshly thawed aliquots were kept at 4°C in equal volume of glycerine (Polypharm Private Ltd., Bombay).

II. Standardization of the ELISA

- 1) Estimation of the enzyme activity in the conjugate.
- 2) Titration of the conjugate with a fixed amount of solid phase antigen (H_{37} Rv cells).

- 3) Titration of the solid phase antigen ($H_{37}Rv$ cells) with a fixed amount of the conjugate (1:10 dilution).
- 4) Standardisation of a competitive inhibition assay using $H_{37}Rv$ sonicate.

1. Estimation of the enzyme activity in the conjugate

Reagents

1) Diethanolamine buffer (1 M)

Diethanolamine (Sigma)	...	97.0 ml
Magnesium chloride, ($6H_2O$)	...	0.1 gm
Sodium azide (Sigma)	...	0.2 gm

The ingredients were dissolved in 900 ml of distilled water. 1 M. HCl was added to get a pH of 9.8 and the volume made to 1 litre with distilled water.

All reagents used were of analytical grade.

The buffer was stored in an amber coloured bottle at 4°C.

2) Buffer-substrate solution : Buffer-substrate solution was prepared just prior to use.

Disodium paranitrophenyl phosphate (Sigma)	...	25 mg.
Diethanolamine buffer	...	25 ml

The final concentration of the substrate disodium paranitrophenyl phosphate was 2.7 mM.

3) Sodium hydroxide solution (NaOH, 1N)

Sodium hydroxide ... 4 gm

Distilled water ... 100 ml

Two ml of buffer-substrate solution were mixed with 50 μ l of different dilutions of the glycerinated antibody-enzyme conjugate. The reaction was allowed to proceed for 15 minutes at room temperature and then terminated by addition of 1.5 ml of 1 N NaOH.

Paranitrophenol, the end product of enzymatic hydrolysis of para-nitrophenyl phosphate by alkaline phosphatase, was measured spectrophotometrically at 405 nm against a buffer-substrate blank employing a standard curve prepared as follows:

To determine the absorbance produced by para-nitrophenol a standard curve was plotted. A series of tubes containing 1.9 ml of the 1 M diethanolamine buffer were prepared and 100 μ l of appropriate dilutions of para-nitrophenol dissolved in the buffer were added so as to give final amounts of 0.72, 0.36, 0.18, 0.09, 0.036, 0.018 , 0.009 and

0.045 μ moles respectively. 1.5 ml of 1 N NaOH were added and the absorbance recorded against a buffer blank. The graph of para-nitrophenol concentrations Vs. absorbance was plotted (Figure 3). Amount of para-nitrophenol generated in the enzyme substrate mixture was directly read from the graph. The enzyme activity in the conjugate was calculated using the following formula:-

Enzyme activity in μ moles/mg of protein/hour.

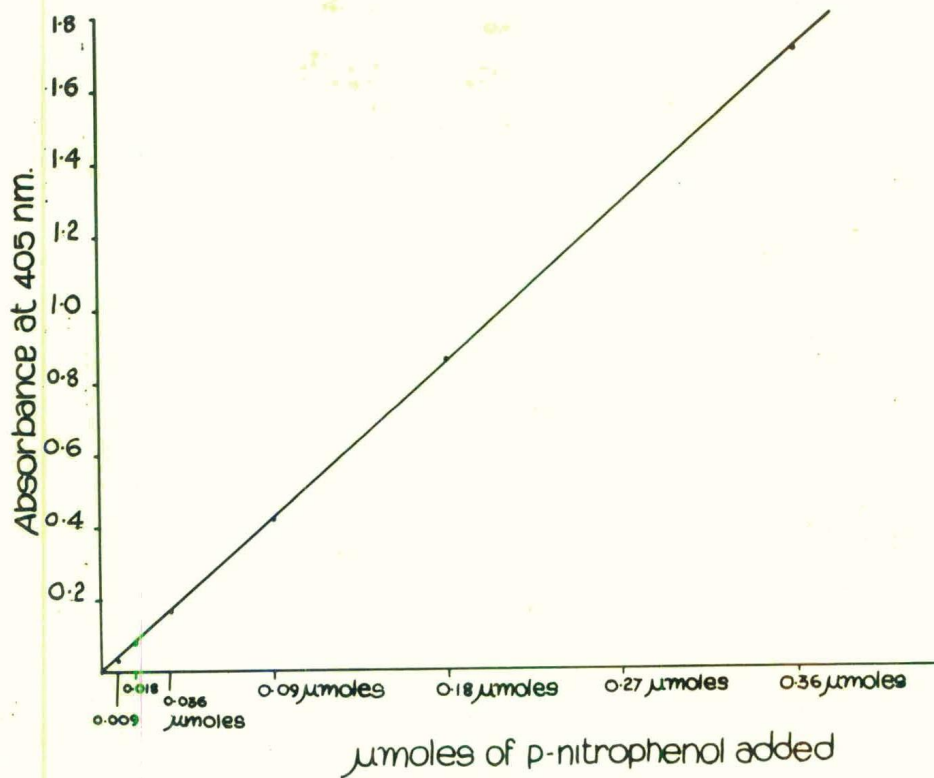
$$= \frac{\text{moles of paranitrophenol liberated}}{\text{mg of conjugate added} \times \text{duration of incubation in hrs.}}$$

2. Titration of the conjugate with a fixed amount of solid phase antigen (H_{37} Rv cells)

One volume of H_{37} Rv irradiated, packed cells was suspended in 2 volumes of PBS so as to give a packed cells volume of 0.33 ml per ml of suspension.

0.1 ml of packed cells of H_{37} Rv suspended in 0.3 ml were transferred into a series of tubes. Enzyme-antibody conjugate was added to each of the tubes to give final dilution of 1:10, 1:50, 1:100 and 1:200 respectively. The reaction volumes were made to 1 ml with PBS. The mixtures were incubated at 4°C overnight on a roller drum

Figure 3 : Absorbance of paranitrophenol
at 405 nm.



(Roller drum apparatus, model SYN 152, Emervee, Pune) at a speed of 12 revolutions per hour. The cells were then washed with PBS containing 0.1% v/v Tween 20 (Mann Research Laboratories, New York, USA). The washes were given on 0.45 μ m membrane filters (Millipore) employing a 25 mm sintered glass filtration assembly (Pushpak, Goring, India). The membrane filters along with the bacterial cells were transferred to tubes, 2.0 ml of the buffer-substrate solution was added to the tubes and the reaction allowed to take place at room temperature. Tubes were shaken intermittently to ensure contact of all the cells with the solution. After 60 minutes the reaction was stopped by addition of 1.5 ml of 1 N NaOH. The cells and the reaction mixture were separated by vacuum filtration using 0.45 μ m membrane filters (Millipore). The filtrate was transferred into cuvettes and absorbance readings were taken in the spectrophotometer against a buffer-substrate blank at 405 nm. On the basis of these readings (See Results), a conjugate dilution of 1:10 was used for standardisation of the inhibition assay.

3. Titration of the solid phase antigen (H_{37} Rv cells) with a fixed amount of the conjugate (1:10 dilution)

In a set of test tubes varying quantities of the bacterial cells were added starting from 0.00835 ml of packed cells to 0.133 ml of packed cells. Each point was assayed in triplicate. To the tubes 0.1 ml of antibody-enzyme conjugate was added and the final volume made to 1.0 ml with PBS. Thus the final dilution of antibody-enzyme conjugate employed in the assay was 1:10. Overnight incubation, washing and the assay was carried out as described above and the absorbance recorded at 405 nm. A graph of amount of cells Vs. absorbance was plotted. On the basis of this graph, 0.1 ml of packed cells were used for further experiments (See Results).

