

ASSESSMENT OF THE VIABILITY OF ADULT
ONCHOCERCA VOLVULUS IN TISSUE
SECTIONS USING ENZYME HISTOCHEMICAL
DEMONSTRATION OF LACTATE
DEHYDROGENASE ACTIVITY

BY

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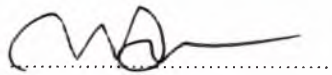
DEDICATION

This thesis is dedicated to my family



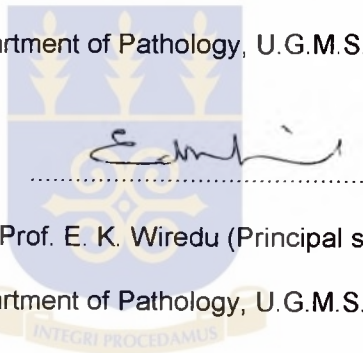
DECLARATION

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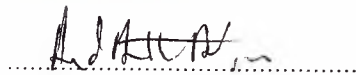
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Current methods of assessing the viability of adult *Onchocerca volvulus* in drug evaluations are mainly based on *in-vitro* and histological techniques. The *in-vitro* method is efficient in detecting live and dead worms while the histological method is more efficient in determining the number of worms in the nodules. Despite their capabilities in some areas, these techniques have certain methodical deficiencies which often lead to inaccurate interpretation of results or marked variations between individuals assessing the viabilities of worms in the same nodules. The deficiencies associated with these techniques are partly responsible for the delay in developing safe and effective drugs for the treatment of onchocerciasis. Based on principles underlying lactate production and tetrazolium reduction tests used in *in-vitro* techniques for testing the viabilities of adult worms, an enzyme histochemical staining technique was employed to determine the presence of lactate dehydrogenase (LDH) in adult *O. volvulus* tissue sections and then used to determine their viabilities.

Eleven onchocerciasis infected volunteers donated a total of sixty-one *O. volvulus* nodules for this study. Out of the total number of nodules donated, unfixed cryostat sections were obtained from thirty-two of the nodules (including two nodules which were heated in distilled water at 60°C to obtain heat-killed worms). The sections were incubated in a substrate containing sodium DL-lactate, nicotinamide adenine dinucleotide (NAD), magnesium chloride and nitro blue tetrazolium compound (NBT) at 37°C for an hour. The same nodules were fixed in phosphate buffered formalin after the cryostat sections were obtained from them and processed by the paraffin

method to obtain paraffin processed sections which were stained by the haematoxylin and eosin technique. The other twenty-nine *O. Volvulus* nodules from the total number of nodules obtained from the volunteers (including two heat-killed nodules) were fixed in phosphate-buffered formalin and paraffin processed sections obtained from them and stained by the haematoxylin and eosin technique. Light microscopic examination of the three sets of sections revealed that worm-sections which were morphologically well preserved in their paraffin sections showed the presence of LDH activity in their corresponding cryostat sections and therefore were adjudged to be sections of live worms. On the other hand worm-sections which were morphologically degenerated in paraffin sections did not show any LDH activity in their cryostat sections and were adjudged to be sections of dead worms. This observation implied that the presence of LDH activity in worm sections were indications that the sections belonged to live worms and those sections which did not show enzyme activity belonged to dead worms.

There was no significant difference between the enzyme technique and histology in determining the number of adult worms in the nodules examined. This suggested that the enzyme histochemical method has the same advantage that the histological method have over the *in-vitro* technique in determining the number of worms in the nodules. All the three worms detected in the heat-killed nodules were morphologically well preserved in their paraffin sections while their cryostat sections did not show enzyme activity. They were therefore alive histologically but declared dead by the enzyme technique indicating that the enzyme method is capable of detecting dead worms which are unable to maintain the activity of their enzymes even without any

significant morphological degenerations. Since degenerative processes that must show on histology to determine dead worms occur several months after the death of the worm, the study indicates that the enzyme method may be able to detect the death of adult worms much earlier than the histological method. This is regarded as a possible advantage of the enzyme technique over the histological method, however, this assertion needs to be confirmed on a larger sample size. The results showed that, the enzyme histochemical technique can be used as a more reliable alternative to the histological technique in determining the viability of adult *O. volvulus* during drug evaluations. It can also be used along with the histological technique in order to obtain early results during drug trials to be followed by histological results which often takes several weeks to process. Comparative analysis of the two sets of paraffin processed sections confirmed that statistical comparisons of the results from different groups of nodules are acceptable for drug investigations

CHAPTER ONE:

