ADENOSINE DEAMINASE ACTIVITY IN THE DIAGNOSIS OF TUBERCULOUS MENINGITIS AND ITS LEVELS IN OTHER DISEASES

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The experimental work described in this project report was performed by me Ofori Mensah at the Department of Chemical Pathology University of Ghana Medical School under the supervision of Professor S. V. Rana and Dr. S. Q. Maddy.

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DEDICATION

This project is dedicated to my wife Ama Agyeiwah and my children Yaw Bawua and Yaa Brempomah.
I express my utmost gratitude to my supervisors Professor S. V. Rana and Dr S. Q. Maddy whose moral support, patience and encouragement helped me to successfully complete this project. My special appreciation goes to Professor S. V. Rana whose original idea made this project a reality. I would also like to thank all the physicians who contributed to the success of the project with special mention of Dr. B. Goka, Dr. J. Welbeck and Dr. Joseph Anthony whose dedicated attention to the paediatric patients included in this project contributed immensely to the success of the project.

I also appreciate Dr. A. G. B. Amoah’s advice on diabetes and the offer of Glycated Haemoglobin test kits. Finally I wish to thank all the members of the Department of Chemical Pathology, especially my student Mr. Vincent Buabeng for their immense contribution to the success of the project.
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<td>ADA</td>
<td>Adenosine Deaminase</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute Lymphatic Leukaemia</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine Aminotransferase</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate Aminotransferase</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacille-Calmette-Guérin</td>
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<tr>
<td>BPT</td>
<td>Bromide Partition Test</td>
</tr>
<tr>
<td>CMI</td>
<td>Cell Mediated Immunity</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CP</td>
<td>Combining Protein</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>d-ATP</td>
<td>Deoxyadenosine Triphosphate</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes Mellitus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DOTS</td>
<td>Directly Observed Treatment Short-course</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed Type Hypersensitivity</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
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<tr>
<td>FBG</td>
<td>Fasting Blood Glucose</td>
</tr>
<tr>
<td>GHb</td>
<td>Glycated Haemoglobin</td>
</tr>
<tr>
<td>GTB</td>
<td>Global Programme on Tuberculosis</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>HD</td>
<td>Healthy Donors</td>
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<td>Adenosine deaminase activity</td>
<td>x</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>INF</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IU</td>
<td>International Units</td>
</tr>
<tr>
<td>IUATLD</td>
<td>International Union Against Tuberculosis and Lung Disease</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi-Drug Resistant</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>NaOCl</td>
<td>Sodium Hypochlorite</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>Type II DM</td>
<td>Non Insulin Dependent Diabetes</td>
</tr>
<tr>
<td>p</td>
<td>Probability (at 5% significant level)</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase-Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PIA</td>
<td>(-)-N(^\text{6})(R-phenyl-isopropyl) Adenosine</td>
</tr>
<tr>
<td>PPD</td>
<td>Purified Protein Derivative</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>Soln.</td>
<td>Solution</td>
</tr>
<tr>
<td>Std.</td>
<td>Standard</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
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<tr>
<td>TU</td>
<td>Tuberculin Unit</td>
</tr>
<tr>
<td>TBM</td>
<td>Tuberculous Meningitis</td>
</tr>
<tr>
<td>NTP</td>
<td>National Tuberculosis Programme</td>
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<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>vs.</td>
<td>Versus</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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<td>Adenosine deaminase activity</td>
<td>xi</td>
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<tr>
<td>Symbol</td>
<td>Description</td>
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<tr>
<td>&lt;</td>
<td>Less Than</td>
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<td>&gt;</td>
<td>Greater Than</td>
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<td>±</td>
<td>Plus or Minus</td>
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The diagnostic value of Adenosine Deaminase was studied to evaluate and establish a cut-off point for the diagnosis of tuberculous meningitis and also to establish its levels in other diseases in the local setting. There were 358 subjects in the study. The mean age of the adults was 29 ± 2; with a range of 13-74 years, while the age of the infants ranged from one day to 13 years. These were made up as follows: ‘Apparently healthy donors’ (30); tuberculous meningitis (230); pulmonary tuberculosis/pleural effusion (41); diabetics (15); Human Immunodeficiency Virus (22); hepatitis (10) and renal failure (10). The cut-off point was 9.0 IU/L with a sensitivity and specificity of 100% and 97% respectively for the diagnosis of tuberculous meningitis. The enzyme activity was compared with glycated haemoglobin in the long term monitoring of diabetics. The mean serum Adenosine Deaminase activity of healthy donors and Type II Diabetes Mellitus patients (before treatment) were 16.0 ± 1.0 and 38.3 ±2.0 International Units/Litre respectively; \( p < 0.001 \) versus healthy donors). After 8 weeks of treatment, the mean Adenosine Deaminase activity was reduced to 27.1 ± 1.6 IU/L significantly less than the initial value before treatment \( p > 0.001 \). The mean Glycated Haemoglobin in healthy donors and Non Insulin Dependent Diabetes Mellitus patients were 5.5% and 14.0% respectively \( P < 0.001 \) versus healthy donors). With respect to the other diseases, the mean serum Adenosine Deaminase for hepatitis, renal failure, Human Immunodeficiency Virus and Acquired human Immunodeficiency Syndrome (tuberculosis positive patients) were 50 ± 2, 38 ± 1, 41 ± 1 and 64 ± 2 IU/L respectively. Pleural effusion Adenosine Deaminase level for pulmonary tuberculosis was 94 ± 1 IU/L. Even though Adenosine Deaminase activity is sensitive for the detection of tuberculosis it was not very specific. The specificity increases when interpreted together with clinical signs and other test results especially Cerebrospinal Fluid protein and cell count. Measurement of total Adenosine Adenosine deaminase activity
Deaminase activity may also be an alternate important tool for monitoring Type II Diabetes Mellitus patients. In 22 patients who tested positive for the Human Immunodeficiency Virus the mean serum Adenosine Deaminase level in 10 tuberculosis positive patients was $75 \pm 1$

The remaining 12 all had full blown aids; ten of whom were TB positives with ADA level of $64 \pm 2$. While the remaining two were TB negative with ADA levels of 46 and 27 IU/L levels respectively. In liver disease 10 patients with acute hepatitis had a mean serum Adenosine Deaminase level of $50 \pm 2$ IU/L. There was no correlation between Adenosine Deaminase and Aspartate Aminotransferase but there was a moderate correlation ($r = 0.5; \ p < 0.02$) between Adenosine Deaminase and Alanine Aminotransferase. In renal disease 10 patient who had renal failure had a mean serum Adenosine Deaminase level of $38 \pm 1$ IU/L. There was no correlation between Adenosine Deaminase and any of the renal diagnostic parameters.
Chapter 1: GENERAL INTRODUCTION

1.1. Tuberculous Meningitis

Miliary tuberculosis is an illness produced by acute diffuse dissemination of tubercle bacilli via the blood stream. Although the overt disease is relatively easily diagnosed, cryptic forms are not at all uncommon. In communities where tuberculosis is now greatly decreased these cryptic forms in older people contribute an important proportion of cases of miliary tuberculosis and are sometimes diagnosed only at post-mortem. When tuberculosis was widespread in a community young children were likely to be infected. The majority of cases of miliary tuberculosis closely followed the primary tuberculous infection, for young children are particularly susceptible to haematogenous spread.

When tuberculosis was common and children were frequently infected, a high proportion of cases occurred in very young children whose resistance to hematogenous dissemination is known to be poor. Measles and whooping cough are well known predisposing factor. There is some evidence that measles may damage the thymus and so affect the T lymphocytes but a direct effect of the virus has not been shown in vitro. Miliary tuberculosis may occur in patients receiving corticosteroid drugs presumably because of lowering of resistance to the disease.

In the commonest type of miliary tuberculosis the classical lesion is a focus the size of a millet seed, a few millimetres in diameter, which gives it its name. These lesions consists of epithelioid cells, lymphocytes and Langhans' giant cells, often with central caseation.
appreciable proportion of patients are first seen with tuberculous meningitis. The disease remains a major problem and an important cause of death in some countries. Human *mycobacteria tuberculosis* is now responsible for most cases of tuberculous meningitis but opportunistic mycobacteria may cause the disease in patients with AIDS.

In the cause of spread from the primary tuberculous focus, or as part of miliary spread, tiny tubercles are seeded into the brain and meninges. Occasionally they may also be seeded into the bones of the skull or the vertebrae. These tubercles may rupture into the subarachnoid space and cause inflammation of the meninges, formation of a grey jelly-like mass at the base of the brain, and inflammation and narrowing of the arteries leading to the brain which may cause brain damage. These three processes produce the clinical picture of tuberculous meningitis.

There is usually a history of general ill-health for 2-8 weeks - malaise, tiredness, irritability, changes in behaviour, loss of weight and mild fever. Then as a result of inflammation of the meninges, there will be headaches, vomiting and neck stiffness. The inflammatory exudate involving the base of the brain may affect the cranial nerves II to VIII giving some specific clinical including deterioration of vision, paralysis of an eyelid, squint, unequal pupils and deafness. Papilloedema is present in 40 percent of patients. Involvement of the arteries to the brain may lead to fits, loss of speech or loss of power in a limb or limbs. Some degree of hydrocephalus is common. This is due to blocking by exudate of some of the cerebrospinal fluid (CSF) connections within the brain. The resulting damage may be permanent and probably account for the bad prognosis in patients who are only seen when they are already unconscious. Spinal block by exudate may cause upper motor neurone weakness or paralysis.
The tuberculin test may be negative especially in advanced stages of disease.

With the development of modern transport and the frequency with which people move about the world, no country can afford to regard tuberculosis as a parochial problem. The relatively high degree of control of tuberculosis in economically developed countries is mainly due to effective chemotherapy and the low prevalence of drug resistance. But in developing countries drug resistance has become a very formidable challenge. With the movement of populations, this problem could well spread to the economically developed countries and result in cases of tuberculosis which would be difficult to control. The complete control of tuberculosis on a world-wide basis is therefore in everyone's interest.

1.2. Clinical Tuberculosis

When pulmonary tuberculosis develops in humans, it often shows a composite picture. Tuberculosis (TB) is a local disease influenced by the local concentration of antigen and the states of activation (and probably sensitisation) of the local macrophages and lymphocytes. Each lesion is handled by the host almost as if the other lesion did not exist. Thus, lesions in one area of the lungs may liquefy and progress, while lesions in other parts may stabilise or regress. Even parts of a single lesion may progress, while other parts remain stable or regress. Finally the disease itself may fluctuate between periods of exacerbation and remission.

Primary infection with virulent mycobacteria induces a state of memory immunity, mediated by T cells. Cells mediating DTH are almost certainly a component of this mechanism. It is the purpose of vaccination, therefore to similarly establish a long-lived state of specific resistance.
to infection retained by a previously clonally expanded population of memory T cells sensitised
to key bacterial antigens that rapidly betray the presence of the secondary infection 4.

Tuberculosis is an infectious disease caused by the tubercle bacilli. Up to about 40 years ago
most people in the world, were infected at sometime in their lives. Fortunately, owing to the
good defences of the host and perhaps to the small number of infecting bacilli, the vast
majority of people overcame the invaders without any evidence of the illness. In a small
proportion the infecting dose was so large, or the host resistance so poor, that clinical
tuberculosis resulted 1. In the United States tuberculosis has become an inner-city disease 5.

With the increased control of malaria tuberculosis has become the world's most important
communicable disease. This is easily forgotten in economically developed countries where
mortality has fallen dramatically, and there has been an appreciable fall in morbidity, although
in fact there are very great differences between different developed countries and within these
countries the problem is often larger than it is recognised. In the economically developing
countries the disease is one of the principal causes of death and suffering. For instance a
survey in Vietnam showed that 0.7% of the population had a sputum positive for the tubercle
bacilli on direct smear. Pulmonary tuberculosis is the most important manifestation of the
disease both because it is by far the most common and because patients with pulmonary
tuberculosis are the principal source of infection 1.
1.3. Aims And Objectives

The principal aims and objectives were:

1 To establish a cut-off point for ADA activity in cerebrospinal fluid for the purpose of making an early diagnosis of tuberculous meningitis.

2 To establish the enzyme levels in other body fluids e.g.
   a. Blood
   b. Pleural effusion
   c. Ascitic fluid, and

3 To establish the enzyme levels in other diseases states e.g.
   a. Diabetes Mellitus
      i. Relationship between ADA and hyperglycaemia
      ii. Comparison between ADA activity and glycated haemoglobin in Type II DM
1.4. Literature Review

1.4.1. History and Epidemiology of Tuberculosis

Tuberculosis is a disease of great antiquity. What were almost certainly tuberculous lesions have been found in the vertebrae of Neolithic man in Europe and in Egyptian mummies perhaps as early as 3700 BC. In early writings pulmonary tuberculosis may well have been confused with other pulmonary diseases. However it seems certain that the disease was common in the Hellenic and Roman periods, and in ancient India and China. Tuberculous lymphadenitis appears to have been widespread throughout medieval Europe. In Europe and North America tuberculosis seems to have increased disastrously during the industrial revolution reaching its peak in the late eighteen century. By 1800 the mortality in New England in the northern USA exceeded 1 percent per year. Until the advent of the Human Immunodefeciency Virus (HIV) disease which predisposes people to the chemotherapy, Bacille-Calmette-Guérin (BCG) and improved medical services have impressively accelerated the decline of the disease.

The present global TB situation indicates that both the prevalence of the disease and the death burden are still very high in developing countries as opposed to the developed countries. Estimates by the World Health Organisation (WHO) and the International Union Against Tuberculosis and Lung Disease (WHO/IUATLD, 1989) show that each year there are about 8-10 million new cases and approximately 3 million new deaths attributable to TB world-wide. More than 75% of these cases and over 95% of deaths occur in developing countries. Furthermore, the number of new cases per 100,000 population per year is about 10 in developed countries compared to between 100-300 in developing countries. In many Adenosine deaminase activity
developing countries, up to about 50% of the adult population aged 15 to 49 years is infected with the tubercle bacilli \textsuperscript{7}, and the average lifetime risk of progression from infection to active disease, which varies with age and immunological status of the affected individual, is about 10\% \textsuperscript{6}.

Further estimates indicate that among disease persons, the rate of death in the absence of treatment is more than 50\% within five years, but this can be reduced to less than 3\% with prompt diagnosis and adequate treatment \textsuperscript{8}. In many developing countries, less than 50\% of patients with infectious pulmonary TB (smear positive cases who are the principal source of infection in the community), are diagnosed, and less than 50\% of these cases are completely cured of the disease with the treatment regimes often used in many of these poor countries \textsuperscript{9}. This relatively low cure rate is attributed to a number of factors including the use of relatively less effective drug regimes taken for relatively long duration (e.g. The 12 month regime with streptomycin, isoniazid and thiacetazone). This often leads to high default and relapse rates. Another factor is the social stigma associated with the disease which often causes delay in patients seeking care \textsuperscript{6}.

With the advent of the HIV/AIDS pandemic, the TB situation has resurfaced in the countries developed whereas in the developing countries of Africa, Asia and Latin America, the already bad situation is getting worse \textsuperscript{6}. Evidence from the United states of America (USA) and many other countries shows an upsurge in the overall incidence of TB which until the mid-1980’s was on a progressive decline \textsuperscript{10}. This contemporary upsurge in the USA and many developed countries have been attributed to the HIV/AIDS pandemic. A similar trend has also been shown in some African countries like Tanzania, which has been operating an effective and
hitherto successful national TB control programme. Again the upsurge in Ghana has been blamed on the HIV/AIDS pandemic.