4. Standardisation of a competitive inhibition assay using H_{37} Rv sonicate

0.1 ml of antibody - enzyme conjugate was mixed with 0.6 ml of sonicate of H_{37} Rv containing 1, 5, 10, 25, 50, 250 and 500 ng of protein. Each antigen concentration employed was tested in triplicate. The

sonicate and enzyme - antibody conjugate mixtures were incubated at 37°C for 1 hour. The mixtures were then transferred to tubes containing 0.3 ml of the solid phase antigen containing 0.1 ml of packed H_{37}Rv cells. The tubes were incubated on the roller drum overnight at 4°C . After incubation, the unbound antibody, excess free antigen and immune complexes free from solid phase, were washed out as described earlier on 0.45 μm membrane filters. The addition of substrate and 1 N NaOH were carried out as described earlier. A graph of the amount of sonicate Vs. % reduction in absorbance was plotted.

III. ELISA on CSF samples

CSF samples were obtained from King Edward VII Memorial (K.E.M.) Hospital, Bombay and Ruby Hall Nursing Home, Pune. Samples were collected in sterile containers and processed as early as possible. As described in the following sections, the samples were processed for routine laboratory investigations and a part of the cell-free supernatant was used for ELISA.

0.5 ml of CSF was mixed with 0.1 ml of antibody enzyme conjugate and incubated at 37°C for 1 hour. 0.1 ml of packed H_{37}Rv cells were delivered in a volume

of 0.3 ml in PBS into the mixture. The final volume was made to 1.0 ml. Each CSF was tested in duplicate. The tubes^a were incubated overnight on a roller drum and the solid phase antigen washed on 0.45 μ m membrane filter as described earlier. After this the substrate and NaOH were added to the solid phase as described earlier. In every batch of tests the following controls were included :-

- 1) 0.3 ml solid phase antigen (0.1 ml packed H₃₇Rv cells) with 0.1 ml of antibody -enzyme conjugate and 0.6 ml PBS.
- 2) 0.3 ml solid phase antigen as above with 0.1 ml of antibody enzyme conjugate and 25 ng sonicate antigen of H₃₇Rv in 0.6 ml PBS.

IV. Processing CSF samples

A) Routine bacteriological and cytological tests

- a) Total and differential cell count : If the fluid was clear in appearance, 50 μ l of CSF were mixed with 50 μ l of CSF diluting fluid (See Appendix) and the mixture run into a modified Neubauer's chamber. In such cases usually all 9 big squares

were counted. If the CSF was turbid white cell counting pipette was used. The CSF was diluted 1:10 with the diluting fluid and then the count was made. The actual count was calculated after taking into consideration the dilution factor.

CSF was centrifuged at 800 g max for 30 minutes and the sediment was used for differential cell count, smear and culture. The supernatant was used for biochemical tests and ELISA.

A drop of the sediment was placed on a clean glass-slide. A smear was made, dried in air and stained with Wright's stain. The cells belonging to different groups such as neutrophils and lymphocytes were counted.

- b) Smear : A loopful of the sediment was used to make a smear. After drying the smear was fixed by passing it through a flame. For staining 0.3% carbol fuchsin was poured on the slide and was heated till the vapour started rising. Hot carbol fuchsin was kept on the slide for 7 minutes. The slide was washed with tap water and dipped in 20%

sulphuric acid for 1½ minutes till the background was adequately destained. After washing it was stained with dilute methylene blue for 30 seconds, washed and dried. At least 100 oil immersion fields were scanned for the presence of acid-fast bacilli in GSF sediment.

- c) Culture : Two slants of Lowenstein-Jensen (L.J.; See Appendix) (85) medium were used for each sample. One loopful of sediment was streaked on each slant and the slants were incubated at 37°C for 4-6 weeks. They were inspected for growth at weekly intervals and if there was no growth at the end of 6 weeks the slants were discarded. If there was any growth it was subjected to Ziehl-Neelsen staining and the neutral red test to determine whether the organisms were classical pathogenic mycobacteria (86).

Neutral red test : (Dubos and Middlebrook test) : A loopful of growth from the L.J. slants was transferred to 5.0 ml of 50% methanol and incubated at 37°C for one hour. The growth was allowed to settle and the supernatant discarded. A second wash was given similarly with 5.0 ml of 50% methanol. 5.0 ml of alkaline barbitol buffer

(See Appendix) and 0.2 ml of 0.05% aqueous neutral red solution were added to the growth sediment and incubated at 37°C for one hour. The tubes were shaken intermittently. Pink to red staining of the clumps of growth indicated a positive test for classical pathogenic mycobacteria.

B. Routine biochemical tests

- a) Protein estimation : Protein content of the CSF was estimated by the method of Lowry et al. (83) as described in the appendix.

0.5 ml of CSF diluted 1:10 and 1:100 were transferred into two tubes containing 2.5 ml of alkaline copper tartrate reagent. 0.25 ml of diluted Folin-Ciocalteu reagent was added to each tube and the absorbance read 30 minutes later at 660 nm. A distilled water blank and a standard protein solution (100 µg/ml) were also run simultaneously. The protein concentration was calculated by referring to a standard graph.

- b) Sugar (Reducing substances) estimation

The estimation was done by the Nelson - Somogyi method (87) (See Appendix).

0.1 ml of CSF was mixed with 3.7 ml of isotonic diluent and 0.2 ml of sodium tungstate solution. The precipitate formed was discarded by centrifugation. 1.0 ml of the supernatant was transferred into a Folin tube containing 1.0 ml of Somogyi's reagent. The mixture was transferred to a boiling waterbath and kept for 15 minutes. After cooling for one minute, 1.0 ml of Nelson's reagent was added and the volume made to 12.5 ml. With each assay of samples a standard of 100 mg% of glucose and a blank were run. The amount of reducing substances present in the CSF was calculated by reference to the standard graph.

c) Chloride estimation

This was done by modified Volhard's method (88).

0.25 ml of CSF were mixed with 2.5 ml of silver nitrate (AgNO_3) solution and a pinch of ferric-alum indicator. After five minutes potassium thiocyanate (KSCN) was added dropwise till the contents showed faint brick-red colour. The amount of KSCN needed to achieve the end point was noted and the amount of

chlorides present in the CSF was calculated using the following formula :

$$\frac{(2.5 - \text{ml of KSCN needed for neutralization})}{\text{ml of CSF added}} \times 100$$

= Chlorides in mg%.

RESULTS

A. Standardization of the ELISA

1. Estimation of the enzyme activity of the antibody

enzyme conjugate : The antibody - enzyme conjugate containing 55 µg/ml of the conjugated antibody, 5 mg/ml of BSA and 50% (v/v) glycerol was assayed for enzyme activity. 50 µl volumes of 1:10, 1:100 and 1:1000 dilutions of glycerinated antibody - enzyme conjugate were mixed with substrate and the enzyme activity assayed in terms of amounts of paranitrophenol generated (See Methods).

The average enzyme activity of the antibody-enzyme conjugate was 18.395 µmoles/mg of protein/hr. (Table 1).