1.1 INTRODUCTION

Onchocerca volvulus is a filarial worm responsible for a chronic parasitic disease known as onchocerciasis. The disease is transmitted by an insect vector of the genus *Simulium* (black flies) which are more than seven hundred species around the world and breed in fast flowing well-oxygenated streams and rivers. This is the reason why onchocerciasis is often referred to as river blindness. In tropical Africa, onchocerciasis is transmitted by *S. damnosum* species complex consisting of thirty-two different forms of which nine species can be found in west Africa (WHO, 1995). The transmission of onchocerciasis is directly associated with the feeding habits of only the female blackflies which suck blood from man and other animals for the maturation of each batch of eggs after fertilization (WHO, 1985; 1995). The male blackflies feed solely on plant juices and do not play any direct role in the transmission of onchocerciasis, limiting the transmission of onchocerciasis further by the population of female black flies in the basins of the rivers where they breed.

When infective *O. volvulus* larvae are introduced into a human host by the female black flies, they mature into adult male or female worms and induce the formation of nodules in which they live. An onchocerca nodule can measure up to 3 cm. in diameter and can contain a dozen or more worms (Shultz-Key, 1977, 1980; Shultz-Key and Kara, 1988). While in the nodules, the adults produce millions of larvae which are directly responsible for the characteristic skin and eye lesions associated with the disease (Walsh, 1984; WHO, 1985, 1995, 2000).

Onchocerciasis can be found throughout tropical Africa and central America with some isolated areas of infections in the Arabian peninsular and some parts of North Africa. It affects mainly small, isolated rural communities along the rivers and streams where the vector breeds so that its impact in these communities destroys their already precarious balance of subsistence economy (WHO, 1985, 1995).

There is no protective vaccine or chemoprophylactic drug against onchocerciasis therefore, the disease can only be controlled by reducing the vector population using biodegradable larvicides and by the administration of safe and effective drugs that will reduce the skin microfilarial density or kill the adult worm (WHO, 1985, 1995, 2000; Forgiione, 2002). Treatment of onchocerciasis requires drugs whose activity should result in the elimination of the adult worm reservoir (macrofilaricides) or permanently disrupt the female worms' ability to produce and/or release microfilariae (microfilaricides). Currently, there are three methods of evaluating the effects of drugs on adult *O. volvulus*. These are based on:

- (1) The use of skin snips as an indirect method (Shultz-Key, 1978, 1980).
- (2) Direct examination of the adult worms after dissection or collagenase digestion of nodular tissue (Shultz-Key, 1977, 1980, 1990; Shultz-Key and Kara, 1988; Büttner *et al.*, 1988; Duke, 1990).
- (3) Histological examination of sections from fixed *O. volvulus* nodules (Shaffie, 1931; Shultz-Key and Kara, 1984; Büttner *et al.*, 1988; Duke, 1990).

The skin snip method is more suitable for the development of microfilaricides than macrofilaricides while dissection and collagenase digestion techniques (*in vitro*) are

mainly suitable for studies in embryogenesis and spermatogenesis as well as studies on the physical features of individual worms (Büttner *et al.*, 1988; Duke, 1990).

Histology is by far the most appropriate and the most widely used technique for the evaluation of drugs directed against the adult worms in infected patients (Büttner *et al.*, 1988; Duke, 1990). However, its application is subjective and often lead to marked variations in results produced by different individuals when they assess the same nodules (Duke, 1996). Any approach that can minimise these variations will enhance the assessment of adult *O. volvulus* during drug trials.

It is suggested that an enzyme histochemical technique may reduce the variations of results from individuals assessing the viability of adult *O. volvulus* because of enzyme specificity. This technique has the ability to indicate the presence or absence of enzymes which may play important roles in the metabolic processes concerning the survival of worms. The technique demonstrates the presence of specific enzymes based on their reactions with specific substrates. To achieve satisfactory enzyme reactions for a reliable demonstration, the substrate must be specific for the enzyme and contain metal ions (often magnesium or manganese) as co-factors. The substrate should also contain compounds such as nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) as coenzymes (Bancroft, 1991; Stevens, 1991). In practice most techniques also require salts which couples with the primary reaction products from the enzyme/substrate reaction to produce final reaction products that can be detected by their colours. Lactate dehydrogenase (LDH, EC: 1.1.1.27) is involved in anaerobic glycolytic action where energy is produced for the *O. volvulus* when they are alive (Stoté, *et al.*, 1993; Stoté, 1998). It is hoped that the

demonstration of lactate dehydrogenase activity in worm tissues using an enzyme histochemical technique can determine whether the worm is dead or alive.