The situation of TB infection in Ghana shows a relatively high prevalence among both the rural (up to 40% in adults, as measured by a tuberculin survey) and urban population (over 60% in adults). Even though the reporting and surveillance systems for TB in this country is poor, available data on the number of reported cases per annum from the regions of the country show a progressively increasing rate over the past ten years from 15 per 100,000 individuals in 1993 to 65 per 100,000 in 1998. It must however be noted that these figures may be low due to the significant degree of underreporting in the present system.

According to the National Tuberculosis Programme (NTP) of Ghana, between 1995 and 1998, new smear positive cases rose from 2,625 to 7,648 per 100,000. All forms of TB rose from 4,118 to 11,154 per 100,000 within the same period. The incidence for smear positive cases rose from 16.4 to 47 per 100,000 while the incidence for all forms of TB rose from 25.7 to 66 per 100,000 within the same period. A number of important factors can be blamed for this state of affairs regarding TB in the country. The first is the long absence of a well organised and functioning National TB control programme. The second factor is the worsening living conditions (i.e. overcrowding and malnutrition), especially in urban slums, due to the worsening of the poverty complex, particularly among the working class. Thirdly, the probable emergence and spread of multi-resistance TB, which has resulted in the use of a rather ineffective and lengthy drug regimen which often leads to poor treatment compliance and high default rates by patients. Finally there is no doubt that this contemporary upsurge may be related to the increasing rate of HIV/AIDS since the progressive immunosuppression...
caused by the HIV enhances the development of TB.

Efforts to ascertain the epidemiology of TB in Europe have been hampered by differences in definitions and in the quality of TB surveillance systems between countries. In order to standardise TB surveillance in Europe, consensus recommendations, including a common case definition and a minimum set of variables, were prepared by a working group set up by WHO and the International Union against TB and Lung disease in 1996, and approved by the countries involved. Based on these recommendations, the European TB project (supported by the Directorate General V of the commission of the European Communities) was initiated in October 1996. The objective was to assess the willingness of countries to participate in an European surveillance system which was to collect information on TB cases reported in 1995, assess the consistency between the information collected and that recommended and to provide baseline data for future evaluation of the impact of the recommendations.

In 1995, 276811 cases of TB were reported in 46 European countries. The number of cases by country varied from one case in Monaco to 96828 in the Russian Federation. National incidence rate varied greatly from 2.7 per 100 000 population in Malta to 101.9 per 100 000 in Romania. The incidence rates were below 20 in twenty two countries. In 32 countries that reported the age of all new and recurrent cases (n=107096), 4% of the cases were children under 15 years of age, 77% adolescent and adults up to 64 years of age, and 19 people aged 65 years or over. The overall male-to-female ratio was 1.8. In thirty four countries that reported new or recurrent status, 10% of the cases were recurrent with small variations between countries.
Dramatic outbreaks of multi-drug resistant tuberculosis (MDR-TB) in HIV-infected patients in the USA and Europe have focussed attention on the emergence of strains of *Mycobacterium tuberculosis* that are resistant to anti-mycobacterial agents. However, resistance of the tubercle bacilli to anti-mycobacterial agents is a world-wide problem in both immunocompetent and HIV-infected populations. The true magnitude of the problem has not been adequately documented; nevertheless, anecdotal data suggest that drug resistance is currently high in Asia and Africa. An adequate assessment of the drug resistance throughout the world and significant comparisons between different countries have not been successful. The reasons include insufficient *M. tuberculosis* culture facilities and inability to perform anti-mycobactericidal susceptibility testing in most countries of the world, absence of standardised or adequate laboratory methodologies and the lack of surveillance systems in many countries. Additionally there is an absence of longitudinal studies and selection bias in many surveys as well as failure to distinguish between initial and acquired drug resistance.

Drug resistant tuberculosis, particularly multi-drug resistant strains of tuberculosis (MDR-TB) is a major potential threat to tuberculosis control because only a few drugs are effective and available especially in low-income countries against *M. tuberculosis*. The spread of strains resistant to the 2 most important drugs, isoniazid and rifampicin, could have serious repercussions on the epidemiology and control of tuberculosis. In addition to patients infected with strains resistant to multiple drugs less likely to be cured, there is also the significant increases in the cost of each patient treated. Moreover, the level of anti-tuberculosis drug resistance can be considered as an indicator of NTP performance. Curing patients rapidly
prevents chronic cases resistant to drugs (acquired resistance), and does not allow resistant
strains to spread to previously uninfected individuals (primary resistance)\textsuperscript{14}.

In early 1994, the WHO Global Programme on Tuberculosis (GTB) joined forces with the
International Union Against Tuberculosis and Lung Disease (IUATLD) and started a global
project on tuberculosis drug resistance surveillance. The overall aim was to improve NTP
performance by contributing to policy recommendations on treatment. The objectives were to
collect data on the extent and severity of anti-tuberculosis drug resistance world-wide in a
standardised manner at country level, particularly in countries identified by WHO and
IUATLD as priorities for assistance; to help countries develop a system of surveillance of drug
resistance and improve the diagnostic capacity of laboratories; and to revise the policy on
tuberculosis treatment only under special circumstances, based on the correlation between
drug resistant level and treatment policies adopted in different countries\textsuperscript{14}.

1.4.3. \textit{Mortality}

The ten-year mortality from tuberculosis since causes of death were first reported in the mid
nineteenth century in England, Wales and Scotland showed a marked overall decrease. The
major factors in the decrease may have been due to improvement of the host resistance by the
gradually rising standards of living and by the survival and procreation of individuals with a
higher natural resistance to the disease. The improvement in housing and working conditions
and the provision of facilities for isolating at least some of the most infectious patients, has
presumably also decreased the risk of receiving a high infecting dose. The increase in mortality
during the two world wars is obvious and the rapid decrease following the introduction of
The fall in mortality has been particularly marked in younger age groups. The fall is less in older people, particularly males, where problems and diseases including diminished respiratory reserve, smoking, alcoholism and misdiagnosis complicate the picture. A survey in Britain showed that many of the residual deaths were preventable. The most important factors were failure of diagnosis, poor treatment and poor social conditions. With the success of chemotherapy residual deaths in developed countries tend to be in those who have escaped diagnosis in life and are only discovered post-mortem, and those who are virtually moribund on diagnosis and whose lungs are so badly damaged before diagnosis that they die of *cor pulmonale*. In a study in England and Wales 20% of tuberculosis were reported after death. According to the NTP of Ghana there were 8.6% deaths in 1995, 5.7% in 1996 and 7% in 1997. At the time of writing there were no figures for 1998 and 1999. Data for these two years would be of interest due to the current reporting of increased HIV prevalence and its association with TB.

Tuberculosis is a major cause of morbidity and mortality world-wide with 95% of new cases in 1990 occurring in the developing countries. In the WHO African Region the incidence of TB in 1990 was 1.4 million cases, the majority of which occurred in sub-Saharan Africa. Eighty percent of cases involved persons in their economically productive years of life (15-59 years). It has been estimated that 26% of avoidable deaths in this category of individuals in sub-Saharan Africa are due to TB. The Human immunodeficiency virus (HIV) infection in sub-Saharan Africa is now strongly associated with TB. In Malawi, in 1989 52% of TB patients were HIV-seropositive and in 1993–1994 in central Hospital in Blantyre 75% were HIV seropositive. As...
a consequence of this strong association between the two infections there has been a big upsurge of TB in many African countries, in the last 10-15 years 15.

1.4.4. Notification

Notification of pulmonary tuberculosis is compulsory in Britain. The patient is supposed to be notified either because he is a danger to other people or when his tuberculosis requires an alteration in lifestyle. The latter is nowadays interpreted to include the necessity of chemotherapy. A notification on the basis of positive bacteriological finding is straightforward although of course it depends partly on the intensity with which tubercle bacilli are sought 1. However there is considerable difficulty in diagnosing TB by the conventional bacteriological and histological method 16 both tests lack sensitivity and culture of tubercle bacilli takes too long to allow an early diagnosis. Meanwhile it is well known that prognosis depend on the interval between the onset of clinical symptoms and the initiation of specific therapy 17.

1.4.5. Prevalence

The number of cases of pulmonary tuberculosis present in a community at any one time is very difficult to estimate accurately. The ideal method will be to institute miniature radiography, tuberculin testing and where relevant bacteriological investigation of the entire population. This has seldom or never been done. A less exacting technique is to carry out sample surveys on a random sample of the population, although this requires careful preparation to ensure that the sample is truly representative. The WHO has sponsored a number of such surveys in different parts of the world, mainly in developing countries. Figures for such countries have
varied from approximately 5 to 50 per 1000 population, the higher figure being characteristic of the Far East. In a study in a rural area without specialised services in south India, the prevalence of culture positive tuberculosis was about 4 per 1000 and the annual attack rate 1 per 1000. About half the diagnosed patients had died within 5 years and a fifth were still excreting bacilli in sputum after 5 years. In developing countries such prevalence studies are the only effective way of measuring the extent of the tuberculosis problem ¹.

1.4.6. **Attack Rate**

As measure of tuberculosis control the attack rate of the disease is theoretically better than the mortality rate which is affected so much by treatment and case fatality, and than the prevalence, which is affected both by the attack rate and case fatality rate. But the attack rate in a community can only be estimated by examining the sputum of, and possibly x-raying, a high proportion of the population on at least two occasions at an interval of one or more years with a subsequent clinical investigation to establish the diagnosis. In a small town in India Frimodt-Møller found the annual attack rate as low as 0.44 per 1000 though the inclusion of ‘inactive’ lesions would have raised it to 0.28% for males and 0.24% for females. The susceptibility of younger people was much less obvious than in Europe, the highest rate being in the 30-49 age group in females and ‘over’ 50 age group in males. There was evidence of a long delay between first infection and the emergence of the disease ¹.

Adenosine deaminase activity
The key to controlling tuberculosis is an effective NTP which has the ability to rapidly detect infectious cases and cure them. In 1991 the World Health Assembly recommended that in order to obtain a favourable impact on tuberculosis control NTPs should work towards achieving the following two targets: (i) to detect 70% new sputum-smear positive cases and (ii) to treat successfully 85% of them. By the end of 1995, the Global Tuberculosis Programme (GTB) had in place a global monitoring and surveillance project which would describe the magnitude of the global tuberculosis epidemic and assess the status of global control measures. Some interesting results emerged when comparing the areas where WHO tuberculosis control strategy was implemented with those where it was not.

For example in those areas where the strategy was implemented 64% of all new pulmonary cases were sputum smear-positive, compared with 33% in those areas where it was not. In areas where the strategy was implemented, 92% of cases registered for treatment were evaluated for outcome of which 33% were treated successfully. In Ghana figures released by the NTP show supervised treatment coverage figures (% of population covered) as follows: 1995 (10%); 1996 (40.5%); 1997 (80%) and 1998 (96%). Of the 80% registered cases in 1997, 51% was recorded as treated successfully. In fact this figure was not very different from the two previous years.

The WHO tuberculosis control strategy produces results which are better than those of any other strategy. Nevertheless only 23% of the world's population currently have access to this strategy. Thus, an NTP's ability to detect and prioritise infectious cases, to cure them and to
evaluate their treatment outcome produces only modest benefits for tuberculosis control on a
global scale. A much wider scale but a sound tuberculosis control strategy is necessary to
control tuberculosis\textsuperscript{18}.

Despite the gloomy predictions about TB epidemic there is a real hope of achieving
satisfactory control by implementing the WHO tuberculosis control programme. This has been
successful in Tanzania, Malawi, Mozambique and Nicaragua. Even in Ghana since the
Ministry of Health embraced the WHO control Programme in 1994 there are signs already of
improved TB services to the various communities. This improvement is threatened by the
emergence of the MDR-TB - defined as resistance to at least Rifampicin and Isoniazid the two
drugs which form the backbone of treatment of tuberculosis. The increase of MDR-TB is real
and very frightening. There are patients who have been taking supervised treatment for two
years or more and are still sputum positive. These patients are free in the community and
anybody unfortunate enough to be infected by them will also have disease resistant to all
drugs. It is therefore vitally important that the TB control programme succeeds and we must
all play our part in this\textsuperscript{19}.

The first reported case of MDR-TB was a 35 year old self employed driver first seen at the
chest clinic of the Korle bu Teaching Hospital in April 1983, and final diagnosis made in 1996
using sputum culture. Over the years he was exposed inappropriately to all the drugs in the
first line anti tuberculosis drugs. His case is not one in isolation. There is the clinical
impression of other such cases and a systemic search for, and documentation of such cases is
needed\textsuperscript{20}.
1.4.7.1. **Bacille-Calmette-Guérin (BCG)**

The theory of BCG vaccination is similar to smallpox vaccination; the artificial induction of a lesion due to a non-virulent organism which gives protection against later infection with a virulent organism. BCG was originally a bovine strain of *M. tuberculosis* which was grown in the laboratory by these French workers for many years. It was found gradually to lose its virulence for laboratory animals and was first used orally in infants in France in 1922. Although a good deal of evidence accumulated regarding its efficacy, the only really well controlled trial carried out in the pre-war years was that by Aronson and his wife in North American Indian children. Highly suggestive results were obtained by Sergent and Ducros-Rougebief who vaccinated the newly born in families which had already lost at least one child from tuberculosis. The major controlled trial by the British Medical Research Council in school leavers, was launched in 1950 and from 1954 most local authorities began the voluntary vaccination of school leavers.¹

Children are now vaccinated in many countries, but at varying ages and with varying completeness of cover. It has been shown that no untoward reaction occurs if individuals already tuberculin positive are vaccinated. This enables mass vaccination to be carried out in developing country without the necessity of a return visit to have a tuberculin test read.¹ It has been suggested that the ingestion and killing of *M. tuberculosis* by human monocyte-macrophages in vitro reflects immunity to infection with mycobacteria.²¹,²² The results of mycobacterial ingestion and survival assessed microscopically and by *M. tuberculosis* colony counts suggest that BCG vaccination increases monocyte viability and uptake of *M. tuberculosis* without increasing the ability of monocytes to killing ingested *M. tuberculosis*.

¹ Adenosine deaminase activity

²¹,²² University of Ghana, http://ugspace.ug.edu.gh
Killing of intracellular mycobacteria by monocytes differs depending on the source of monocytes, the length of incubation and maturation of monocytes to macrophages, the source of the serum added, and the virulence of the mycobacterial strain used to determine intracellular killing. However BCG immunisation has been shown to enhance mycobacteria killing by monocyte monolayers in the presence of autologous lymphocytes activated with whole dead tubercle bacilli.

Observations made by Sepulveda RL et al, suggest that BCG has an important effect on monocyte when given in the new-born period and that BCG immunisation may accelerate a maturational process leading to an increased survival in unstimulated cultures. But they noted that further studies are required to determine the possible influence of age, other stimuli of the immune system, individual variability, and the development of acquired specific T-cell immunity on monocyte viability and function in the first month of life in humans. Thus the increased killing capacity of mononuclear phagocytes seems to depend on the acquisition of T-cell mediated immunity after BCG vaccination.

An effective BCG vaccination apparently does not prevent infection, but interferes with the hematogenous spread of tubercle bacilli, thus reducing the risk of severe primary disease and its complications. i.e. uncontrolled replication and dissemination of *M. tuberculosis* from primary foci to other parts of the lungs and body. For developing countries, BCG vaccination is an attractive policy to protect children against tuberculosis although it does not significantly decrease transmission of the disease. In their study Güler et al noted that half of their patients with CNS tuberculosis had been vaccinated with BCG in the first few days of life. This ratio is very high and gives considerable doubt about the effectiveness of BCG vaccine.
against severe infantile forms of tuberculosis\textsuperscript{25}.