Table 1

Enzyme activity of the antibody - enzyme conjugate

<u>Dilution of the conjugate</u>	<u>Protein content</u>	<u>Absorbance at 405 nm*</u>	<u>Total amount of paranitrophenol released</u>	<u>Enzyme activity**</u>
1:10 (50 µl)	275 ng	> 2	-	-
1:1000 (50 µl)	27.5 ng	0.58	0.1224 µmoles	18.13
1:1000 (50 µl)	2.75 ng	0.06	0.0126 µmoles	18.66

* Absorbance at the end of 15 minutes.

** Enzyme activity in µmoles of paranitrophenol liberated/ng of protein/hour.

In order to develop a sensitive assay to detect the mycobacterial antigens in biological fluids, it was necessary to use optimum concentrations of solid phase antigen and antibody - enzyme conjugate. Titrations were done to arrive at the requisite concentrations of the solid phase antigen and the conjugate to be used by varying one of the components at a time.

2. Titration of the antibody - enzyme conjugate with a fixed amount of the solid phase antigen (H_{37} Rv cells)

0.3 ml of H_{37} Rv cell suspension containing 0.1 ml of packed cells was mixed with appropriate amounts of the antibody enzyme conjugate to get a final dilution of 1:10, 1:50, 1:100 and 1:200 and the final volume was made to 1.0 ml with PBS.

After the requisite period of incubation, the solid phase was washed and substrate added. Following incubation with substrate, the solid phase was discarded and the supernatant was read in a spectrophotometer at 405 nm (See Methods). The amount of antibody combining with the solid phase antigen decreased with each dilution of the antibody enzyme conjugate used. The

results of this assay are shown in Table 2.

Table 2
Decrease in absorbance with various
dilutions of antibody - enzyme conjugate

<u>Dilution of the antibody</u> <u>- enzyme conjugate used</u>	<u>Absorbance at 405 nm</u> <u>(M $\pm$ S.E.)</u>
1:10	0.72 $\pm$ 0.0245
1:50	0.43 $\pm$ 0.01633
1:100	0.25 $\pm$ 0.00814
1:200	0.15 $\pm$ 0.00592

The final dilution of 1:10 of the conjugate was employed in further experiments.

3. Titration of the solid phase antigen (H_{37} Rv cells)
with a fixed amount of the conjugate (1:10 dilution)

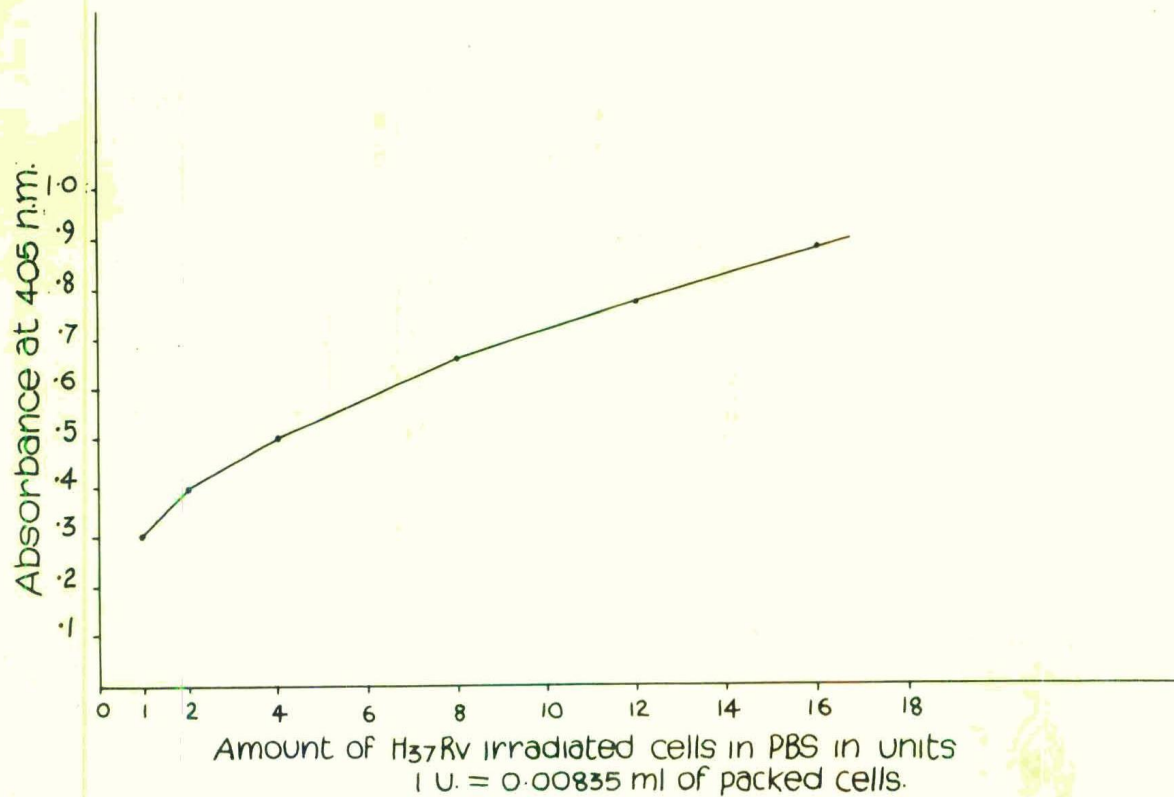
Packed cells of H_{37} Rv, the solid phase antigen, were mixed in amounts of 0.00835, 0.0167, 0.033, 0.066, 0.10, 0.133 ml with a requisite volume of the antibody enzyme conjugate and PBS to give a final dilution of the conjugate 1:10. The incubation, washing, addition of substrate and assay of liberated

paranitrophenol were carried out as described earlier (See Methods). With a fixed amount of the antibody - enzyme conjugate added, varying amounts of solid phase antigen showed a linear increase in absorbance with the increase in the amounts of solid phase antigen added (See Figure 4). For the standardization of the competitive inhibition assay, 0.1 ml of the $H_{37}Rv$ cells suspended in 0.3 ml was selected as the optimal amount of solid phase antigen because the absorbance of 0.7 obtained with 0.1 ml of solid phase antigen was considered fairly adequate and in the linear range for carrying out competitive inhibition assay.

4. Standardization of a competitive inhibition assay using $H_{37}Rv$ sonicate (nonparticulate antigen)

To determine the amount of the mycobacterial antigen present in the biological fluids it was necessary to quantitate the amount of inhibition produced by a known quantity of nonparticulate protein antigen. The nonparticulate protein in the form of crude $H_{37}Rv$ sonicate competed with the solid phase antigen for a limiting amount of the

Figure 4 : Increase in absorbance with increase in quantity of the solid phase antigen ($H_{37}Rv$ irradiated cells)



antibody added in the form of the antibody - enzyme conjugate. The soluble immune complexes formed were washed off on the membrane filters and only the solid phase antigen with the antibody attached to it participated in the colour reaction by the enzyme. One ng to 25 ng of protein of the H₃₇Rv sonicate caused a linearly increasing reduction in the absorbance because of competitive inhibition. The inhibitory capacity reached a peak at 50 ng of the sonicate antigen and there was no further decrease in absorbance with further increase in the amounts of the competitive inhibitor. A graph of per cent reduction in absorbance with increasing amounts of inhibitor antigen is shown in Figure 5. Reduction in absorbance brought about by different concentrations of the inhibitor were tested statistically (Table 3), employing Student's 't' test (89). Ten ng of inhibitor antigen could reduce the absorbance by 0.15. This was highly significant by Student's 't' test ('p' value < 0.01). Hence a reduction in absorbance by 0.15 or more was taken as an indication for the presence of the mycobacterial antigen in the fluid under test. It was decided to report the OSF

Figure 5 : Percentage reduction in absorbance with increase in the quantity of nonparticulate antigen ($H_{37}Rv$ sonicate).