To develop drugs against onchocerciasis, sensitive laboratory techniques are required to indicate minor changes in parasite densities due to the effects of drugs under investigation. However, there are technical difficulties in assessing the effects of promising compounds on the adult *O. volvulus*. These difficulties are partly responsible for the delays in developing safe and effective drugs for the treatment of onchocerciasis (Büttner *et al.*, 1988). It is known that enzymes play an essential role in the survival of adult *O. volvulus* (Acqaah, 1995). We therefore believe that the presence or absence of some of these enzymes could affect their survival and LDH may be one of them.

1.2 OBJECTIVES

The objectives of this project are;

- (1) To demonstrate the presence of LDH in adult *O. volvulus* tissue sections using an enzyme histochemical staining technique.
- (2) To use an enzyme histochemical staining technique to locate areas of LDH activity in adult *O. volvulus* tissues.
- (3) To demonstrate that only live *O. volvulus* worms show LDH activity in their tissue sections.
- (4) To use the enzyme histochemical staining technique to assess the viabilities of adult *O. volvulus*.
- (5) To compare the use of enzyme histochemistry in the detection of the number of worms in nodules as well as the number of live and dead worms with the

histological and *in-vitro* techniques (P= 0.05 level of significance) using the Chi square test.

1.3 OUTPUT OF THE STUDY

The study is aimed at introducing a new method of assessing the viability of adult *O. volvulus* based on the demonstration of LDH which may improve the quality of assessing them during drug evaluations. By so doing, there will be some consistencies in reports during drug evaluations. The study will also indicate the areas of LDH activity in adult onchocerca worms which are vital areas for their survival and can therefore be targeted for future drug developments.

1.4 BENEFICIARIES OF THE STUDY

The project may benefit people suffering from onchocerciasis by helping to develop safe and effective macrofilaricides for their treatment. This will reverse the negative socio-economic problems such as poverty and economic stagnation due to migration of the working population from these areas. The technical committee of WHO (Tropical Diseases Research Group) will be able to improve the method of assessing the effects of promising compounds on the adult *O. volvulus*. This may be able to speed up the search for an effective macrofilaricide which is essential for the total eradication of onchocerciasis in the world (Alley *et al.*, 2001). It is also hoped that the Ministry of Health, Ghana and the country as a whole will benefit from an early development of a macrofilaricide which will help to eliminate onchocerciasis and prevent an increase in the number of people blinded by onchocerciasis in the country. By this, the number of blind people will reduce which will then reduce the number of people who are totally

dependent on the working population as well as government subventions.



CHAPTER TWO:

2.1 ONCHOCERCA VOLVULUS

Like all filaria worms the *O. volvulus* exists in two stages, the adult stage (macrofilariae) and the larval stage (microfilariae). Its adults are normally found entangled in fibrous host tissue and placed subcutaneously at various sites of the body especially over bony prominences (Shultz-Key and Kara, 1988). At full maturity the entangled worms together with the fibrous tissue from the host known as onchocercoma or onchocercal nodules are painless, firm and usually palpable. A disentangled male *O. volvulus* can measure up to 5 cm in length and 0.02 mm in diameter, while the much larger females can measure between 30 and 80 cm with a diameter of about 0.04 mm. The larvae on the other hand are about 220-360 μ long and usually found moving freely and sometimes in large numbers in the dermis of the skin (Shultz-Key and Kara, 1988; Forgione, 2002).