Questions about BCG vaccination that remain unsolved include its overall efficacy in selected population, the duration of protective immunity and how age at vaccination affects protection. A trial in Malawi showed BCG to have no efficacy against tuberculosis at any age, but a 50-70\% protection against leprosy\textsuperscript{26}. In Turkey, children are revaccinated if their tuberculin skin reaction fail to convert to positive within 2-3 months of vaccination, and a second BCG is given during campaigns at primary schools. In their study in Turkey Güler et al\textsuperscript{25} found that none of their patients with CNS tuberculosis had repeated BCG vaccination. This finding, although may well be due to chance needs to be properly evaluated. A small case-controlled study in Chile found no evidence of protection associated with one, two or three BCG scars. A recent trial in Malawi (the Karonga Prevention Trial) also showed that a second vaccination can add appreciably no protection against leprosy, without providing any protection against tuberculosis\textsuperscript{26}.

At the present there is no laboratory test that correlates with the protective efficacy of BCG vaccine. Protection by BCG vaccination is mediated by memory T lymphocytes induced by the first encounter with BCG. Although tuberculin sensitivity occurs after BCG vaccination there is poor correlation between tuberculin reactivity and protection against active tuberculosis. The size of the reaction depends on a number of factors related to both the vaccine and the host. Reasons for differences in BCG efficacy include the use of different strains of BCG, genetic differences, age at vaccination and prevalence of other mycobacteria infections in the population. There is experimental and epidemiological evidence of the impact of nutritional level on the efficacy of the BCG vaccination\textsuperscript{25}.
In conclusion Güler et al did not find any difference in the tuberculin reactions between vaccinated and non-vaccinated children with CNS tuberculosis. They suggest that the presence of BCG vaccine does not influence immunity against *M. tuberculosis* in children with severe forms of tuberculosis. The implications of these findings for public health are that the absence of a tuberculous focus is more important in preventing the disease than BCG vaccination.

1.4.7.2. *Technique of administration*

Both types of vaccine have in the past usually been given by intradermal injection of 0.1 mL in the lower deltoid area. In infants the dose may be divided into two 0.05 mL injections given in on either arm. A papule appears in 3-4 weeks which usually remains for a number of weeks and may ulcerate slightly and discharge. There is sometimes a slight enlargement of the draining lymph glands. The tuberculin test should become positive within three months with ‘wet’ vaccine usually within 6 weeks, with ‘freeze dried’ rather later.

1.4.7.3. *Evidence of efficacy*

There has been 4 well controlled trials of BCG. The first was carried out by Aronson and his wife on North American Indians in 1936-38 and showed a high degree of protection which persisted for 20 years. The mortality rate for tuberculosis in the unvaccinated control group was reduced by 82%. The second by Rosenthal and his colleagues in Chicago infants in 1937-48 gave a 74% protection over 12-23 years. In 1950-52 The British Medical Research Council launched a control trial in 56000 urban school leavers who were followed for 20 years. A 79% reduction in tuberculosis in the vaccinated group was initially achieved, the annual attack rate...
of tuberculosis in this group being 0.40 per 1000 participants compared with 1.91 among those in the initially tuberculin negative unvaccinated group. A 15-year follow-up had shown very similar results, with only a slight waning of protection\(^1\).

In South India a large scale control trial by the WHO in children and young adults, at first showed no benefit regarding the rates of sputum positive tuberculosis. But a moderate benefit appeared in the 15-year follow-up. But of course children seldom develop positive sputum. It can therefore be concluded the Indian trial was not a good trial of the possible benefit of BCG given to children. But other research in several poorer countries has shown increased protection in children by BCG, especially protection against miliary tuberculosis and tuberculous meningitis\(^2\).

The present recommendation by WHO and the International Union against Tuberculosis and Lung Disease is that in countries with high tuberculosis prevalence BCG should be given as a routine to all infants (but with few exception, such as active AIDS). The effect of BCG probably lasts about 15 years, at least in a well-nourished population. It will help to protect in childhood but will loose some of its protection later. Some countries therefore try to repeat BCG about the age of 15. But it is difficult to cover the whole population at this age; and its value when given at this age in tropical countries have not yet been scientifically proved\(^2\). A recent statement made by the WHO concluded that for persons who have received BCG vaccination, repeat vaccination is not recommended\(^28\).

As has been stated earlier in section 1.6 above, an effective BCG vaccination apparently does not prevent infection, but interferes with the hematogenous spread of tubercle bacilli, thus
reducing the risk of severe primary disease and its complications, i.e. uncontrolled replication and dissemination of *M. tuberculosis* from primary foci to other parts of the lungs and body. And again it has been suggested that the presence of BCG vaccine does not influence immunity against *M. tuberculosis* in children with severe forms of tuberculosis. The implications of these findings for public health are that the absence of a tuberculous focus is more important in preventing the disease than BCG vaccination.

1.4.7.4. **Complications**

The complications of BCG are very few. Local secondary infections may occur and occasionally give rise to abscess or to swollen and tender draining lymph gland, sometimes with a resultant abscess. Erythema nodosum and urticaria have occasionally been recorded. In the world literature there have been 13 recorded deaths form disseminated BCG among more than 200 million vaccinated. In at least ten of these subjects there were previous disturbance of immunity. BCG should not be given to such individuals or patients with extensive dermatoses. A case has been described of apparent pulmonary tuberculosis due to BCG. Complications of any kind should occur in well under two percent of vaccinated individuals.

1.5. **Measurement of infection (tuberculin testing)**

The basis of the tuberculin test is the fact that coincident with the development of the primary tuberculous lesion, the patient becomes hypersensitive to a protein fraction of the tubercle bacillus. This hypersensitivity can be detected by introducing tuberculin into the skin with a resultant reaction of the delayed type. A positive reaction of any degree was formerly...
considered to indicate a previous infection with tubercle bacillus, although not necessarily the presence of tuberculous disease. This view has now been modified. In the course of the investigations by the WHO it was shown that there was a variation in different parts of the world in the proportion of the population showing weak reactions to tuberculin, although patients with actual tuberculous disease almost always showed strong reactions. If the size of the reaction among the general population was plotted against the frequency it was found that in many countries there was a bimodal distribution, a large number of the population having small reactions, a relatively small number having intermediate reactions, and a second peak of frequency of larger reactions.

Consequently it was suggested that there were two sorts of reaction to the tuberculin tests: a specific reaction, indicating previous infection to the tubercle bacilli and manifested by a larger skin response, and a non-specific reaction manifested by a smaller response to tuberculin possibly due to infection by some unknown organism. As mentioned, there is considerable geographical variation. The WHO studies suggested that non-specific sensitivity ranged from 10% in Denmark and North USA to over 90% in the Philippines, the Sudan and Vietnam. It was relatively high with a figure of 70-80%, in some parts of India, and relatively low between 20 and 30%, in England and Mexico. Though in the temperate and subtropical regions it was easy to differentiate between weak (non-specific) and strong (specific) reactions, there was considerable overlap in tropical areas. These studies were done in children. It was found that as the children grew older an increasing number of them transferred into the group with specific hypersensitivity, in which the greater reaction will obscure any co-existing non-specific hypersensitivity.
A number of techniques for tuberculin testing have been used in the past. The great majority of testing for epidemiological purposes which have been carried out, especially by the WHO, have employed the Mantoux test. In Britain the multi-puncture test devised by Heaf has been very widely used. The use of the Tine, patch and von Pirquet has been abandoned. The Mantoux test is performed on the volar surface of the forearm by injecting intradermally with a fine needle (no. 26G) the appropriate dose of tuberculin contained in 0.1 mL of diluent (normally a buffer solution). The WHO recommends use of an isotonic phosphate saline at pH 7.83 to which 0.05 part per 1000 of a detergent (Tween 80) has been added. For epidemiological work the standard dose used in most international work has been 5 tuberculin units (TU), though WHO has recommended 1 TU or more recently, 2TU. A separate syringe must be used for each tuberculin. The test is read at 48 to 72 hours. The reaction consists of both induration and erythema. Erythema is disregarded and the diameter of the induration is measured. The thickness of the induration must be at least 1mm. At one time a diameter of 6 mm was regarded as due to previous tuberculous infection but now it has been realised that some of these reactions may be non-specific and only reactions above 10 mm are suggestive of tuberculous origin. Smaller reactions must be recorded because of possible significance with regard to infection with non-tuberculous mycobacteria.

The Heaf employs a simple instrument - a Heaf gun. It consists of a six spring loaded needles which when fired pierce the skin through a drop of undiluted purified protein derivative (PPD). But after a lot of use the points of the needle may get bent. There is now a ‘magnetic’ Heaf gun with blades which do not get bent. Using a dropper, a drop of undiluted PPD is
placed on the clean dry skin at the junction of the mid and upper third of the anterior (front, smooth-skinned) surface of the forearm. Adjust the length of the needles (2 mm for adults, 1 mm for young children). Dip the end-plate and needles of the gun into a shallow dish containing spirit and ignite them in a flame of a spirit lamp. Cool for not less than 10 seconds. Place the plate firmly over the drop of tuberculin. Depress the handle of the gun thus causing the needles to pierce the skin.

The test should be read at 48-72 hours and the results recorded as follows: Grade 0, shows no reaction; Grade I shows at least 4 papules; Grade II the papules are confluent to show a ring; Grade III the ring is filled in the center to form a solid area of induration in Grade IV there are shiny vesicles over the solid area and there may be erythema.

1.5.2. Interpreting a Tuberculin Test

If the test is to be used for epidemiology (to measure the number of infected people in community) there will probably be a national rule about what is regarded as positive. It will recommend that the diameter of the induration should be recorded for each test. Positives will probably include a diameter of 10 mm or more to the doses in the Mantoux test or Grades I, II, III or IV in the Heaf test. But the WHO recommends that the Heaf test should not be used for epidemiological surveys.

If the test is to be used as a help in diagnosis the problem is more difficult: A diameter of induration of less than 10 mm should not be regarded as ‘negative’. This does not exclude a
diagnosis of tuberculosis. The reaction may be suppressed by malnutrition, other conditions or by the severity of the tuberculosis. A diameter of 10 mm or more in a child or adult who has not had a BCG (look for scar) is positive. A diameter of over 15 mm in a child who has had a BCG is positive (smaller reactions may be due to the BCG): this means the child has also been infected with TB. The larger the diameter (above 10-15 mm) the greater the support of a diagnosis of Tuberculosis. The younger the child the stronger this support.

1.6. Diagnosis and prognosis

The main conditions to be distinguished are bacterial, viral and HIV-related cryptococcal meningitis. In the first two the onset is much more acute. Cryptococcal meningitis may have a much slower onset. A family history of tuberculosis, or the finding of tuberculosis somewhere else in the body makes tuberculosis more likely. But the best evidence comes from examination of the CSF obtained by a lumber puncture. Death is certain if the disease is untreated: the earlier it is diagnosed and treated, the more likely is the patient to recover without serious permanent damage. The clearer the state of consciousness when treatment is started the better the prognosis. If the patient is comatose the prognosis for complete recovery is poor. Unfortunately 10-30 percent of survivors are left with some damage, such as paralysis, epileptic attack or some intellectual damage. Because of the fatal outlook if diagnosis is missed treatment must be given if the diagnosis is at all likely.

1.6.1 Tuberculous Meningitis

Tuberculous meningitis is difficult to diagnose especially in its early phase. The clinical
presentation and the CSF abnormalities vary. The diagnosis is usually based on a history of contact, clinical features, characteristic CSF changes, chest roentgenogram and corroborative presence of extra cranial tuberculosis, like primary complex, cervical or mediastinal lymph nodes, segmental lesions in the lungs or miliary tuberculosis. A positive tuberculin test lends further support to the diagnosis. The demonstration of Acid Fast bacteria has been disappointingly infrequent, and are shown rarely in direct Ziehl Neelson smears of CSF specimens and are cultured only in 42-75% of all patients.

The CSF findings however are often ambiguous, especially in children, and interpretation of the tuberculin skin test is difficult as it may be reactive because of immunisation or falsely negative due to malnutrition or severe infection. Therefore the diagnosis of tuberculous meningitis (TBM) is often delayed and can adversely affect the outcome. In recent years various biochemical and serological tests have been evaluated for the early diagnosis of tuberculous meningitis, including detection of tuberculostearic acid and 3-(2'-ketohexyl) indoline by gas chromatography, measurement of adenosine deaminase (ADA) activity, the bromide partition test (BPT) and the detection of the mycobacterial antigens and antibodies by enzyme-linked immunosorbent assay (ELISA).

At the time of the report of Coovadia et al., none of the tests listed had gained widespread acceptance for the rapid diagnosis of tuberculous meningitis because the methods gave disappointing results. Coovadia and his colleagues decided to evaluate ADA, BPT and the ELISA methods. Their conclusion was that at the cut-off point of 10 IU/L the sensitivity and specificity of ADA assay were 73% and 71% and therefore did not recommend the assay as compared with the BPT which was 92% sensitive and specific. What they did not say or do...
was whether or not they experimented with different cut-off points to arrive at their conclusion since the literature on this subject indicate different cut-off points from different populations or workers.

1.6.2. Adenosine Deaminase

Adenosine deaminase (ADA) is an enzyme of the purine salvage pathway which catalyses the deamination of adenosine and deoxyadenosine which are its main substrates to inosine and deoxyinosine respectively. The enzyme is involved in the degradative pathways leading to the formation of uric acid. It is widely distributed in animal and human tissues and enzyme activity is higher in leukocytes than in erythrocytes. Three isoenzymes of ADA have been isolated. They are ADA₁, ADA₂ and ADA₁+CP. ADA₁ is abundant in the spleen, lymphocytes, monocytes and neutrophils. ADA₂ is the predominant form in normal sera where it is only detected in monocytes, which showed 82% ADA₁ and 18% ADA₂. ADA₁+CP is dominant in liver, lung, muscle and pancreatic tissue; kidney has only ADA₁+CP.  

In all sera with increased ADA, the ADA₂ isozyme predominates except in acute lymphatic leukaemia (ALL) and other lymphogenic diseases where lymphoblasts are apparently the source of the increased serum ADA. The reported reference values at 95% significance for normal human serum are 10-25 U/L at 37° C. The origin of serum ADA and the mechanism by which serum activities are increased have not yet been fully elucidated but there is an indication that ADA₂ originates exclusively from monocyte/macrophage cell lineage and therefore reflects the involvement of the cellular immune system.  

Since normal sera contains ADA₁+CP and ADA₂ but no ADA₁, and ADA₂ can only be
demonstrated in monocytes, it has been assumed, although not confirmed that the serum ADA originates from monocytes. However the reason why ADA₂ is the predominant serum enzyme, in contrast to the intracellular monocyte ADA pattern (82% ADA₁ and 18% ADA₂), still has to be determined. It has been suggested that perhaps ADA₂ is actively secreted by monocytes or the half life of ADA₂ is longer. The absence of ADA₁ in the serum can be explained by the presence of excess combining protein (CP), so that any ADA₁ would readily be converted to ADA₁+cp.

The main biological role of Adenosine deaminase is related to the proliferation and differentiation of lymphocytes. Inherited defects of ADA lead to abnormalities in purine nucleotide metabolism which result in the production of substance(s) that are selectively toxic to lymphocyte. This culminates in severe combined immunodeficiency involving T-cell and B-cell dysfunction. The activity of ADA increases during antigenic and mitogenic responses of lymphocytes and it is considered as a marker for cell mediated immunity (CMI). The mechanisms by which the lack of the enzyme leads to immune dysfunction are not known.