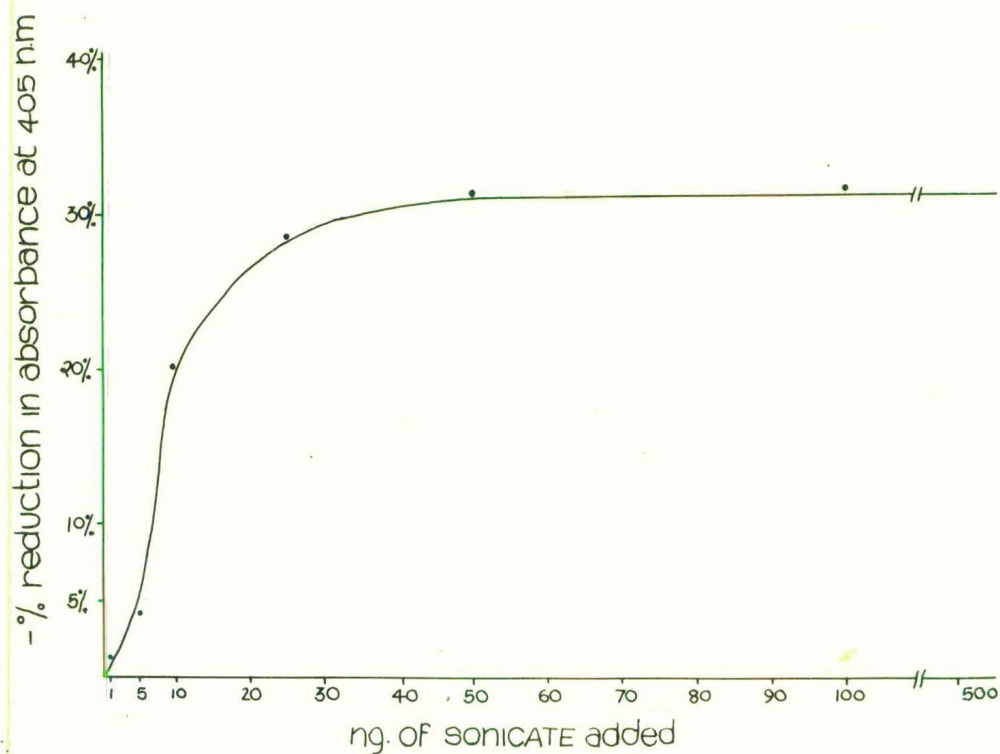


Table 3

Inhibition of conjugate binding to solid phase by nonparticulate antigen of H₃₇Rv

Protein content of nonparticulate antigen added	Mean absorbance at 405 nm	Decrease in absorbance	% Reduction in absorbance	'p' value
0 ng	0.74	-	-	-
1 ng	0.73	0.01	1.352	NS *
5 ng	0.71	0.03	4.054	p < 0.05
10 ng	0.59	0.15	20.27	p < 0.01
25 ng	0.53	0.21	28.38	p < 0.001
50 ng	0.51	0.23	31.09	p < 0.001
100 ng	0.51	0.23	31.09	p < 0.001

* Not significant

samples that brought about reduction in absorbance by 0.15 or more as positive. For every batch of tests 10 ng of sonicate antigen was used as an internal positive standard for inhibition.

B. ELISA on CSF samples

For this study, CSF samples were collected from K.E.M. Hospital, Bombay and Ruby Hall Nursing Home, Pune. Except for the cases of febrile convulsions all the others were inpatients. Based on the history, clinical examination and examination of the CSF, cases were divided into various groups. It was not possible to collect CSF from normal healthy individuals because of the obvious practical difficulty. For the purpose of present study, children who had a normal CSF profile and had a febrile convulsion served as normal controls. The profiles of the various groups are as follows:

1. Tuberculous meningitis : 15 cases diagnosed as tuberculous meningitis on the basis of clinical findings and laboratory profile of the CSF are the test cases of the study. Eleven patients were

females and four males. All the patients belonged to the paediatric agegroup with the age range of 6 months to 10 years.

Based on the mode of onset and progress the above 15 cases were divided into two groups namely-

a) Acute tuberculous meningitis - 10 cases

and

b) Subacute tuberculous meningitis - 5 cases.

Acute TBM : These patients were admitted for high fever, vomiting, disorientation and convulsions for a few days. All the patients gave history of low grade fever, cough, loss of appetite for a duration of a few weeks to a few months prior to admission.

Clinical examination revealed signs of meningeal irritation such as neck rigidity, presence of Kernig's sign and Brudzinski's sign in all the ten cases. In addition six patients showed clouding of consciousness. All the patients showed evidence of hypertonia, exaggerated deep reflexes and showed Babinski's sign. One patient showed hemiparesis.

Subacute TBM : All the five cases had symptoms of central nervous system involvement for a few weeks to a month. Four cases were admitted for clouding of consciousness. One patient was admitted for convulsions. Three patients were unconscious at the time of admission. One patient gave a history of headache, fever and vomiting for three weeks. One patient showed, choreo-athetoid movements of the right hand.

Clinical examination revealed signs of meningeal irritation in all the five cases. Hypertonia, extensor planter and exaggerated deep reflexes were present in all the five cases. There were no localizing signs.

Examination of CSF showed the following profile:-

Appearance	: Clear (8 cases) cloudy (7 cases)
Proteins	: Average 278 mg% (Range 45-837 mg%)
Sugars	: Average 32 mg% (Range 14-48 mg%)
Chlorides	: Average 627mg% (Range 520-740 mg%)

Total cell count : 120 cells/cmm (Range
15-820/cmm).

Predominant cell type : 65% lymphocytes (Range
50-100%)

35% Neutrophils (Range
0-30%)

Smears prepared from cobweb and the sediment were stained by Ziehl-Neelsen's technique. No acid-fast bacilli were seen in any sample.

The sediment and the cobweb were cultured on Lowenstein-Jensen medium. From three CSF samples slow-growing, acid - fast bacilli could be isolated after 4-6 weeks' incubation. All the three samples gave positive neutral red test.

ELISA tests for mycobacterial antigens were carried out on all the fifteen samples. Twelve CSF samples gave positive results.

Out of 15 patients six patients died during the stay in the hospital. In all these six cases CSF examination for mycobacterial antigens by ELISA test had yielded positive results. One case was autopsied and the diagnosis of TBM confirmed. Three of the

patients who expired had AFB positive CSF by cultural examination. Six patients showed distinct clinical improvement following treatment with antitubercular drugs (Streptomycin, Isonicotinic acid hydrazide and Rifampicin). The CSF picture had returned to normal after therapy for one month in these cases. No follow-up data is available for the remaining three cases.

Out of twelve ELISA positive cases, diagnosis of TBM could be confirmed in three cases by culture of CSF, in one case by autopsy studies and in six cases by virtue of clinical improvement and recovery following treatment with antitubercular drugs.