Several hypotheses have been put forth to explain the biochemical consequences of a lack of ADA. Firstly, high levels of deoxyadenosine triphosphate (d-ATP) inhibit ribonucleotide reductase activity and as a consequence inhibit deoxyribonucleic acid (DNA) synthesis. Secondly deoxyadenosine inactivates S-adenonsyl homocystein hydroxylase, leading to decreased S-adenosylmethionine required for methylation of bases in ribonucleic acid (RNA) and DNA and increased levels of adenosine resulting in increased cyclic adenosine monophosphate (cAMP) levels. It is possible that each of these contribute to the overall effects of immune dysfunction. There is however no suitable explanation for the specificity of

Adenosine deaminase activity
the effects on only T and B cells.

The association between reduced serum ADA activity and impairment of cellular immunity in humans has been well described. Conversely, elevated ADA levels (> 25 IU/L) have been described in conditions involving stimulation of lymphocyte proliferation. Acute viral hepatitis (79 ± 22 IU/L), active cirrhosis (70 ± 21 IU/L) and to a much lesser extent other hepatic diseases, e.g. obstructive jaundice (43 ±16 IU/L) are typical examples. Very high serum ADA levels have been observed in typhoid fever (112 ± 31 IU/L) It is also increased in various diseases such as, tuberculosis, infective mononucleosis, and certain malignancies, especially those of haemopoietic origin. ADA levels in CSF have also been found to be elevated in tuberculous pleural and peritoneal effusion and mycosis fungoides.

Serous inflammation is quite common in tuberculous disease and differential diagnosis is often a clinical challenge. There is considerable difficulty in diagnosing TB by conventional bacteriological and histological methods. Final diagnosis is established when *M. tuberculosis* is isolated from certain of the patient’s body fluids. However both tests lack sensitivity and exhibits additional limitations: histological examination is invasive (moreover, not practicable in TBM), and culture of the tubercle bacilli takes too long to allow early diagnosis. Meanwhile it is well known that prognosis depend on the delay between clinical symptoms and the beginning of specific therapy, especially in tuberculous meningitis. Thus tests allowing reliable early diagnosis would be greatly appreciated by both physicians and patients.

TBM is difficult to diagnose with certainty, especially in its early phase. The clinical

Adenosine deaminase activity
presentation and CSF abnormalities vary, and smears for acid-fast bacilli yield few positive results. Usually growth of *M. tuberculosis* is not recognised in culture media until after 4 weeks, and the frequency of positive cultures is only about 15% \(^{43}\). In sharp contrast, Coovadia et al \(^{31}\) also found positive cultures in 42-75% of patients. They further observed that CSF findings are often ambiguous especially in children and interpretation of the tuberculin skin test is difficult as it may be reactive because of immunisation or falsely negative because of malnutrition or severe infection. To improve diagnosis enzyme linked immunosorbent assays (ELISA) have been developed to demonstrate anti-microbial antibodies in CSF and serum, but the sensitivity and specificity of this ELISA method is low \(^{43}\).

Earlier studies \(^{16, 17, 32 - 46}\) have established and confirmed the sensitivity and the value of estimating ADA in the diagnosis of pleuropericardial, meningeal and ascitic involvement. Studies by Segura *et al* \(^{17}\) showed that in exudative pleuroperitoneal and pericardial effusions, an ADA decision level of 43 IU/L displayed a sensitivity of 1.00, and was higher than those of histologic (0.83) and bacteriologic (0.62) studies. At this level, ADA reached a specificity of 0.92 and efficiency of 0.94.

Even though Maartens and Bateman \(^{46}\) concluded that Adenosine deaminase does not provide as valuable a diagnostic test of pleural tuberculosis as has been suggested, they concede that median adenosine deaminase activity was significantly higher in tuberculous effusions than in any of the other categories, but there was considerable overlap between the groups. Gakis *et al* \(^{38}\) who were the first to report on the activity of the enzyme in biologic fluids in 1973 have been prompted to react to the inaccuracies in several papers leading to misinterpretations of the enzyme activity. Moreover they observed that ADA activity increases in many infectious
presentation and CSF abnormalities vary, and smears for acid-fast bacilli yield few positive results. Usually growth of *M. tuberculosis* is not recognised in culture media until after 4 weeks, and the frequency of positive cultures is only about 15%\(^3\). In sharp contrast, Coovadia et al\(^3\) also found positive cultures in 42-75% of patients. They further observed that CSF findings are often ambiguous especially in children and interpretation of the tuberculin skin test is difficult as it may be reactive because of immunisation or falsely negative because of malnutrition or severe infection. To improve diagnosis enzyme linked immunosorbent assays (ELISA) have been developed to demonstrate anti-microbial antibodies in CSF and serum, but the sensitivity and specificity of this ELISA method is low\(^4\).

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diseases caused by micro-organisms infecting mainly the macrophages, in HIV infections, and in some malignant conditions.

Further points of interest involve children under one year of age who are extremely ill with miliary disease. ADA results have been found to be totally misleading in this condition as well as in proven pulmonary tuberculosis. This is believed to be the result of impaired cellular immunity found in this age as evidenced by the less effective response to BCG vaccination. In addition high ADA levels were occasionally found in non-tuberculous neurological conditions in this age group. Other rare cases of false positive results have arisen from primary lymphatic neoplasms in this age group.

1.7. Other Diseases

Diabetes mellitus is a heterogeneous group of diseases, which is characterised by an elevation of fasting blood glucose caused by a relative or absolute insulin deficiency. According to the World Health Organisation (WHO), a single random blood glucose level in excess of 11.1 mmol/L establishes the diagnosis of diabetes mellitus with or without clinical symptoms. Levels between 5.5-11.1 mmol/L makes diabetes uncertain, while levels below 5.5 mmol/L makes the disease unlikely. In the event of uncertainty, it is sufficient to measure the fasting plasma glucose levels and at 2 hours after a 75 g oral glucose load, using specific enzymatic procedure. In the case of venous plasma as in the present study, diabetes mellitus is established with a fasting venous plasma glucose concentration ≥ 7.8 mmol/L and ≥ 11.1 mmol/L 2 hours after the glucose load. The estimation of fasting blood glucose has been one of the major tools in the diagnosing and monitoring of diabetic patients.
Glycated haemoglobin (GHb) is an important tool in the long term monitoring and treatment of diabetic patients. GHb is derived from haemoglobin A (Hb A), the major component of adult haemoglobin, which also consists of a small amount of Hb A₂ and traces of haemoglobin F (Hb F). Formation of glycated haemoglobin is a non enzymatic process which begins during erythropoiesis and continues through the 120 day life span of the red blood cells by a post translational modification of haemoglobin. There are various forms of glycated haemoglobin A, these include Hb A₁ₐ, Hb A₁₉ and Hb A₁c. Hb A₁c is the most abundant form and it is a sub-fraction of Hb A₁. Increased amounts of Hb A₁c are found in patients with prolonged hyperglycaemia, as in uncontrolled diabetes mellitus since the extent of glycation depends on the plasma concentration of a particular hexose during the 120-day life span of the red blood cell of such individuals.

Since the level of GHb in the red blood cells depends on the concentration of blood glucose, and its formation is irreversible, measurement of glycated haemoglobin provides an index of glycaemic control over a period of 120 days which is the life span of the red blood cell, this has been proven to evoke changes in diabetes treatment resulting in improved metabolic control. A series of studies have confirmed the utility of glycated haemoglobin measurement as a monitor of glucose control in diabetes. It was demonstrated in 1976 that when patients with insulin dependent diabetes mellitus were brought under control in hospital, it took 8-12 weeks for the elevated glycated haemoglobin concentration to return to normal. Thus the concept that a single glycated haemoglobin determination reflects the average serum glucose concentration for the preceding 8-12 weeks was established.

As mentioned earlier, adenosine deaminase’s main biological role is related to the proliferation

| Adenosine deaminase activity | 33 |
and differentiation of lymphocytes. It is suggested to be an important enzyme for modulating the bioactivity of insulin but its clinical significance in diabetes mellitus is not yet characterised. In a study by Tomoaki et al.\textsuperscript{52} to characterise the significance of the isozymes of ADA in hospitalised diabetes mellitus patients who had had the condition for more than 5 years, they estimated total ADA, ADA\textsubscript{1} and ADA\textsubscript{2} (~ 72% of serum total ADA activity\textsuperscript{32}) and observed a significant correlation between ADA\textsubscript{2} and Hb A\textsubscript{1c} (P<0.002) in poorly controlled Non Insulin Dependent Diabetes Mellitus (Type II DM) patients. For total ADA a significant correlation of P< 0.005 was noticed, but ADA\textsubscript{1} activity did not correlate well with HbA\textsubscript{1c}.\textsuperscript{52}

It is not economically possible to control and monitor diabetes in this part of the world as has been described above. Furthermore the use of the isozymes for the purposes of routine testing would also be very expensive. In view of the fact that serum ADA\textsubscript{2} activity constitutes about two thirds of the total serum activity, and the fact that isozyme assays will add on to the already expensive monitoring of the disease, it was believed that a comparison of serum total ADA activity with GHb in outpatient subjects for any correlation may be interesting. A good correlation would make ADA assay a useful alternative to GHb assay because it will be relatively affordable. Furthermore since ADA is a marker for infection it would be expected to be an important adjunct to the control of the disease with respect to diabetic gangrene.

Several cut-off points for ADA levels have been suggested by different authors for the diagnosis of TBM. These cut-off points were obviously relevant to the population for which the studies were made. In this study the unique cut-off point for the Ghanaian population was determined for making early diagnosis of TBM. ADA levels in some other disease conditions
have also been determined to establish any relationships between ADA and the existing diagnostic parameters with the aim of further improving their diagnostic value.
Chapter 2: EXPERIMENTAL

2.1. Materials and methods

2.1.1. Equipment

Standard laboratory equipments

Weighing balance (Bosch S2000) Gerb. Bosch, D-7455 Jungeingen Germany
Waterbath (Kottermann) Kottermann, D 3165 Hanigsen, West Germany

pH meter (Mettler Toledo 355 ion analyser) Ciba Corning Diagnostic Ltd. Sudbury Suffolk,

Ace autoanalyser Schiapparelli, Biosystems, Inc. USA.

2.2. Chemicals and Reagents

Ammonium Sulphate (analar BDH chemicals Ltd. Poole England)

Sodium dihydrogen orthophosphate anhydrous (NaH$_2$PO$_4$) “

Disodium hydrogen orthophosphate anhydrous (Na$_2$HPO$_4$.) “

Sodium nitroprusside “

Sodium Hydroxide “

Sodium hypochlorite (Randox Laboratories Ltd UK)

Adenosine (Sigma)

Phenol (Sigma)

Adenosine deaminase activity
2.3. Preparation of sample

Approval for this study was obtained from the ethical and protocol committee of the University of Ghana Medical School.

After obtaining informed consent from the subjects, 5 mL of both venous blood specimen and pleural effusion were collected into plain sample tubes for ADA, total protein and albumin analysis, while 1 mL aliquot of the pleural effusion were collected into fluoride bottles for glucose analysis.

Blood samples were centrifuged at 1000 x g for 5 min; while CSF samples were centrifuged at 1200 x g for 15 min. Fifty (50) µL of the resultant supernatant of both samples were required for analysis. Because erythrocytes contain ADA only sera free from haemolysis and pleural effusion free from blood contamination were used. The enzyme is stable in serum for at least 24 h at 25° C, 7 days at 4° C and 3 months at -20° C 32, however in all cases in this study analyses were performed within 30 min. after sample separation. All ADA analyses were performed spectrophotometrically.

2.4. Procedures

2.4.1. Subjects:

The study included patients attending paediatric, internal medicine and chest clinics with particular interest in those with confirmed diagnosis of pulmonary tuberculosis by the existing criteria. ADA levels of patients with other forms of infections which predispose them to Adenosine deaminase activity
pleural effusion as well as those with HIV infection were assessed.

The subjects in this study included 358 individuals between ages of a few days to 74 years. These were made up as follows: Controls for serum ADA (30); TBM (230); Pulmonary TB/Pleural effusion (41); Diabetics (15); HIV (22); Hepatitis (10) and Renal failure (10). The control population was taken from ‘apparently healthy subjects’ i.e., those without any form of infection or undergoing any form of treatment for any infection. Further, additional control subjects for pleural effusions were taken from those patients who produced effusions from conditions other than TB. Subjects in this study should not have been on antituberculous therapy for the previous fifteen days since these are known to reduce the level of ADA. 17.

Samples from all patients who were clinically diagnosed as TBM cases were taken before they were put on antibiotic therapy. Those who did not make any progress as expected were quickly switched onto anti tuberculous therapy. A second testing was offered to those patients who stayed in hospital for more than fifteen days. This was done after 15 days on antituberculous therapy to obtain data for retrospective definitive diagnosis for the disease since there is no definitive laboratory test presently. Results of post mortem on those who died were obtained to confirm or rule out TBM as the case may be.

In diabetes, a total of 33 newly diagnosed Type II DM out patient between the ages of 19 and 70 years attending clinic at the diabetes centre at the Korle-Bu Teaching hospital were selected as the subjects for this study. Out of the number selected, 22 (66.7%) were females, and 11 (33.3%) were males. Four (12.1%) of the selected patients were below 40 years and 29 (87.9%) were forty years and above.
Blood samples were collected from patients for the initial estimation of fasting blood glucose, glycated haemoglobin and adenosine deaminase respectively. Patients were given two months to come back for re-testing after initiation of diabetic therapy. Out of the 33 patients selected for the base line testing, 15 (8 females and 7 males) turned up for the follow-up testing.

2.4.2. Assay

ADA activity was determined in all samples according to the method of Giusti and Galanti. Total protein and albumin analyses were carried out on the ACE autoanalyser using the Biuret and the BCG dye binding methods respectively. Fasting blood glucose analysis was carried out using the glucose oxidase/peroxide method by Barham and Trinder. CSF culture and sensitivity tests were carried out in the department of microbiology of the University of Ghana Medical School, using chocolate and blood agar media. Culture positive samples would help to identify false positives cases of bacterial meningitis from true positive TBM cases. The main purpose of the protein and glucose estimations in this study was to help in the differential diagnoses.

ADA analysis: The assay is based on the Berthelot’s reaction. The substrate in this method is adenosine (20 mmol/L). In the presence of water, adenosine is hydrolysed to ammonia. The ammonium ion reacts with phenol and hypochlorite in the alkali medium to form indophenol. Nitroprusside is used to catalyse the reaction. Absorbance of the dissociated indophenol is measured at 623 nm. A control sample of ADA was used to determine the precision of the assay.
Briefly, 50 μL of sample was incubated at 37 °C with 1 mL of a 20 mmol/L buffered adenosine solution (phosphate buffer 50 mmol/L, pH 6.5). After a 60 min incubation period the reaction was stopped with phenol/nitroprusside solution and indophenol was formed with 3 mL of sodium hypochlorite solution (11 mmol/L in 125 mmol/L sodium hydroxide). Absorbances were measured at 623 nm. Ammonium sulphate standard solution (75 μmol/L) which corresponds to the ammonia produced by a sample with enzyme activity of 50 IU/L, was used to calculate sample concentrations. Reagent and sample blanks without adenosine, were processed in all series and the absorbances were subtracted from those of standards and blanks respectively (Appendix A).

Albumin levels were determined in both serum and pleural effusion for the purpose of differentiating malignancy from other causes of the pleural effusion. Total protein determination was also expected to help differentiate between exudates and transudates. Pleural fluid was considered an exudate when the protein concentration ratio between fluid and serum was higher than 0.5. Since exudative effusion usually results from infection or malignancy, the glucose levels were also determined to help differentiate bacterial infection from tuberculous infection or malignancy. Glucose in the fluids was expected to be low in bacterial infections as compared to infections due to other causes; since bacteria is known to use up the glucose in the fluid.

Glycated Hb analysis: The kit method used is based on the affinity which boronate groups have for cis-diols structure found in all glycated proteins. Glycated proteins were eluted from boronated columns and their absorbances determined with a spectrophotometer.
Two calibration curves were plotted; one at a lower concentration from 0-150 μmol/L (fig. 1), and the other at a higher concentration from 0-280 μmol/L. (fig. 2) The two curves were necessary for two reasons: (a) to compare the two salts (old and fresh) by way of their absorbances and (b), to observe the linearity of the assay at both the lower and upper concentrations of the salts.

In both cases the (NH₄)₂SO₄ was diluted in the phosphate buffer to the various concentrations as indicated on the graphs (figs. 1&2) The calibration was then carried out according to the assay procedure (Appendix A). The co-efficient of variation (CV) of the assay was also performed using two different serum samples one at the lower end (15 IU/L) and the other at the higher end (50 IU/L). The assay was then repeated for both samples in 11 replicates for the calculation of the CV.

2.6. Statistics

All results were analysed by parametric methods including student t test, simple regression analysis and a probability of less than 5% (P< 0.05) was considered to be statistically significant. With the exception of data for CV (appendix B) and data for all TBM cases (appendix C) the geometric mean and standard deviation, were calculated for all other sets of data. All results are reported as ±1 standard deviation. The geometrical mean and standard deviation were computed after the logarithmic transformation of data (necessary for data; n < 20). This method compensates for skewness which otherwise distorts the true mean.
3.1. Calibration Curves

The two calibration curves showed the linearity both at a lower concentration of the (NH₄)₂SO₄ salt from 0-150 μmol/L (fig. 1), and at a higher concentration from 0-280 μmol/L. (fig. 2) They also helped to show that the purity of the salt is crucial to the assay since there is no availability of reference material yet.

The co-efficient of variation (CV) of the assay ranged from 4.4% at the lower end (15 IU/L) to 9.0% at the higher end (50 IU/L). It was observed that the CV increased with higher ADA concentration (appendix B&E.)

3.2. Tuberculous Meningitis

The initial diagnosis was based on the cut-off point of 12 IU/L. This decision was based on cut-off points from other research groups which ranged from 2-12 IU/L. But the initial results which were computed revealed that there were going to be many false negatives. It was therefore lowered to 10 IU/L and finally settled on 9 IU/L upon which final diagnosis were made. On the other hand there would have been many more false positives at the cut-off point of 7 IU/L. Thus the final cut off point of 9 IU/L was used for the pathognomonicity of CSF ADA levels (table 1). Based on the same tentative cut-off point of 12 IU/L, a baseline CSF ADA level of 2 IU/L ± 2 was also established for the assay. The base line value included all patients whose CSF ADA levels were due to causes other than TBM as diagnosed clinically and confirmed by the assay at the established cut-off point.
3.2.1. Pathognomonicity

At the cut-off points of 9 and 12 IU/L the sensitivity, specificity, efficiency, and the negative predictive values, were the same. The positive predictive values were however different. At 9 IU/L the positive predictive value was 92% while it was 87% at 12 IU/L (table 1).

Out of the 230 patients who were tested 188 (82%) were below age 13. The mean age of the adults was 29 ± 2 years; with a range of 13-74 years, while the age of the infants ranged from one day to age 13 years. Using the cut-off point of 9 IU/L, out of the 230 patients there were 55 (24%) positives, and 175 (76%) negatives. Of the 55 positives there were 5 (9%) false positives. The 5 false positives were culture and sensitivity positive for bacteria meningitis.

The mean CSF ADA level of the 50 true positives was 18 ± 2.0 IU/L. Out of these true positives the 12 patients whose ADA levels were monitored before and 15 days after treatment had their ADA levels considerably decreased from the mean of 18.0 ± 3.0 and a range of 10-47 IU/L to a mean of 3.4 ± 2.0 and a range of 0.1-8 IU/L (fig. 3). The post mortem report of the three patients who died confirmed TBM as the cause of death. These results which were obtained retrospectively confirmed the diagnosis definitively since all these patients as well as the rest of the positive cases made good recovery on the anti-tuberculous therapy and were discharged for later reviews.

The mean CSF protein of those diagnosed as tuberculous meningitis positive was 1.0 ± 1.0g/L and that of the glucose was 2.1 ± 1.0 mmol/L. While there was no correlation between the glucose and the ADA results there was a correlation between the protein and the ADA.
3.3. Pulmonary tuberculosis

The mean total ADA activity in the serum of the 'apparently healthy' donors (HD) was 16.0 ±1.0 IU/L (n=30). Out of the 41 patients with pulmonary related complaints 15 were previously confirmed by sputum smear test as positives for TB on at least two occasions. Their mean serum ADA concentration was 37 ± 1.0 IU/L. Out of this number 10 produced pleural effusions with a mean ADA concentration of 94 ± 1.0 IU/L. Another 5 out of the 41 patients had pneumonia with a mean serum ADA concentration of 14 ± 2 IU/L. but their mean pleural effusions ADA concentration was 37 ± 1.0 IU/L. Therefore out of the 41 patients 15 produced pleural effusions which were due to causes other than pulmonary TB. This combined group (table 2) had a mean pleural effusion ADA concentration of 13 ± 1.0 IU/L and they were used as the control for the 15 positive cases of pulmonary tuberculosis (table 3). There was no correlation between the serum ADA concentration and ascitic/pleural fluids of the TB positive cases.

3.4. Diabetes

Of the 33 volunteers who took part in the study, 15 returned for the follow-up testing after 8 weeks.

3.4.1. Blood Glucose determination

The mean FBG concentration of the 15 poorly controlled patients was 12.5 ± 1.4 mmol/L. This
was more than 7.8 mmol/L before treatment. After 8 weeks of drug/diet management the mean FBG concentration was reduced to 7.8 ± 4.1 mmol/L, and was significantly less than the initial value before treatment ($p > 0.001$; Fig. 4). The only well-controlled patient had an initial equivocal FBG value of 6.6 mmol/L. However his two hour postprandial result was 11.6 mmol/L. His FBG concentration after treatment was 3.0 mmol/L.

3.4.2. *Adenosine Deaminase determination*

The mean total ADA activity in the serum of ‘healthy donors’ (HD) was 16.0 ±1.0 IU/L (appendix D). The mean initial ADA activity in the serum of the poorly controlled patients was 38 ± 2 IU/L. After 8 weeks of treatment, the mean ADA activity was reduced to 27.1 ± 1.6 IU/L significantly less than the initial value before treatment ($p>0.001$; fig. 5). The only well controlled patient had ADA value of 12 IU/L after treatment as compared with the initial ADA value of 24 IU/L.

3.4.3. *Glycated Haemoglobin determination*

The mean GHb level in the 20 apparently healthy donors was 5.5 ± 1.1%. The initial mean GHb levels in the poorly controlled diabetics was 14.0 ± 1.3% ($p<0.001$ Vs HD). After 8 weeks of treatment, it decreased to 10.9 ± 1.4 ($p > 0.001$; fig. 6). The result as compared to the apparently healthy donors showed that only one patient was well controlled for both GHb and ADA (12 IU/L) for the 8 weeks period of study. His GHb level after treatment was 5.7% as compared to the initial GHb of 14.9%; while his ADA level reduced from 24 IU/L before treatment to 12 IU/L after treatment.
3.4.4. Correlation Test:

There was a good correlation between the FBG and ADA before treatment ($r = 0.78$ or $P<0.001$); as well as after treatment ($r = 0.60$ or $P<0.01$; Fig. 7 a and b). The after treatment correlation between GHb and FBG; GHb and ADA were $r = 0.56$ ($P<0.02$) and $r = 0.70$ ($P<0.005$) respectively. (Fig. 8 a and b).

3.5. HIV/AIDS

The mean age of the 22 patients who were diagnosed as HIV positive by the Public Health reference laboratory was 27 ± 2 years. Out of the 22 patients there were 12 (55%) females and 10 (45%) males. Eleven (50%) of the 22 patients were diagnosed as TB positive. Out of this number 7 (63%) were females and 4 (36%) were males (table 4).

In 22 patients who tested positive for the HIV the mean serum ADA level in 10 tuberculosis positive patients was 75 ± 1. The remaining 12 all had full blown aids; ten of whom were TB positives with ADA level of 64 ± 2. While the remaining two were TB negative with ADA levels of 46 and 27 IU/L levels respectively.

3.6. Hepatitis

Ten patients comprising 6 males and 4 females were clinically diagnosed as having acute hepatitis. Their mean age was 30 ± 2 years. Their liver enzymes were estimated to confirm diagnosis and to compare and contrast the ADA levels. (table 5). Their mean serum ADA concentration was 50 ± 2 IU/L; AST - 306 ± 2 IU/L; ALT - 282 ± 3 IU/L and ALP - 436 ± 2
IU/L. There was no correlation between the ADA concentrations and AST or ALP but there was a moderate correlation ($r = 0.5; p < 0.02$) between ADA and ALT.

### 3.7. Renal failure

Ten patients comprising 6 males and 4 females were clinically diagnosed as having renal failure. Their mean age was $20 \pm 3$ years. Their renal profiles were estimated to confirm diagnosis and to compare and contrast the ADA levels. (table 6). Their mean serum ADA concentration was $38 \pm 1$ IU/L; Urea $38 \pm 1$ mmol/L and Creatinine $-1056 \pm 2$ mmol/L.

There was no correlation between ADA and any of the renal parameters. A summary of the mean serum/fluid ADA activity in the various clinical groups is presented in table 7.
Table 1. The pathognomonicity of CSF ADA levels at the cut-off points of 7, 9 and 12 IU/L

<table>
<thead>
<tr>
<th>CUT-OFF POINT IU/L</th>
<th>SENSITIVITY (%)</th>
<th>SPECIFICITY (%)</th>
<th>EFFICIENCY (%)</th>
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<tr>
<td>7</td>
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<td>92</td>
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</tr>
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<td>9</td>
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<table>
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<tr>
<th>CUT-OFF POINT IU/L</th>
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<th>NEGATIVE PREDICTIVE VALUE (%)</th>
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<td>9</td>
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<tr>
<td>12</td>
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Table 2. ADA levels of control subjects for pleural effusions
e.i.e. effusions due to causes other than tuberculosis. n=15

<table>
<thead>
<tr>
<th>REFERENCE VALUES</th>
<th>AGE (Years)</th>
<th>ADA (IU/L)</th>
<th>(&lt; 33)</th>
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</tr>
<tr>
<td>Mean</td>
<td>31 ± 2</td>
<td>13 ± 1</td>
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</table>
Table 3. Serum and effusion ADA levels of confirmed pulmonary tuberculosis patients. Five patients did not produce any effusion. n =15

<table>
<thead>
<tr>
<th>PATIENT ID.</th>
<th>AGE (Years)</th>
<th>SERUM ADA (IU/L)</th>
<th>EFFUSION ADA (IU/L)</th>
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<td>JH</td>
<td>43</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>55</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>HC</td>
<td>17</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>JA</td>
<td>35</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>27 ± 2</td>
<td>37 ± 1</td>
<td>94 ± 1</td>
</tr>
</tbody>
</table>
Table 4. Serum ADA levels of HIV sero positives and AIDS patients. * = with tuberculosis; A = AIDS; P = HIV sero positive. n =22

<table>
<thead>
<tr>
<th>PATIENTS ID.</th>
<th>SEX</th>
<th>AGE (Years)</th>
<th>STATUS</th>
<th>ADA (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RD*</td>
<td>F</td>
<td>7</td>
<td>A</td>
<td>95</td>
</tr>
<tr>
<td>SO*</td>
<td>M</td>
<td>19</td>
<td>A</td>
<td>58</td>
</tr>
<tr>
<td>SA*</td>
<td>M</td>
<td>25</td>
<td>A</td>
<td>84</td>
</tr>
<tr>
<td>BM*</td>
<td>F</td>
<td>28</td>
<td>A</td>
<td>76</td>
</tr>
<tr>
<td>SB*</td>
<td>F</td>
<td>30</td>
<td>A</td>
<td>90</td>
</tr>
<tr>
<td>BY*</td>
<td>M</td>
<td>30</td>
<td>A</td>
<td>100</td>
</tr>
<tr>
<td>CT*</td>
<td>F</td>
<td>33</td>
<td>A</td>
<td>41</td>
</tr>
<tr>
<td>ES*</td>
<td>F</td>
<td>36</td>
<td>A</td>
<td>68</td>
</tr>
<tr>
<td>EO*</td>
<td>F</td>
<td>37</td>
<td>A</td>
<td>54</td>
</tr>
<tr>
<td>EA*</td>
<td>F</td>
<td>42</td>
<td>A</td>
<td>81</td>
</tr>
<tr>
<td>KA*</td>
<td>M</td>
<td>27</td>
<td>P</td>
<td>116</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td></td>
<td><strong>26 ± 2</strong></td>
<td></td>
<td><strong>75 ± 1</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ID.</th>
<th>SEX</th>
<th>AGE (Years)</th>
<th>STATUS</th>
<th>ADA (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>M</td>
<td>36</td>
<td>P</td>
<td>34</td>
</tr>
<tr>
<td>MA</td>
<td>F</td>
<td>6</td>
<td>P</td>
<td>36</td>
</tr>
<tr>
<td>AF</td>
<td>F</td>
<td>40</td>
<td>P</td>
<td>27</td>
</tr>
<tr>
<td>TO</td>
<td>F</td>
<td>39</td>
<td>P</td>
<td>38</td>
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<tr>
<td>ND</td>
<td>M</td>
<td>35</td>
<td>P</td>
<td>32</td>
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<td>KK</td>
<td>M</td>
<td>30</td>
<td>P</td>
<td>49</td>
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<tr>
<td>BB</td>
<td>M</td>
<td>31</td>
<td>P</td>
<td>64</td>
</tr>
<tr>
<td>EM</td>
<td>M</td>
<td>19</td>
<td>P</td>
<td>56</td>
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<tr>
<td>FA</td>
<td>F</td>
<td>36</td>
<td>P</td>
<td>57</td>
</tr>
<tr>
<td><strong>FT</strong></td>
<td>F</td>
<td>42</td>
<td>A</td>
<td>46</td>
</tr>
<tr>
<td><strong>MA</strong></td>
<td>M</td>
<td>32</td>
<td>A</td>
<td>27</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td></td>
<td><strong>33 ± 1</strong></td>
<td></td>
<td><strong>41 ± 1</strong></td>
</tr>
</tbody>
</table>

Adenosine deaminase activity
Table 5. Serum ADA levels of patients with Hepatitis. Liver enzymes are shown for comparison. Sub mean does not include extremely high values in asterisks. \( n = 10 \).