2. Patients with normal CSF profiles : There were six cases in this group. Out of the six cases two were male children and four female children. Their respective ages were 1½, 1½, 1½, 2, 2½ and 4 years. All the six children were admitted for fever and convulsions. None of the six patients had signs of meningeal irritation. Two of the female children showed signs of malnutrition. One patient was unconscious, had generalized hypotonia and right

hemiparesis. This patient developed severe diarrhoea and died in the hospital.

CSF examination showed the following profile:

Appearance	... Clear
Proteins	... Average 42 mg% (Range 28-57 mg%)
Sugar	... Average 60 mg% (Range 41-80 mg%)
Chlorides	... Average 675 mg% (Range 628-760mg%)
Total cell count	... 0-2 cells/cmm
Predominant cell type..	Lymphocytes

ELISA tests on the CSF samples for the presence of mycobacterial antigen were negative in all six cases.

Four patients were discharged on the day of admission as cases of febrile convulsion. One patient died. The remaining patient was kept in the hospital for treatment of malnutrition.

3. Suspected meningoencephalitis : Six cases of suspected viral meningoencephalitis have been included in the study. Two of these were female children and four male children. Their respective ages were 3½ months, 8, 3, 7, 2, and 7 years. One patient gave a history

of having received antirebics vaccine, following a dog-bite, 3 months prior to admission. All the patients presented with mild to moderate fever of 2-8 days' duration. Three cases had generalized convulsions. One patient had clouding of consciousness and disorientation. Two patients were admitted in unconscious state. On examination of the nervous system three patients showed hypertonia, one showed hypotonia, four showed exaggerated deep reflexes.

Examination of CSF showed the following profile:

Appearance	: Clear
Proteins	: Average 59 mg% (Range 23-110 mg%)
Sugar	: Average 56 mg% (Range 40-80 mg%)
Chlorides	: Average 634 mg% (Range 580-700 mg%)
Total cell count	: 8 cells/cmm (Range 0-24/ cmm)
Predominant cell type	: Samples with high cell count showed lymphocytic predominance.

ELISA tests on the CSF samples were negative for the presence of mycobacterial antigens in all six cases.

Two out of six patients died in the hospital. One of them was clinically diagnosed as post-vaccination encephalopathy due to anti-rabies vaccine and the other as Reye's syndrome because of derangement of liver functions and high levels of blood ammonia and blood urea. Out of the six, remaining four were discharged after clinical improvement following treatment with corticosteroids, mannitol and intravenous fluids.

4. Pyogenic meningitis : Seven cases diagnosed as pyogenic meningitis on the basis of clinical findings and the laboratory profile of CSF were also included in this study. All the patients were males. Their respective ages were 10, 22, 28, 29, 30, 35 and 48 years. All of them presented with severe headache and high fever of 3-8 days' duration. Two patients also had disorientation for 1-2 days, two were unconscious at the time of admission. All of them showed signs of meningeal irritation.

Examination of the CSF showed the following profile :

Appearance : Turbid

Protein	: Average 580 mg% (Range 152-1790 mg%)
Sugars	: Average 21 mg% (Range 8-61 mg%)
Chlorides	: Average 630 mg% (Range 580-720 mg%)
Total cell count	: 160/cmm (Range 100-500/cmm)
Predominant cell type	: 80-95% neutrophils.
Smear	: Gram positive cocci seen in 2 cases

All the patients were treated with antibiotics (Ampicillin, chloromycetin and cotrimoxazole). Three patients died in the hospital, the remaining recovered with the treatment.

ELISA tests on the CSF samples for the presence of mycobacterial antigens were negative in all the cases.

5. Meningismus with uncertain diagnosis : Three cases showing signs of meningeal irritation without definite evidence of meningitis were also included in the study. The details of the cases are as follows :

- 1) One year old female child was brought in for fever, vomiting and convulsions. She was conscious, had increased tone in all four limbs

and brisk deep reflexes. Examination of CSF showed 52 mg% proteins, 80 mg% sugar, 660 mg% chlorides and 3 lymphocytes/cmm.

- ii) Two year old female child had dry cough, breathlessness, fever and tonic spasms. Examination of lungs showed bilateral crepitations and harsh bronchial sounds suggestive of pneumonitis. Examination of the nervous system did not show any abnormality. CSF examination showed 74 mg% protein, 58 mg% sugar and 680 mg% chlorides. No cells were seen.

- iii) Four year old male child was admitted for headache, moderate fever, convulsions and disorientation. He had neck rigidity, hypertonia and Babinski's sign. CSF showed 58 mg% proteins, 44 mg% sugar and 720 mg% chlorides. No cells were seen.

Examination of CSF for the presence of mycobacterial antigens by ELISA in all three cases was negative.

DISCUSSION

Tuberculous meningitis is one of the commonest causes of meningeal inflammation in the paediatric population in India. In spite of the very high morbidity and mortality rates (3) the diagnosis of TBM is based mainly on clinical presentation of the patient and laboratory examination of the CSF. A patient presenting with constitutional symptoms suggestive of tuberculosis like fever, cough, lethargy along with signs and symptoms of meningitis is diagnosed as a case of TBM. Laboratory examination of CSF usually shows moderate increase in protein content, a decrease in sugar content and a reduction in chloride content along with lymphocytic exudate. All these criteria are relative criteria. Definite limits cannot be set as the diagnostic finding, hence there is an overlap in findings suggestive of TBM and those suggestive of pyogenic meningitis. McGee and Kaiser (24) have mentioned that there is an overlap of 30-40% in cases of TBM, pyogenic meningitis and viral encephalitis as far as the CSF profile is concerned. In late stages of pyogenic meningitis the cell count may show predominance of lymphocytes rather than neutrophils which are associated

very commonly with acute inflammation. In a few cases of acute TBM also, neutrophils are found to predominate the cellular picture. From the cell count it is very difficult to differentiate a case of viral encephalitis from a case of TBM as again in both the cases cellular exudate is supposed to be prominently lymphocytic. A relatively higher reduction in sugar content of CSF is found in cases of pyogenic meningitis but it is difficult to define a definite limit to differentiate between CSF of pyogenic meningitis and of TBM. Since all the constituents of CSF mentioned above vary nonspecifically as a result of inflammation, it is very difficult to assess the usefulness of these investigations as the diagnostic method. Due to this, demonstration of the causative organisms is the only definitive way for the diagnosis.

In cases of TBM chances of detecting acid-fast bacilli in smears prepared from the sediment of CSF are very low (6), therefore it is not possible to arrive at the bacteriological diagnosis by examination of a smear alone. Culture and guineapig inoculation for the diagnosis are also unsatisfactory. In the first place the procedures require 6-8 weeks for a definitive

diagnosis. TBM being an acute illness, early diagnosis is absolutely mandatory. Secondly the two tests are not highly sensitive because the isolation rates even in the best of the hands are very poor. However, Kennedy and Fallon (23) have reported positive bacteriological diagnosis in 87% of cases provided the CSF is cultured at least on four different occasions. The general experience, though, is to the contrary.

Serological tests were employed for the diagnosis of tuberculosis. Whole-cell agglutination test developed by Nicholls (35) was not found to be useful in the diagnosis of active cases of tuberculosis because the test was positive in healthy persons with positive tuberculin reaction also (36). Antibody detection tests using RIA were developed by Winters and Cox (37). These were also not useful for the specific diagnosis of tuberculosis.