<table>
<thead>
<tr>
<th>REFERENCE VALUES (IU/L)</th>
<th>(10-25)</th>
<th>(1-37)</th>
<th>(1-42)</th>
<th>(114-294)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PATIENT ID.</td>
<td>SEX</td>
<td>AGE (Years)</td>
<td>ADA (IU/L)</td>
<td>AST (IU/L)</td>
</tr>
<tr>
<td>EE</td>
<td>M</td>
<td>26</td>
<td>121</td>
<td>208</td>
</tr>
<tr>
<td>EA</td>
<td>M</td>
<td>30</td>
<td>38</td>
<td>85</td>
</tr>
<tr>
<td>CA</td>
<td>F</td>
<td>36</td>
<td>93</td>
<td>925</td>
</tr>
<tr>
<td>FQ</td>
<td>M</td>
<td>38</td>
<td>35</td>
<td>93</td>
</tr>
<tr>
<td>PS</td>
<td>M</td>
<td>29</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>LY</td>
<td>F</td>
<td>41</td>
<td>29</td>
<td>75</td>
</tr>
<tr>
<td>FG</td>
<td>M</td>
<td>50</td>
<td>35</td>
<td>89</td>
</tr>
</tbody>
</table>

Sub Mean          35 ± 1  48 ± 2  139 ± 2  171 ± 2  360 ± 2

<table>
<thead>
<tr>
<th>Total Mean</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>EO</td>
<td>F</td>
<td>24</td>
<td>59</td>
<td>1956*</td>
</tr>
<tr>
<td>BB</td>
<td>F</td>
<td>6</td>
<td>106</td>
<td>2900*</td>
</tr>
<tr>
<td>KO</td>
<td>M</td>
<td>56</td>
<td>27</td>
<td>1230*</td>
</tr>
</tbody>
</table>

Adenosine deaminase activity 52
Table 6. Serum ADA levels of patients with renal failure. Urea and creatinine levels are shown for comparison. $n = 10$

<table>
<thead>
<tr>
<th>PATIENTS ID.</th>
<th>SEX</th>
<th>AGE (Years)</th>
<th>ADA (IU/L)</th>
<th>UREA (IU/L)</th>
<th>CREATININE (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>F</td>
<td>41</td>
<td>47</td>
<td>32</td>
<td>643</td>
</tr>
<tr>
<td>JF</td>
<td>M</td>
<td>1</td>
<td>30</td>
<td>54</td>
<td>483</td>
</tr>
<tr>
<td>DA</td>
<td>M</td>
<td>12</td>
<td>43</td>
<td>53</td>
<td>734</td>
</tr>
<tr>
<td>EA</td>
<td>M</td>
<td>28</td>
<td>42</td>
<td>67</td>
<td>2037</td>
</tr>
<tr>
<td>PA</td>
<td>M</td>
<td>19</td>
<td>42</td>
<td>80</td>
<td>3108</td>
</tr>
<tr>
<td>QE</td>
<td>M</td>
<td>42</td>
<td>25</td>
<td>33</td>
<td>1199</td>
</tr>
<tr>
<td>AM</td>
<td>F</td>
<td>27</td>
<td>39</td>
<td>67</td>
<td>1863</td>
</tr>
<tr>
<td>PO</td>
<td>F</td>
<td>35</td>
<td>42</td>
<td>40</td>
<td>700</td>
</tr>
<tr>
<td>AK</td>
<td>F</td>
<td>26</td>
<td>38</td>
<td>52</td>
<td>820</td>
</tr>
<tr>
<td>ME</td>
<td>M</td>
<td>30</td>
<td>40</td>
<td>36</td>
<td>930</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>$20 \pm 3$</td>
<td>$38 \pm 1$</td>
<td>$49 \pm 1$</td>
<td>$1056 \pm 2$</td>
</tr>
</tbody>
</table>
Table 7. A summary of the mean serum/fluid ADA activity in clinical groups.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>No.</th>
<th>SERUM (IU/L)</th>
<th>CSF (IU/L)</th>
<th>EFFUSION (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (serum)</td>
<td>30</td>
<td>16 ± 1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Controls (pleural effusion)</td>
<td>15</td>
<td>-</td>
<td>13 ± 1</td>
<td></td>
</tr>
<tr>
<td>Controls (baseline for CSF)</td>
<td>184</td>
<td>-</td>
<td>2.2 ± 1</td>
<td>-</td>
</tr>
<tr>
<td>TBM</td>
<td>50</td>
<td>-</td>
<td>18 ± 1</td>
<td>-</td>
</tr>
<tr>
<td>Pulmonary TB (no effusion)</td>
<td>15</td>
<td>37 ± 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pulmonary TB (producing effusion)</td>
<td>10</td>
<td>37 ± 1</td>
<td>-</td>
<td>94 ± 1</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>5</td>
<td>14 ± 2</td>
<td>-</td>
<td>37 ± 1</td>
</tr>
<tr>
<td>HIV TB negative</td>
<td>11</td>
<td>41 ± 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIV TB positive</td>
<td>11</td>
<td>75 ± 2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIV AIDS (TB negative)</td>
<td>2</td>
<td>36 ± 2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIV AIDS (TB positive)</td>
<td>12</td>
<td>64 ± 2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>10</td>
<td>50 ± 2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Renal Failure</td>
<td>10</td>
<td>38 ± 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Diabetes</td>
<td>15</td>
<td>38 ± 2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. 1. A calibration curve relating the (NH₄)₂SO₄ standard concentration and absorbance. It also shows the linearity of the method at lower concentrations. The lower absorbances also reflect the low grade of the chemical used, and the dotted line represents the linear regression of the data.
The higher absorbances (cf. Fig. 1) reflect the higher grade chemical used, and the dotted line represents the linear regression of the data. The linearity of the method at higher concentrations (cf. Fig. 1) reflects the higher absorbance. Fig. 2. A calibration curve relating (NH₄)₂SO₄ standard concentration and absorbance. It also shows the linearity of the method at higher concentrations.
Those with asterisk died before the second lesion.

Fig. 3 CSF ADA levels of 12 of the patients who tested positive for TBM.
The dotted line represents the reference value of 5.5 mmol/L.

Figure 4. The relationship between serum FBG before and after

PATIENT

FBG before

FBG after

CONCENTRATION OF FBG

0.0

10.0

20.0

30.0

40.0
The dotted line represents the mean control value of 16.0 IU/L, n=15.

Fig. 5  The relationship between serum ADA before and after treatment.
The relationship between serum GHb before and after treatment.

The dotted line represents the mean control value of 5.5%, n=15.
Fig. 7a  The correlation between serum ADA and FBG before treatment. n=15

Fig. 7b  The correlation between serum ADA and FBG after treatment. n=15
Fig. 8a  The correlation between serum FBG and GHb after treatment. n=15

Fig. 8b  The correlation between serum ADA and GHb after treatment. n=15
Chapter 4: **DISCUSSIONS**

4.1. **Assay**

The process of standardisation of the ADA assay requires a lot of patience and meticulousness. A major issue is the fact that there are no reference standard materials as yet. This requires that the standard Ammonium Sulphate salt must be of pure quality. It does not necessarily mean only acquiring the salt from a known reliable source. It is very important to experiment with more than one and select the one with an absorbance equal or closer to what has been suggested in the literature. Comparing the two salts that were used in this project, the salt of high concentration as in fig 2., produced absorbances which were about ten times that of the one in fig. 2. This means if the one with the low absorbances had been used the results of the assay would have been spuriously high.

The assay itself measures the ammonia which is produced in the reaction. Therefore several precautions had to be adhered to. The process of water distillation removes ammonia among other gases like carbon dioxide from the tap water. Therefore this assay should not be run when distillation of water is in progress especially in areas where the ammonia content of the water is known to be very high. Distillation of water increases the ammonia content of the surrounding air. This ambient ammonia inevitably interferes with the assay by contaminating any previously washed test tubes meant for the assay. Therefore tubes had to be cleaned and dried immediately before the assay. Tubes which had previously been cleaned and stored for later use were found to be contaminated. During the assay itself mixing reagent added to tubes could not be done using the bare fingers since the small amount of ammonia on the fingers was also found to contaminate the assay. It was therefore imperative to cap tubes with parafilm at
all times of the assay to achieve the desired results.

The preparation of the adenosine substrate was also critical in that it did not readily dissolve in the buffer. It was necessary to heat the mixture before it dissolved into a solution. Any overheating resulted in the degradation of the adenosine. The heating process had to be closely observed to the point where the salt started to dissolve and then quickly mixed and cooled under the tap water to prevent any degradation of the salt. The colour development did not create much problem. The blue colour which was produced by the assay was found to be relatively stable for 1 hour.

One major problem for the assay emanated from the wards. And that was using proper sample tubes. Because most clinicians are used to sending CSF samples in fluoride bottles a lot of CSF samples were sent as such even after repeated education on the use of the appropriate plain sample bottles. Fluoride was found to inhibit the ADA activity by about 30%. And this was quite considerable and therefore efforts were made to this known to the clinicians. Sample storage was not a problem since it could be stored in the refrigerator at 4°C for up to 72 hours or as indicated under sample preparation in section 2.3.

The standardisation of the assay required all the above precautions to be meticulously put in place for achieving good results. The assay was shown to be fairly linear within the required assay concentration range. The purity of the ammonium sulphate standard however was very critical. It is very easy to pick up any salt from the shelves and use. But again even though the old or low grade salt produced a linear relationship it could not be used in the standardisation of the assay (figs. 1 & 2).
The need for a serum matrix control is also very important. Using ammonium sulphate solution as a control is not the best. The effect of the assay reaction on the serum will be slightly different from that of the pure salt due to the matrix effect. Therefore the coefficient of variation (CV) of the assay was performed with serum both at a low (15 IU/L) and at the higher end (50 IU/L). It was observed that the CV increased with higher ADA concentration (appendix B.). However the CV range of 4.4% to 9.9% was within the acceptable range for an enzyme assay. The importance of a good CV cannot be overemphasised. This is especially so, as the ADA assay although not very specific depends on the differing levels in various diseases for its diagnostic value.

4.2. Tuberculous Meningitis

As indicated in section 1.7.1 tuberculous meningitis is difficult to diagnose especially in its early phase. The clinical presentation and the CSF abnormalities vary. The diagnosis is usually based on a history of contact, clinical features, and other conventional methods. The demonstration of Acid Fast bacteria by direct Ziehl Neelson smears of CSF specimens have been disappointing infrequent, and positive cultures have been obtained only in 42-75 % of all patients with TBM.

For these reasons none of the tests so far reported in the earlier studies have gained widespread acceptance for the rapid diagnosis of tuberculous meningitis. When Mann et al evaluate the ADA activity by, BPT and the ELISA methods, their conclusion was that at the cut-off point of 10 IU/L the sensitivity and specificity of ADA assay were 73% and 71% respectively and therefore did not recommend the ELISA method as better option compared
with the BPT which was 92% sensitive and specific. However these authors did not indicate whether or not they experimented with different cut-off points to arrive at their conclusion since the literature on this subject indicate different cut-off points from different populations or workers. They however conceded that the difference between the two tests was not significant.

It has to be pointed out as stated by Coovadia et al.\textsuperscript{31}, that most of the listed tests unlike ADA assay require sophisticated expensive equipment such as gamma counters which are not readily available in most laboratories. Furthermore they require longer assay time which is not convenient for rapid results. Several authors\textsuperscript{29, 62, 63, 64}, who evaluated the ELISA method for either antigen or antibody have very diverse conclusions on the reliability of the method. Chuan - Zhen et al.\textsuperscript{29} observed that even though the method demonstrates anti-mycobacterial antibodies in the serum and CSF, the sensitivity and specificity of ELISA are low. They have developed a new method - a nitrocellulose immunospot assay which they believe is useful in the early diagnosis of TBM. But their method which is also involving by way of procedures such as overnight incubation may not be suitable for rapid routine work.

Even though Kadival et al.\textsuperscript{62} observed the usual cross reactivity by other mycobacterial species they believe that the ELISA test shows promise as a specific adjunct to the early diagnosis of tuberculous meningitis. Krambovitis\textsuperscript{63} on the other hand found a sensitivity of 85% for the assay and Vineeta et al.\textsuperscript{65} thought with some improvement the test appears to be a promising approach to a definitive diagnosis for TBM.
The conflicting views about the diagnostic value of ADA test prompted Gakis et al. who were the first to report the high level of the enzyme activity in biologic fluids in 1973, to react to the wrong interpretations they observed in several articles on the enzyme including those whose description of the activity of the ADA was surprisingly incomplete. They noted that a lot of inaccuracies had led to misinterpretation of results and that in their view did not explain the reasons for the high activities. They therefore published an article to give the correct interpretation of high ADA activity in biologic fluids. For the correct interpretation it must be taken into account that ADA activity originates from the action of two isozymes, ADA1 and ADA2. These two isozymes have different pH, Michaelis constant (Km) and relative substrate specificities. ADA1 has a low Km, optimal pH of 7.0 to 7.5 and a similar affinity for both adenosine and 2'-deoxyadenosine (2'-deoxyadenosine/ADA ratio of approximately 0.75). It is present in all tissues and it is essential for effective immune response. The congenital absence of ADA1 in lymphocytes and erythrocytes lead to a severe combined immunodeficiency syndrome.

ADA2 has a very high Km, an optimal pH of 6.5, a poor affinity for 2'-deoxyadenosine (2'-deoxyadenosine/ADA ratio of approximately 0.25) and its absence is not related to severe combined immunodeficiency syndrome. It is found only in monocytes/macrophages and not in any other cells and is released by these cells when they are stimulated by the presence of live microorganisms in their interior. The serum activity is approximately 72% of the total ADA activity. This explains why ADA2 increases in biologic fluids in the course of infectious diseases characterised by microorganisms infecting the macrophages. It is therefore very important to establish whether the high ADA activity in biologic fluid is due to the presence of ADA1 or ADA2. When in doubt this may be done using the substrate specificity ratios.
The pros and cons for ADA assay as well as the correct interpretation as offered by the originators of the test indicate that it is the best among all the tests on offer so far. Hence the choice for ADA. In this project as in many other articles already cited the total ADA activity has been used due to the fact that ADA\textsubscript{2} constitutes approximately two thirds of the total activity. When the ADA test is interpreted with other test results then its value as a specific adjunct to the early diagnosis of tuberculous meningitis is better than any of the existing tests already mentioned. In the study by Thora et al\textsuperscript{66} to evaluate the effect of BCG vaccination on serum ADA levels, they also evaluated the levels in CSF intracranial tuberculosis in children and observed that the ADA test can be used to judge cell mediated immune response after BCG vaccination.

Segura \textit{et al} \textsuperscript{17} reported that ADA assessment in CSF were few and contradictory. They quoted that whereas Zotti and Ipata \textsuperscript{67} ascribed relevance to CSF - ADA activity in patients with nervous system neoplasias, Gakis C. \textit{et al} \textsuperscript{38} failed to confirm this. In contrast they described not only patients suffering from TBM but also a clear distinction between TBM and viral lymphocytic meningitis. Other authors who evaluated TBM in paediatric patients reached different conclusions. Mann \textit{et al} \textsuperscript{61} comparing the reliability of ADA and bromide partition tests for the diagnosis of TBM found similar accuracy for both tests.

In their assessment, Segura \textit{et al} \textsuperscript{17} agreed more with Gakis \textit{et al} \textsuperscript{38} and Mann \textit{et al} \textsuperscript{61} than with other authors. The results of this project also agree with the above authors. However some indirect evidence support the idea that it could be due to local synthesis of the enzyme. In patients with sepsis caused by \textit{Salmonella typhi} extremely high serum ADA have been observed, while CSF levels of the enzyme remained within reference values \textsuperscript{17}.
This brings into focus the question of overlap of CSF-ADA results between TBM and bacterial meningitis. Mishra et al.\textsuperscript{68} observed that it appears complete differentiation between these two diseases is not possible on the basis of CSF ADA activity alone at lower cut-off points. Although other authors as in this project have also found similar results, the specificity of the assay for this differentiation has been seen to be better at slightly higher cut-off points. In this project although there was some overlap by way of false positivity, it was found that at a lower cut-off point than the decision level of 9 IU/L there would have been more false positives suggesting a higher degree of overlap.

Donald et al.\textsuperscript{69} in their contribution towards the utility of CSF-ADA determination noticed that there was a correlation between the CSF ADA activity and protein as was also observed in this project ($r = 0.63$, $p < 0.01$). They believed that the increased CSF-ADA activity may be due to increase of the protein permeability of the vascular barrier between the blood and other body fluids. But as has been pointed out earlier Segura et al.\textsuperscript{17} showed that this argument was not tenable. Further in response to this assertion Ribera et al.\textsuperscript{70} remarked that to date, they had determined CSF-ADA activity in more than 400 patients and in 57 patients with bacteria meningitis only 3 had levels greater than 9 IU/L. These three had very high neutrophil counts (>15,000/mm\textsuperscript{3}). Gakis et al.\textsuperscript{38} reported only 2 patients of 16 cases of bacteria meningitis who had ADA activity > 9 IU/L; in this project it was 5 out of 50 false positive cases. Those patients also had an elevated CSF cell count (>20,000/mm\textsuperscript{3}).

These results are in agreement with levels found in pleural fluids. In pleural empyema (high neutrophil counts), levels of fluid ADA have been found to be very high as was found in this project. In these cases the overlap of such values is not clinically important because pleural
empyema and bacteria meningitis with very high cell counts in the CSF do not present a
diagnostic problem \(^7^0\). In fact Tsushima and Kubo \(^7^1\), after treatment with antituberculcous
therapy, performed a serial brain magnetic resonance imaging (MRI) and examined the
cerebrospinal ADA activity. After 4 months treatment levels of ADA became normal and the
patient regained consciousness. They concluded that both cell counts and the level of ADA in
the cerebrospinal fluid are good indicators of the activity of tuberculous meningitis and
reflected its clinical course. These extra parameters when employed undoubtedly increase the
specificity of the assay.

The differences in paediatric and adult populations in TBM has also been an issue but in most
of the reports it appears that the sample population some authors used was too small to be
statistically tenable. Donald et al \(^6^9\) obtained a mean of 26.2 IU/L in 3 adults as compared with
10.4 IU/L in 27 children. Ribera et al \(^7^0\) studied 10 children with a mean of 9.8 as compared to
15.8 IU/L in adults. In this project there were 188 adults and 42 children and there was no
significant difference between the two groups. Perhaps further studies need to be done in this
regard especially on the toddlers where the increased activity due to leucocytes proliferation
may make a difference as far as the activity of ADA\(_1\) is concerned.

4.3. Pathognomonicity

The merits and demerits of the ADA assay makes it necessary for a proper scientific evaluation
in subsequent studies on the subject. Really the scientific diagnosis of tuberculous meningitis
can be established only when we demonstrate the presence of the tubercle bacilli in the CSF
either on direct examination or culture; smears of acid fast bacilli yield only few positive
results. Usually the growth of *M. tuberculosis* is not recognised in culture media until after about 4 weeks and the frequency of positive cultures is only about 15%.\textsuperscript{43,72} From the above premise a retrospective diagnosis approach was used in this project as though it were a novel study on the subject. The sensitivity and specificity etc. were based on patients who were identified on the basis of clinical diagnosis and ADA at the tentative cut-off point of 9 IU/L. Samples were taken from these patients before they were put on initial antibiotic therapy. Some of these patients were too ill for the complete antibiotic regimen. These patients were immediately switched onto anti-tuberculous therapy by the attending physician. Those who were not too ill were made to finish the drug regimen and on the evidence of no improvement were switched onto anti-tuberculous therapy.

The CSF ADA before and after the anti-tuberculous therapy of the latter were used in computation of the pathognomonicity. This was the only way the diagnosis could have been done definitively. The two patients who died before the second sampling could be done were diagnosed at post-mortem of having died of tuberculous meningitis. Those who responded to the anti-tuberculous therapy had their CSF ADA reduced quite considerably at the second testing and responded to the therapy enough to be discharged for later reviews.

Referring to table 1 and at the cut-off points of 9 and 12 IU/L the sensitivity, specificity, efficiency, and the negative predictive values, were the same. The positive predictive values were however different. At 9 IU/L the positive predictive value was 92% while it was 87% at 12 IU/L. Segura \textit{et al}\textsuperscript{17} found a sensitivity and specificity of 100% and 99% respectively at the same cut-off point but according to them the sensitivity decreased to 89% when patients younger than 7 years were included. Their positive and negative predictive values were 93%
and 100% respectively. Mann et al \(^6\) \(^1\) had a sensitivity of 87% and a specificity of 84%. The positive and negative predictive values were also 87% and 84% respectively.

The interesting point about these figures is the number of patients (33 and 35 respectively) in these two studies as compared to 230 in this project. Mishra et al \(^3\) \(^3\), also using 35 patients had a sensitivity and specificity of 62.5% and 88.9% respectively. The small number of patients in this case was made worse by using a very low cut-off point of 5 IU/L. The low cut-off point was likely to include a lot more false positives which accounts for the low sensitivity. These results tend to suggest that statistically the sample size and the cut-off point for the ADA assay are very crucial in arriving at meaningful results. Nevertheless the pathognomonicity results of this project agree with that of Segura et al \(^1\) \(^7\).

The sensitivity is a measure of the incidence of positive results in patients known to have a condition i.e. ‘true positives’ and the specificity is a measure of the incidence of negative results in patients known to be free of a disease i.e., ‘true negatives’ \(^7\) \(^3\). The implication of 100% sensitivity in this project means that all those known to have TBM will be diagnosed as having it on the basis of the ADA test alone. The specificity of 97% implies that 3% of disease-free people will be classified as having TBM (false positives) on the basis of the test result.

An ideal diagnostic test would be 100% sensitive, giving positive results in all diseased subjects, and would be 100% specific, giving negative results in all disease-free subjects. But in reality no tests achieve such high standards; all generate false positives and false negatives. Factors which tend to increase the specificity of a test tend to decrease the sensitivity and vice
versa as there is always an overlap between test results seen in health and in disease. If it were decided to diagnose TBM only and if CSF ADA activity was set at a cut-off point of 15 IU/L, the test would have 100% specificity and positive results (>15 IU/L) would only be encountered in TBM. On the other hand the test would have a low sensitivity in that many patient with mild TBM would be misdiagnosed. If a concentration of 5 IU/L was used, the test result would be very sensitive and all those with TBM would correctly be diagnosed but very non specific because many people with bacteria, or purulent meningitis and other normal people would be diagnosed as having TBM.

A highly specific and sensitive test does not necessarily perform well in the clinical context. This is because the predictive value of a test result which is equal to the percentage of all positive results that are true positive, is dependent on the prevalence of the disease. If a common condition has a low prevalence and the test is less than one hundred percent specific, many false positives will result. A high predictive value for a positive test is important if the appropriate management of a true positive would be potentially dangerous if applied to a false positive, as is the case in TBM. However this discussion applies to the use of a single test for diagnostic purposes but in practice, the clinician will combine clinical information and results of several investigations to make the diagnosis.

The positive predictive value of the assay at the cut-off point of 12 IU/L in this project was 87% as compared to that of 92% at the cut-off point of 9 IU/L; hence the choice of 9 IU/L as the cut-off point for the test. If the test is used rationally together with others as has been pointed out, especially with the protein and the cell count as was done by Ribera et al.,
predictive value of the positive result will be higher in the patient who has other features suggesting a particular diagnosis.

4.4. Other Diseases

4.4.1 Diabetes Mellitus

4.4.1.1 ADA activity in Hyperglycaemia

ADA activity was estimated in newly diagnosed non insulin dependent diabetic patients with the view of establishing the level of the serum enzyme activity and also any relationship between raised serum total ADA activity and hyperglycaemia. All patients who volunteered to participate in the study were confirmed diabetics without any control at all since they were all newly diagnosed. The mean initial total ADA activity (38.0 ± 2.0 IU/L) in the Type II DM patients was significantly higher than that of the healthy donors (16.0 ± 1.0 IU/L). The elevated ADA decreased significantly after 8 weeks of treatment even though not enough to bring all of them except one into the reference values.

The results of the FBG also showed a significant difference between the initial testing (12.5 ±1.4 mmol/L) and after treatment (7.8 ± 4.1 mmol/L) (fig. 4). This indicated some degree of patient compliance to the doctors' prescribed drugs or good dietary management. Despite the modest control of hyperglycaemia, only four patients were able to control their FBG to the reference value. This may indicate treatment inadequacy or non-compliance, especially regarding the difficulty in diet restriction as confessed by some of the volunteers. This indicated some degree of patient compliance to the doctors’ prescribed drugs or good dietary management.
There was also a significant correlation between the FBG Vs ADA; before treatment ($r = 0.78$ or $P<0.001$) as well as after treatment ($r = 0.60$ or $P < 0.01$). The correlation between the above is suggested to be due to the apparent role ADA plays in the modulation of insulin action on blood glucose metabolism $^{52}$. This observation agrees with the findings of other research workers. Insulin administration was noticed to decrease ADA activity in lymph nodes in diabetic BB rats; and in tissue of streptozotocin diabetic rats which were known to have elevated ADA activity, therefore elevated ADA activity in the serum of DM patients may be directly correlated with impaired insulin activity in glucose metabolism $^{52}$.

The significance of elevated ADA levels in the sera of DM patients is not understood at the present time. For the most part nucleosides have no biological role other than to serve as precursors for the nucleotides. Adenosine is an exception. In mammals adenosine function as an autocoid or ‘local hormone’. It circulates in the blood stream, acting locally on specific cells to influence such diverse physiological phenomena as blood vessel dilation, smooth muscle contraction and neurotransmitter, inhibiting neuronal firing and synaptic transmission. It also inhibits platelet aggregation. Adenosine acts by binding to membrane receptors and affecting adenylate cyclase activity, leading to a change in cAMP concentration. It activates adenylate cyclase in certain cells, producing an increase in cAMP, whereas it inhibits adenylate cyclase in other cells. In the brain membranes each of these actions is mediated by a separate receptor. The A1 receptor mediates a decrease in adenylate cyclase activity, while the A2 mediates an increase $^{74}$.

In a previous study in which adenosine inhibited adenylate cyclase through the A1 adenosine receptors resulting in the inhibition of lipolysis, removal of endogenous adenosine by ADA
resulted in an immediate rise in lipolytic activity and therefore an increase in glucose level. Elevated ADA levels in DM patients may therefore augment hyperlipidaemia by increasing lipolysis however there was no significant correlation between the ADA levels in DM patients and hyperlipidaemia.

The explanation to this discrepancy is that even though adenosine inhibits lipolysis through its A1 receptor via the G1 protein lowering effect on cAMP, inhibition of insulin by adenosine in starvation or diabetes does not occur as demonstrated by Koopmans et al and Londos et al with starved and streptozotocin induced diabetic rats; because insulin binding is through tyrosine kinase receptors and autophosphorylation at Tyr residues allows the receptor tyrosine kinase to remain active even after the activating hormone has been dissociated from receptor.

Koopmans et al have shown that any direct effect of insulin on adenylate cyclase system would be mediated by other circuits than the G1 circuits because rat adipocytes were highly responsive to insulin in the presence of maximal concentrations of (-) N(R-phenyl-isopropyl) adenosine (PIA). There is further evidence by Mooney et al who demonstrated that the inhibitory effect of insulin on lipolysis is independent of adenylate cyclase.

4.4.1.2. **Comparison between ADA activity and glycated haemoglobin in Type II DM**

The results so far have clearly demonstrated that serum total ADA activity and FBG were elevated in the Type II DM patients. In a study by Tomoaki et al to characterise the significance of the isozymes of ADA in hospitalised diabetes patients who had diabetes for more than 5 years, they estimated total ADA, ADA1 and ADA2 and observed a significant correlation (P<0.002) between ADA2 and Hb A1c in poorly controlled Type II DM patients.
For total ADA a significant correlation ($P < 0.005$) was noticed, but ADA$_i$ activity did not correlate well with HbA$_{1c}$. In the study 11 out of 12 hospitalised poorly controlled Type II DM patients (HbA$_{1c} > 8.5$) normalised their glucose level after 42 days, indicating that hospitalisation or metabolic control must be a better way of managing Type II DM patients, although not a very practical option at least in Africa.

In the present study the mean GHb level in the 20 apparently healthy donors was $5.5 \pm 1.1\%$. The initial mean GHb levels in the poorly controlled diabetics of $14.0 \pm 1.3\%$ was significantly higher than that of the apparently healthy donor. After 8 weeks of treatment, it decreased to $10.9 \pm 1.4$ ($p > 0.001$; fig. 6). There was a good correlation between the FBG and ADA before treatment ($r = 0.78$ or $P < 0.001$); as well as after treatment ($r = 0.60$ or $P < 0.01$; Fig. 7 a and b). The after treatment correlation between GHb and FBG; GHb and ADA were $r = 0.56$ ($P < 0.02$) and $r = 0.70$ ($P < 0.005$) respectively. (Fig. 8 a and b).

The only well controlled patient happened to be in the high socio-economic class and was very eager to control his diabetes. He was also well informed on the complications of the disease. Thus it is obvious that patients’ eagerness to control the disease as well as their socio-economic status may be factors that can influence the out-patient control of diabetes mellitus. This also brings into question the whole idea of diabetic education. It appears the majority of the patients may not be taking the diabetic awareness programme serious or simply, find strict dietary control very difficult.

The positive correlation between ADA and GHb as a function of hyperglycaemia in the present study is very important. Even though the comparison is between the only well-
controlled patient vs. the other fourteen poorly controlled, the observation agrees with that of Tomoaki et al. The difference here is the hospitalisation of the poorly controlled diabetics for metabolic control with insulin. However, this does not reflect the real situation in most diabetic clinics such as the Korle Bu hospital diabetic clinic where consultation is purely on out-patient basis.

The positive correlation again suggests that under conditions of uncomplicated DM, i.e. without any obvious infections which will be a function of ADA release, the patient’s serum ADA activity may be used to monitor the long-term effect of diabetic control. This may be very important in situations (such as in Ghana) where most patients cannot obviously afford the relatively very expensive GHb testing. Furthermore, there is the added advantage of using ADA activity as an adjunct to the differential diagnosis of any such complications of DM since it is readily a non-specific marker for infections. Under this circumstance, the DM may be brought under control, yet the serum ADA activity will remain elevated. The serum GHb level however may be used as a confirmation test only.

4.5 Pulmonary tuberculosis

Pleural tuberculosis is often difficult to diagnose since mycobacterium in pleural fluid is scanty and rarely observed on direct observation. In addition, pleural biopsy and cultures are positive in less than 50 percent of investigations. In this context, attempts have been made to identify markers which allow more rapid diagnosis. One such marker is adenosine deaminase which has been proposed to be a useful diagnostic tool for tuberculous disease in pleura, pericardium and peritoneum.
Ocaña et al. showed that there is a selective increase in adenosine deaminase activity in pleural effusions of tuberculous origin, with a sensitivity of 100% and a specificity of 97%. They also concluded that the test is of great value in the differential diagnosis of neoplastic and lymphomatous pleural effusions. A tuberculous aetiology can be assumed if adenosine deaminase activity is found to be high in a lymphocytic pleural exudate but if it is lower than 50 IU/L and other non-invasive methods prove negative, a pleural biopsy should be done.

In tuberculous pleural effusions both the absolute number and percentage T-lymphocytes are significantly higher than that in the peripheral blood, a fact which could explain the increase in adenosine deaminase activity, an enzyme predominantly detected in T-cells and macrophages. An increase in T-lymphocytes is also found in neoplasms and connective tissue diseases. In a previous study Ocaña et al., found no correlation between enzyme activity and total number of T-lymphocytes in tuberculous pleural fluid. Therefore the specific enzyme increase in tuberculous lymphocytic exudates must be related to the maturative stage T-lymphocytes which are presumably more immature and reactive than in other diseases as part of the cell-mediated response to mycobacterial antigens.