Detection of specific antigens in various biological fluids in patients suffering from various diseases has been used very widely in diagnostic medicine. Methods are available to diagnose meningococcal, pneumococcal and H. influenzae infections of the nervous system (90).

These methods like counter-immunoelectrophoresis and latex agglutination test can help in the early diagnosis of meningitis (90,91). Both these methods can detect antigens in microgram quantities. Mycobacteria being slow-growing organisms it was thought that mycobacterial antigens might not be present in large quantities so as to give positive results with counter-immunoelectrophoresis or latex agglutination test. ELISA is known to be more sensitive than both these tests and usually as sensitive as RIA. Therefore utility of ELISA in the diagnosis of TBM was investigated.

The antigen that is being detected by this method is present in $H_{37}Rv$ sonicate, M.bovis-BCG sonicate and PPD in appreciable amounts. It is also present in the culture filtrate of $H_{37}Rv$. Since it can be easily extracted in aqueous medium from the $H_{37}Rv$ cells, it was expected that it would be released into a biological fluid like CSF very easily.

The ELISA test employed in this study is extremely sensitive because it can detect 10 ng of antigen. This order of sensitivity is definitely in the range of sensitivity of radio-immunoassay. However the competitive

inhibition assay shows some peculiar features that need to be discussed. When the antibody-enzyme conjugate in the amounts employed in the assay (0.1 ml, i.e. 1:10 final dilution) was mixed with varying amounts of the solid phase antigen, absorbance varied from 0.31 to 0.88 (Figure 4). Based on this relationship one would expect that a reasonably adequate amount of soluble antigen employed for competitive inhibition should inhibit binding of the antibody-enzyme conjugate to solid phase antigen. Surprisingly this is not so. The inhibitory capacity of the sonicate is saturated at 30% inhibition and that too with as little as 50 ng of inhibitor antigen. The likely explanation for this phenomenon is in the nature of the soluble antigen employed, namely the sonicate of H₃₇Rv. The latter contains a large amount of lipid and hydrophobic antigens (84). It is possible that in the aqueous buffers the antigen forms micelles and as the concentration of micelles increases they sterically hinder the antigen antibody interaction. In fact if 0.22 µm membrane filters are employed instead of the usual 0.45 µm filters for conducting washes in the assay system, the soluble immune complexes can no more pass through the filters

indicating the larger size of the complexes. (Data not shown in the results section). This is very much in favour of the explanation that the lipid in the sonicate, in fact, forms micelles.

In the present study, 37 CSF samples were analysed for the presence of mycobacterial antigens, by ELISA. Out of the 37 cases 15 cases were diagnosed and treated as TBM on the basis of clinical presentation and the routine examination of CSF. Twelve cases out of 15 were positive for the presence of mycobacterial antigens by ELISA. One case was proved to have TBM after autopsy, three cases were proved by culture and six cases showed distinct clinical improvement at the end of one month. All the ten cases were positive by ELISA. A few patients with normal CSF profiles, a few with a definite diagnosis other than tuberculosis (e.g., proved cases of pyogenic meningitis) and a few with abnormal CSF profiles without any definitive diagnosis were studied. All these cases served as control CSF samples. In none of these cases ELISA was positive. From this discussion it is clear that the present enzyme-linked immunosorbent assay can detect the mycobacterial antigens in clinically or

bacteriologically proven cases of TBM. In cases of meningitis of other etiologies, meningeal irritation and in cases with normal CSF profiles the test remains negative.

These data clearly indicate that there are no false positive results with the test system and that it detects fairly large number of cases (12 out of 15).

Another important feature which needs to be discussed is specificity. Mycobacteria are known to share some antigenic specificities with nocardia and corynebacteria (92). The antiserum employed in the test system has not been studied for reactivity against the antigens of nocardia and corynebacteria. It is also important to note that the antiserum is not necessarily specific for M.tuberculosis-H₃₇Rv. In fact, preliminary studies indicate that it does react with antigens of other mycobacteria including the saprophytic species. However, in body fluids such as CSF, pleural and peritoneal fluid, synovial fluid where nocardia, corynebacteria and saprophytic mycobacteria are not likely to occur as contaminants one may employ the test effectively. In contrast the utility of the test is

doubtful in samples of sputum and urine. In the former corynebacteria and in the latter saprophytic mycobacteria may pose a problem. It is also important to note that the test detects antigens of mycobacteria, without identification of the species, hence positive test should not be interpreted as an evidence for the presence of Mtuberculosis.

In conclusion, presence of mycobacterial antigens in CSF, as detected by ELISA, can be employed as an evidence of mycobacterial meningitis. The test is quite sensitive and it can give a rapid diagnosis.

SUMMARY AND CONCLUSIONS

An enzyme-linked immunosorbent assay employing the principle of competitive inhibition was established to evaluate its utility in detecting mycobacterial antigens in the CSF in place of bacteriological diagnosis of tuberculous meningitis. M.tuberculosis-H₃₇Rv cells were employed as solid phase antigen. Rabbit antibody against rapidly moving, water soluble protein antigen of the sonicate of M.tuberculosis-H₃₇Rv was conjugated with a high specific-activity alkaline phosphatase enzyme preparation employing the 'one-step' glutaraldehyde method. The binding of the antibody enzyme conjugate to the solid phase antigen could be inhibited by as little as 10 ng of protein antigen making the assay quite sensitive.

Thirty seven samples of CSF were tested for the presence of mycobacterial antigens. Significant inhibition of binding of the antibody-enzyme conjugate to the solid phase antigen by 0.5 ml of undiluted CSF was treated as a positive test. Fifteen samples of CSF tested were from cases diagnosed on clinical examination and CSF profile, to be of tuberculous meningitis. Out of

these twelve cases gave positive results. In three of the cases diagnosis could be confirmed by isolation of acid-fast bacilli from CSF; in one by autopsy and in six cases by follow-up studies including follow-up CSF examinations after treatment and clinical recovery. Seven CSFs from cases of pyogenic meningitis, six cases of viral meningoencephalitis, six CSFs from cases of febrile convulsions and three cases of meningismus were also investigated and were found to yield negative results by ELISA for mycobacterial antigens. Thus the data shows that the test does not give false positive results and that it is quite sensitive (12/15 suspected cases gave positive results).

APPENDIXA) Preparation of media1) Youmans' medium (82)

Asparagine	...	0.5 gm
Potassium dihydrogen phosphate (anhydrous)	...	0.5 gm
Potassium sulphate	...	0.05 gm
Glycerol	...	2.00 ml
Magnesium citrate	...	0.15 gm
Distilled water	to make	100 ml

All chemicals used were of analytical grade.

The ingredients were dissolved in the order given, in glass distilled water. Each ingredient was dissolved completely before the next one was added. Magnesium citrate was added after the pH was adjusted to 7.0 with 40% sodium hydroxide. The medium was filtered through Whatman No.1 filter paper (Whatman Ltd., Maidstone, England) to remove any turbidity. The medium was dispensed either as 10 ml aliquots in test tubes or in larger volume in conical flasks as necessary and autoclaved at 121°C for 15 minutes.

2) Lowenstein-Jensen medium (85)

Salt solution -

Potassium dihydrogen phosphate	...	2.4 gm
Magnesium sulphate, (7H ₂ O)	...	0.24 gm
Magnesium citrate	...	0.6 gm
Asparagine	...	3.6 gm
Glycerol	...	80.0 ml
Distilled water	...	600.0 ml

Potato flour	...	30.0 gm
Homogenized whole eggs (20)	...	1000.0 ml
Malachite green (2% aqueous)	...	20.0 ml
Penicillin (28,000 I.U./ml)	...	4.0 ml
Nalidixic acid	...	5.6 mg

The medium was purchased from Haffkins

Bio-Pharmaceutical Corporation Ltd., Bombay; as slants in sterile 25 mm x 100 mm screw capped tubes (Corning, India).

Preparation of buffers

1) Phosphate buffered saline (PBS)

Sodium chloride	...	8.0 gm
Di-sodium hydrogen phosphate (Na ₂ HPO ₄ , 2H ₂ O)	...	1.44 gm
Potassium dihydrogen phosphate (anhydrous)	...	0.2 gm

Dissolved in 1 litre of distilled water; pH adjusted to 7.2.

2) Tris - HCl buffer

Tris (hydroxymethyl) aminomethane (0.2 M)	... 50 ml
HCl (0.2 M)	... 26.8 ml
Distilled water	to . 200 ml

pH adjusted to 8.0 with HCl or Tris solution as necessary.

3) Alkaline barbital buffer (pH 8.5)

Sodium barbital	... 1.0 gm
Sodium chloride	... 5.0 gm
Distilled water	... 100 ml

All chemicals used for the preparation of buffers were of analytical grade.

C) Preparation of reagents and standardization of biochemical tests

1) Lowry's method for estimation of protein (83)

In this method various amino acids mainly tyrosine and tryptophan bring about a reduction of

tungstate and/or molybdate from the Folin-Ciocalteu reagent to produce coloured compounds.

Preparation of reagents

a) Folin Ciocalteu reagent (2 N) was purchased from Centron Research Laboratories, Bombay. The reagent was diluted 1:1 with distilled water just before use.

b) Alkaline copper tartrate reagent -

1) Copper sulphate solution (1%)

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	...	1 gm
Distilled water	...	100 ml

The solution was stored at 4°C.

2) Sodium potassium tartrate solution (2%)

Sodium potassium tartrate	...	2 gm
Distilled water	...	100 ml

The solution was stored at 4°C.

3) Sodium carbonate (Na_2CO_3) in sodium hydroxide solution (2% in 0.1 N)

Na_2CO_3	...	2 gm
NaOH	...	0.4 gm
Distilled water	...	100 ml

This solution was prepared fresh every day just before use.

0.5 ml of (1) 0.5 ml of (2) and 50 ml of (3) were mixed just before use to give the alkaline copper tartrate reagent.

c) Stock bovine serum albumin solution (2 mg/ml)

Ten mg of bovine serum albumin (BSA) (Sigma) were dissolved in 5 ml of glass distilled water and kept frozen. This was used as a stock solution and appropriate dilutions were made when needed.

d) Standard protein solution - Stock BSA solution was diluted to give a concentration of 100 µg/ml and used as a standard for every assay.

All chemicals used were of analytical grade.

For estimation of proteins first a standard curve was prepared using BSA as a standard protein.

The stock BSA solution was diluted with distilled water to give concentrations of 10 µg, 40 µg, 60 µg, 80 µg and 100 µg per ml respectively. 0.5 ml of these protein solutions was transferred into a series

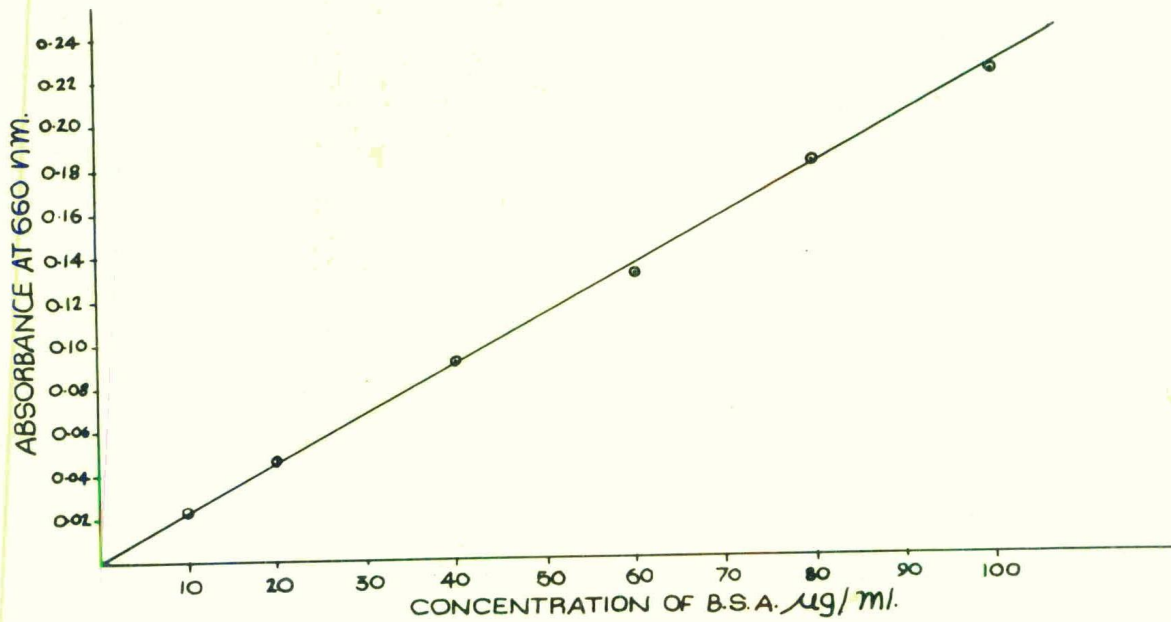
of test tubes. 0.5 ml of distilled water was used as blank. To these tubes, 2.5 ml of alkaline copper tartrate reagent was added. The contents were mixed thoroughly and allowed to stand for 15 minutes. Subsequently 0.25 ml of diluted Folin - Ciocalteu reagent was added and the absorbance read 30 minutes after addition on a PMQ II spectrophotometer at 660 nm in cuvettes of 1 cm path length. All samples were run in duplicate. The graph of protein concentration Vs. absorbance was plotted and used as the standard curve (Figure 6).

For every assay, a blank and a standard protein solution were run.

2. Nelson-Somogyi's method for estimation of reducing substances (57)

The reducing substances in protein-free filtrate reduce alkaline cupric salts to cuprous salts, which react with the arsenomolybdate reagent in acidic medium to give blue colour.

Figure 6 : A standard reference curve for estimation of protein using BSA as standard.



Preparation of reagents**1) Isotonic diluent**

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	...	6.0 gm
Sodium sulphate (anhydrous)	...	12.6 gm
Distilled water	...	1000 ml

2) Sodium tungstate solution

Sodium tungstate	...	10 gm
Distilled water	...	100 ml

3) Somogyi's reagent**Solution A -**

Sodium carbonate (anhydrous)	...	24.0 gm
Sodium potassium tartrate	...	12.0 gm
Distilled water	...	250.0 ml

Solution B -

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	...	4.0 gm
Distilled water	...	50.0 ml
Sodium bicarbonate	...	16.0 gm

Copper sulphate was dissolved in water completely.
Sodium bicarbonate was added to the solution with
constant stirring.