Several authors who have already been cited have found very high sensitivity and specificity for the test for the diagnosis of tuberculous pleurisy. In this project the levels of serum and pleural fluid ADA were estimated. As for the serum level it has been observed to be increased in several infectious diseases caused by microorganisms infecting mainly the macrophages, HIV and some malignant diseases therefore its level may only be for academic interest. The control level of 13 IU/L (effusions due to causes other than TB) also agrees with levels in conditions such as cirrhotic and malignant ascites as was found by several author including...
Mishra et al.\textsuperscript{33}, who found ADA levels in patients with tuberculous, cirrhotic and malignant ascites to 98.8 ± 20.1, 14 ± 10.6 and 14.6 ± 6.7 IU/L respectively. The level of 94 IU/L for tuberculous effusion in this project agrees with that of several authors already cited.

4.6. Renal failure

Though serum ADA concentration is not very specific, the assay is very useful in the accurate diagnosis of many pathological diseases\textsuperscript{38}. The activity of ADA increases during antigenic and mitogenic responses of lymphocytes. As T-cell dysfunction is seen in acute nephrotic syndrome, it could affect ADA level\textsuperscript{33}. Mishra et al.\textsuperscript{33} have shown that cell mediated immunity is greatly depressed by way of low absolute lymphocyte count, decreased response to PPD skin reaction, and T-lymphocyte percentage in acute nephrotic syndrome patients. On the other hand the serum ADA activity was significantly elevated with a level of 40.9 ± 11.5 IU/L; which compares with 38.0 ± 1 IU/L of this project report.

They contend that the elevated serum ADA activity in the presence of decreased lymphocyte hypo-activity and decreased T-lymphocyte percentage, points towards a mechanism leading to lyses of T-lymphocytes, causing the release of ADA and raising its serum levels. This contrasts the findings of other authors who observed that the specific enzyme increase in tuberculous lymphocytic exudates must be related to the maturative stage of T-lymphocytes which are presumably more immature and reactive than in other diseases as part of the cell-mediated response to mycobacterial antigens\textsuperscript{78}.
The view of Mishra et al.\textsuperscript{33} is also supported by Ooi et al.\textsuperscript{79} who demonstrated the presence of circulating lymphocytotoxins in nephrotic patients. Mishra et al.\textsuperscript{33} also observed that in remission the absolute lymphocyte count and T-lymphocyte percentage improved significantly, but the latter was still lower compared with controls. The serum ADA activity remained unchanged. The persistence of decreased T-lymphocytes percentage and elevated ADA activity indicate abnormal CMI even when the patient is in remission. Thus the depressed CMI may also lead to impairment of T-cell dependent B cell activation accounting for the immunoglobulin abnormality both in active state and in remission of the disease. It is fair to conclude that serial estimation of serum ADA in renal disease may be an important tool for the management of the disease.

4.7. HIV/AIDS

The average age of 27 ± 2 years of the HIV patient in this project is consistent with the current trend in the general population. The serum ADA concentration of the 11 TB - negative HIV positive patients was 41 IU/L, and this was not significantly different from the 37 ± 1 IU/L of the pulmonary TB patients without HIV. But significantly both levels were higher than that of the controls but not as high as that of the 10 AIDS patients who were TB positive with a mean serum ADA concentration of 64 ± 2 IU/L.

The most significant thing about this group is the contribution of tuberculosis to the high level of serum ADA concentration. It also highlights the difference in the level of ADA in HIV sero positive patients compared to the HIV/AIDS patients. ADA activity has been established by several authors on this subject, as a function of cell mediated immune response. Therefore it is
generally expected that patients with full blown AIDS will have low levels of ADA due to the lowered immune response attributed to the cytotoxic effect of the HIV virus. In this project it was found not to be entirely correct. While those with the full blown disease had a level of 64 ± 2 IU/L, those who were sero positive but without AIDS had a value of 75 ± 2 IU/L, even though slightly lower this was not statistically different from the latter.

It is believed that depending on the stage of the disease an AIDS patient may still have some ability of immune response which means mitogenic from the CMI point of view and cytotoxic from the HIV point of view. Both these processes according to Mishra et al. 33 and Ooi et al. 79, will result in raising the serum ADA concentration. This explains why AIDS patients with tuberculosis were found to have almost the same level as the sero positive cases instead of being much lower. It is even more interesting to notice that the two patients with full blown AIDS but TB negative had serum ADA levels of 46 and 27 IU/L respectively.

This position appears to lend some credence to the findings of Mishra et al. 33 who contend that the elevated serum ADA activity in the presence of lymphocyte hypo-activity and decreased T-lymphocyte percentage, points towards a mechanism leading to lyses of T-lymphocytes, causing the release of ADA and raising its serum levels. Since HIV destroys these immunocompetent cells it will be interesting to show the presence or level of circulating lymphocytotoxins in HIV/AIDS patients as Ooi et al. 79 found in the case of nephrotic disease.
4.8. **Hepatitis**

In hepatitis the level of $50 \pm 2$ IU/L is consistent with what other authors found\(^3\). There was no correlation between ADA and the liver enzymes except ALT. Incidentally increased activity of ALT in serum reflects hepatocytes damage with enzyme leakage. The moderate correlation ($r = 0.5;\ p < 0.02$) between ADA and ALT suggests that some adenosine deaminase may originate from the cytoplasm of the hepatocytes since ALT is confined there. Thus in hepatitis serum ADA activity may not necessarily reflect monocyte activity or origin as has been suggested by Ungerer et al\(^3\). It will be of interest to carry out subcellular studies of the enzyme to establish its concentrations in some of the organelles such as the cytoplasm and mitochondria to ascertain the source of the enzyme particularly in the hepatocytes.
Chapter 5: CONCLUSIONS

In conclusion, in most of the diseases studied in this project serum ADA seems to originate from the monocyte/macrophage origin and therefore reflects involvement of cellular immune system. However the demonstration of lymphocytotoxins in primary renal disease and in hepatitis, the modest correlation between serum ADA and ALT in this project suggest that the source of serum ADA may be from lymphocytes and hepatocytes as well and may indicate the involvement of ADA₁₊ₑᵣ. It may also be concluded that since there is a significant negative correlation between T-lymphocyte percentage and ADA activity the mechanism may be from both lymphocyte proliferation by mitogenic stimulation as observed by Hovi et al and a process leading to the lyses of T-lymphocytes, causing the release of ADA and raising its serum levels as indicated by Ooi et al.

Furthermore since T-Lymphocytes are precursors of ‘effector’ cells responsible for specific cell mediated immunity as well as control both humoral (B-cell) and cell mediated immune responses one may conclude that total ADA estimation in body fluids is the best parameter for the diseases which are a function of cell mediated immunity. If and when necessary the isozyme determinations could be used to differentiate between diseases of lymphomatous origin such as acute lymphatic leukaemia and those of cell mediated immune response.

The results suggest that in cerebrospinal fluid tubercular infection is behind the altered activity and that with the combination of other parameters especially protein and cell count, the specificity of the test would be improved tremendously in the diagnosis of TBM at the established cut-off point of 9 IU/L. As has been stated already serum total ADA levels together with other parameters at the same time can be very useful in the diagnosis and
especially prognosis of diseases states.

In diabetes it has been demonstrated that ADA levels were high in Type II DM patients as compared with healthy donor, ADA activity decreased significantly following blood glucose control and the decrease in the enzymatic activity correlated significantly with decrease in FBG and glycated haemoglobin levels. However, the levels could not be controlled well enough to the reference values except for one patient whose eagerness was such that all the three parameters were controlled to the reference values.

Total ADA activity in the serum of diabetic patients could be an important adjunct in the monitoring of Type II DM patients. But the level of ADA also increases in all inflammatory conditions in which the immunocompetent cells are stimulated to produce ADA. This requires that clinicians should rule out infections that may activate the activity of the enzyme before it could be used to monitor DM patients effectively. ADA activity estimation may serve as an alternative assay to glycated haemoglobin in Ghana since both serve the same purpose but the cost per test is far cheaper than the cost of glycated haemoglobin test.

Finally, this project has clearly established an unique cut-off point for ADA activity in CSF for the Ghanaian population for early diagnosis of TBM; and the levels in other body fluids vis-à-vis the varying cut-off points as documented in the literature. In HIV/AIDS it has also been demonstrated that even though it is generally believed that the enzyme activity is low due to lysis activity of the virus on the immuno competent cells; in TB this notion is not entirely correct. Depending on the stage of the disease an individual may still possess some ability of immune response and therefore a moderately raised level of ADA as opposed to being low in
the AIDS patient without TB.

In hepatitis it has also demonstrated a correlation between ADA activity and ALT and therefore the possibility of the assay in monitoring the disease. In diabetes it has demonstrated a correlation between ADA activity and hyperglycaemia and glycated haemoglobin. This suggest that ADA activity may be an alternate to glycated haemoglobin in the long term monitoring of Type II DM patients.
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- 9


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APPENDIX A

A layout of the assay procedure for ADA determination.

<table>
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<tr>
<th></th>
<th>Reagent Blank</th>
<th>Standard</th>
<th>Sample Blank</th>
<th>Sample</th>
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</thead>
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<td>Phosphate buffer</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Adenosine soln.</td>
<td>-</td>
<td>-</td>
<td>1 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ Std. Soln.</td>
<td>1 mL</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50 µL</td>
</tr>
<tr>
<td>Water</td>
<td>50 µL</td>
<td>50 µL</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

cap tubes with parafilm, incubate for 60 min. in 37° C water-bath

<table>
<thead>
<tr>
<th>Nitroprusside Soln</th>
<th>3 mL</th>
<th>3 mL</th>
<th>3 mL</th>
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<tbody>
<tr>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>50 µL</td>
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</tr>
<tr>
<td>Hypochlorite soln</td>
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<td>3 mL</td>
<td>3 mL</td>
<td>3 mL</td>
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Incubate for 30 min. in a 37° C water-bath, measure absorbance against water at 623 nm

Addition of Water can be omitted without causing any appreciable error
APPENDIX B

The results of Coefficient of Variation (CV) for the (NH₄)₂SO₄ standard solution at low and high concentrations of the salt.

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</thead>
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<td>High (50 IU/L)</td>
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<td>0.296</td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>4</td>
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<td></td>
</tr>
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<tr>
<td>CV (%)</td>
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</table>
## CSF ADA levels of all TBM patients. n=230

| No. | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | 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ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU/L) | No. ADA(IU(L): No
APPENDIX D

Serum ADA levels of all ‘Apparently Healthy’ Donors (HD). n=30

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APPENDIX E

Statistical Analysis

\[
\bar{X} = \frac{\sum x}{n}
\]

Standard Deviation (SD) = \( \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}} \)

Correlation coefficient (r) = \( \frac{\sum (x_1 - \bar{x})(y_1 - \bar{y})}{\sqrt{\sum (x_1 - \bar{x})^2 \sum (y_1 - \bar{y})^2}} \)

Where \( x_1 \) and \( y_1 \) = Variable counts of individual specimen by the comparative and test methods, respectively

\( \bar{x} \) and \( \bar{y} \) = Mean of variable counts of all the specimens of the comparative and test methods, respectively

\( d \) = Difference between paired measurements

\( \bar{d} \) = Mean of differences

\( n \) = Number of paired measurements

\( \Sigma \) = The sum of

Coefficient of Variation (CV) = \( \frac{SD}{\bar{x}} \times 100\% \)

- Adapted from Statistics at Square One by TDV Swinscow, printed by Latimer Trend and Company Ltd. Plymouth.
Reagent Preparations

All solutions were prepared with specified double distilled water as follows:

1) **Phosphate buffer (50 mmol/L; pH 6.5):** *(Dark bottle)*
   Dissolve KH$_2$PO$_4$ .H$_2$O (4.73 g; anhydrous 4.1g) and Na$_2$HPO$_4$.12H$_2$O (5.62 g; anhydrous 2.2 g) in water dilute to 1,000 mL with boiled water.

2) **Substrate**
   Adenosine (280 mg) + 30 mL Phosphate buffer, warm in a hot water bath to dissolve, cool under running H$_2$O. Dilute to 50 mL with phosphate buffer. Prepared fresh.

3) **Ammonium Sulphate Stock Solution (15 mmol/L):** *(Dark bottle)*
   Dissolve anhydrous ammonium sulphate (0.1982 g) in distilled H$_2$O and make up to 100 mL.

4) **Ammonium Sulphate Standard Solution (75 µmol/L):** *(Dark bottle)*
   Take 0.5 mL stock Ammonium sulphate solution, make up to 100 mL with phosphate buffer.

5) **Phenol/nitroprusside solution (phenol, 106 mmol/L; nitroprusside, 0.17 mmol/L):** *(Dark bottle)*
   Dissolve 5g phenol, 25 mg sodium nitroprusside in approximate 250 mL water. Dilute to 500 mL.

6) **NaOH (1 mol/L):**
   Weigh 10 g and dissolve in 250 mL H$_2$O

7) **Clorox (5% NaOCl)**

8) **Alkaline Hypochlorite Solution (NaOCl, 11 mmol/L; NaOH, 125 mmol/L)**
9) Weigh 2.5 g NaOH and dissolve in water. Add 8.2 mL of (7) or 4.1 mL (10%) NaOCl and dilute to 500 mL with H₂O.

Very important precautions; All glassware must be acid washed (0.1 mmol/L HCl) to remove Ammonia.

Store all solutions at 0°- 4°C.

Solutions 1, 3, 4, 5, 6 are stable for at least 2 months.

Discard solution 5 if it becomes brown.

Solution 2 must be prepared fresh.
PROBLEMS ENCOUNTERED WITH ASSAY

The preparation of the 5% NaOCl from the powder proved difficult since it would not dissolve. A 10% solution was acquired for this purpose. It was also found that the purity of the ammonium sulphate for standard preparation was very critical in this assay. The first salt which was known to be very old gave very weak absorbances. It was therefore decided to use a newly acquired salt which interestingly gave absorbances which were about five times that of the old one. The older salt would have given spuriously higher results. Since there is no availability of reference materials on this assay it was necessary to carry out experiments with the purity of the salt with samples from normal subjects and compare them with reference values obtained by other researchers on the subject.

The absorbance of the reagent blank measured at 630-640 nm. against water should be below 0.030. It was found that this was not the case if glassware was not thoroughly rinsed in distilled water after the acid wash. Most probably traces of acid in the tubes interfere with the assay by affecting the pH. Consequently the HCl wash concentration was found to be ideal at 50 mmol/L solution. Tubes should be dried in ammonia free environment. Experience has shown that in a room where a distilled water plant is in operation ammonia gas released from the process interferes with the assay resulting in higher blanks. Likewise tubes should be freshly washed and dried immediately before use. Tubes that had been washed and kept for more than a day were also shown to be significantly contaminated with ammonia.

Samples collected into fluoride tubes were shown to be about 33% lower in concentration than those collected into clean plain tubes. Thus fluoride inhibits the enzyme activity by this
magnitude.

Phenol tends to react with plastics therefore it could not be weighed in a weighing boat made of plastic. It should be weighed in a glass beaker.

The colour development depends on correct preparation of the alkaline hypochlorite solution. NaOH (1 mol) was prepared as stock from which a known quantity was used to prepare the required concentration. The concentration of the NaOH had decreased after about five weeks at 4°C presumably due to its hygroscopic nature. It was therefore decided to prepare the alkaline hypochlorite solution by directly weighing the right amount of the NaOH pellets and the right amount of the clorox as indicated in section (appendix F).