Solution C -

Sodium sulphate (anhydrous)	...	180.0 gm
Boiled cooled distilled water	...	500.0 ml

Solutions A (250 ml), B (50 ml) and C (500 ml) were mixed and the volume made to 1 litre and stored at room temperature.

4) Nelson's arsenomolybdate reagent**Solution A -**

Ammonium molybdate	...	25.0 gm
Distilled water	...	450.0 ml
Concentrated sulphuric acid	...	21.0 ml

Solution B -

Sodium arsenate	...	3.0 gm
Distilled water	...	25.0 ml

Solution A (471 ml) and B (25 ml) were mixed and the volume made to 500 ml. The solution was kept in the incubator at 37°C for 24-48 hours and stored in an amber coloured bottle thereafter at room temperature.

5) Standard dextrose (D-glucose) solution -

Dextrose	...	100 mg
Saturated solution of benzoic acid	...	100 ml

6) Saturated benzoic acid solution -

Benzoic acid	...	300 mg
Distilled water	...	100 ml

The solution was prepared and the undissolved benzoic acid settled at the bottom was removed by filtration. The solution was stored at room temperature.

All chemicals used for the preparation of various reagents were of analytical grade.

Preparation of a standard curve

The standard dextrose solution (100 mg%) was diluted in saturated solution of benzoic acid to get 10, 30, 50, 70 and 100 mg per 100 ml concentrations. 0.1 ml of each was transferred to a series of test tubes. 3.7 ml of isotonic diluent and 0.2 ml of sodium tungstate solution were added and the precipitate formed was sedimented by centrifugation at 800 g max for 10 minutes. One ml of

the supernatant was transferred into a Folin tube containing 1.0 ml of Somogyi's reagent. One ml of distilled water served as the blank.

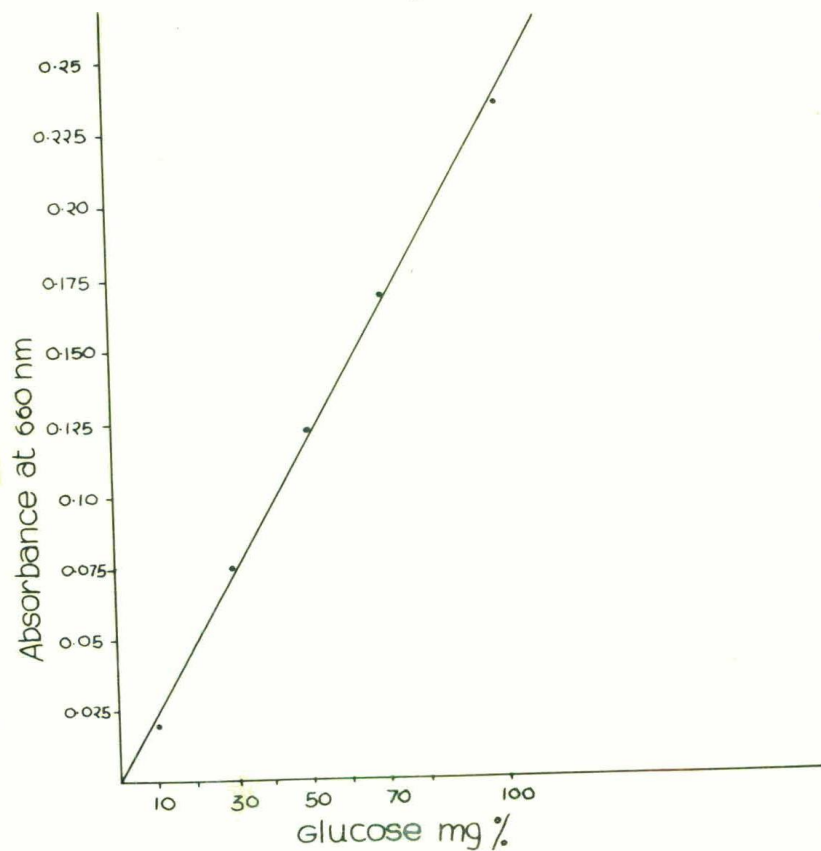
The mixture was heated in a boiling waterbath for 15 minutes. After cooling for one minute, 1.0 ml of Nelson's reagent was added. The volume was made upto 12.5 ml with distilled water. The colour was read at 660 nm on the spectrophotometer in a cuvette of 1 cm path length. The graph of glucose concentration Vs. absorbance was plotted (See Figure 7).

With each assay of samples a standard of 100 mg% of glucose and a blank were run. Reducing sugar concentrations as low as 10 mg% were detectable by this method.

3. Chloride estimation by modified Volhard's method (88)

Chlorides were precipitated as silver chloride with silver nitrate (AgNO_3) solution. The excess of AgNO_3 was back titrated with potassium thiocyanate (KSCN) using ferric-alum as indicator.

Figure 7 : A standard reference curve for estimation of sugar (reducing substances) using glucose as standard.



Preparation of reagents1) AgNO_3 solution

AgNO_3	...	2.907 gm
Distilled water	...	1000 ml

Stored at room temperature in an amber coloured bottle.

2) KSCN solution

KSCN	...	1.8 gm
Distilled water	...	1000 ml

3) Ferric-alum indicator powder

All chemicals used were of analytical grade.

The AgNO_3 solution and the KSCN solution were titrated against each other using ferric-alum indicator.

1.0 ml AgNO_3 neutralised 1.0 ml of KSCN .

4. Preparation of GSF diluting fluid

Crystal violet	...	0.2 gm
Glacial acetic acid	...	10.0 ml
Distilled water	...	100.0 ml

D) Miscellaneous

1) Gel diffusion in agarose

100 mg of agarose (Sigma) were mixed with 10 ml of PBS and the mixture heated in a boiling waterbath with intermittent shaking till the agarose was completely dissolved. The solution was allowed to cool to 70°C and 2.5 ml were poured on a 25 mm x 75 mm microscope slide. The slide was cooled to obtain a well-formed gel. Wells (3.0 mm diameter) were punched with a gel-punch and a template. Interwell distance was 7 mm. After filling the wells with antigen and antibody solutions, the slide was kept in a moist chamber for 24-48 hours at 4°C. The slide was then washed in saline overnight to remove excess protein from the gel matrix. It was then pressed and dried overnight at 37°C. The dried gel slide was stained with 0.05% solution of Coomassie Brilliant Blue R 250 (Sigma) for 15 minutes after which it was put in the destainer solution overnight. The immunoprecipitates were stained blue.

Preparation of reagents

a) Coomassie Brilliant Blue stain -

Coomassie Brilliant Blue R 250 (Sigma) ... 50 mg

Methanol	...	10.0 ml
Glacial acetic acid	...	10.0 ml
Distilled water	...	80.0 ml

b) Destainer solution -

Methanol	...	10.0 ml
Glacial acetic acid	...	10.0 ml
Distilled water	...	80.0 ml

All chemicals used were of analytical grade.

2. Preparation of saturated solution of ammonium sulphate

550 gm of ammonium sulphate were weighed and added to 600 ml of distilled water. The mixture was heated gently till all the salt was dissolved. It was filtered through a coarse filter paper. The clarified solution was made to 990 ml after it had attained room temperature. The pH was adjusted to 6.5 with ammonium hydroxide and the volume made to 1000 ml. Some amount of undissolved salt remained at the bottom.

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