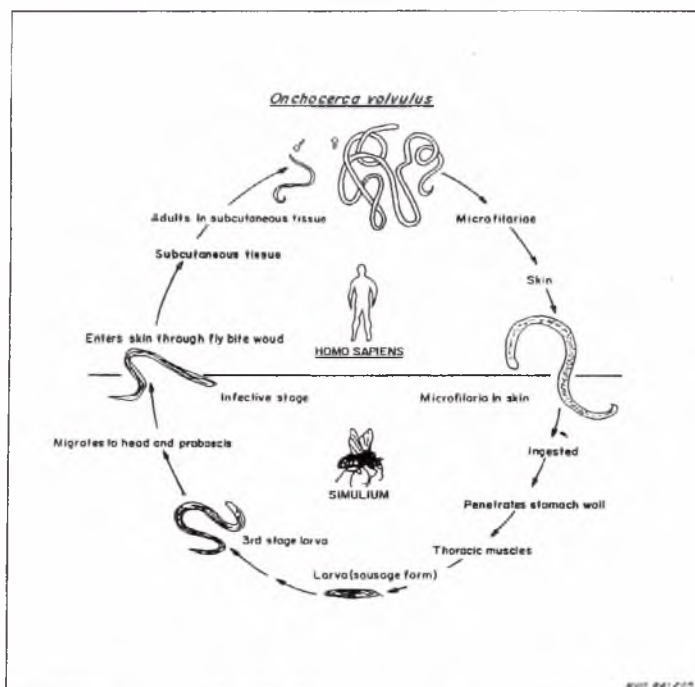
A cycle of transmission begins when the female black fly ingests some larvae from an infected person during a blood meal. A female fly can ingest several hundreds of microfilariae from a single bite of a highly infected person but most of the microfilariae ingested may end up in the fly's stomach and become digested. A few however, penetrate the stomach wall and into the abdominal and thoracic cavities where they transform into intermediate forms which can be morphologically distinguished from the non-infective larvae by their sausage shaped appearances. These then develop into more mature and infective forms (known as L3) which can measure up to 650 μ in length. The number of larvae that eventually transform into infective forms in one fly is generally less than ten after several bites. The infective larvae then find their way into

the head and proboscis of the fly so that in subsequent blood meals, they are introduced into a new host or even the same host (WHO, 1985, 1995). The maturation or transformation into the infective forms inside the black flies, depends on the temperature conditions existing in the breeding site of the flies. At temperatures between 27°C and 37°C it takes about seven days for the larvae to transform into infective forms and may last up to twelve days in lower temperatures. It is known that the development of the larvae in the fly stops completely when the temperature in the breeding sites fall below 16°C (WHO, 1995). In the human host, the infective larvae migrate along the lymphatic channels to subcutaneous sites where they mature into adult male or female worms (Figure 1). A matured female worm can release up to one thousand microfilariae a day with an average life span of one and a half years. These microfilariae then migrate through the nodular tissue to the dermis of the hosts' skin (Shultz-Key, 1978). At this rate of release, it is estimated that an adult individual living in an endemic area may have up to seventeen female worms which can maintain a steady overall skin microfilarial load of between 50 and 200 million (Shultz-Key, 1978; WHO, 1995).

Experiments carried out on volunteers and chimpanzees on the varied capacity of *O. volvulus* to cause disease, show that there are two forms of *O. volvulus* in West Africa. These are designated savanna and forest forms each of which is associated with different vectors of the *S. damnosum* species complex (De Sole *et al.*, 1989; Meredith *et al.*, 1989; Poltera, 1991; WHO, 1985, 1987). The microfilariae of the savanna strain develop well in *S. damnosum* subspecies of the savanna regions but develop poorly in the forest subspecies of the *S. damnosum* and vice versa (Duke, *et al.*, 1966). A

clinically established difference between the two strains is the association of the savanna strain with a higher incidence of blindness and severe eye lesions than the forest strain (Anderson *et al.*, 1974; Dadzie *et al.*, 1989, 1990; Duke and Anderson, 1972; Femme *et al.*, 1989). Physically, the savanna strains (including the L3 forms) are shorter than the forest forms and their microfilarial densities are higher in the superficial parts of the dermis than the forest strains (Botto *et al.*, 1988; Voung *et al.*, 1988; Eichner and Ranz, 1990). The staining patterns of acid phosphatases and isoenzyme analysis in the two strains also reveals that there are some biochemical differences between the forest and savanna subspecies (Omar, 1978; Cianchi *et al.*, 1985; Fockart *et al.*, 1986). The antigenicity and host antibody responses also differ between the two subspecies (Lobos and Wiess, 1985; Lucius *et al.*, 1987). Other differences between the forest and savanna strains exist in the analysis of a variable, tandem repeat 0-150 DNA sequences and hybridization to strain specific DNA probes (Erttmann *et al.*, 1987, 1990; Meredith *et al.*, 1991; Zimmerman *et al.*, 1994; Fischer *et al.*, 1996). It has been shown that when *O. volvulus* DNA fragments are amplified by the polymerase chain reaction (PCR) technique using forest strain specific primers FA (5'GCGGCATAAATCTGCAAATTC3') and FB (5' GATTTTCCGACGAACAGCGC3'), only the DNA from the forest strain hybridizes with a forest strain specific oligonucleotide probe (PFS1-107). The PFS1-107 with a sequence 5'CTGTTCGTCGG AAAAATCGCGGCATAAATCTGCAAATTCACCAAAATATAGTCGAATATTTTTTTTAG CACTCAATTTGAAGGTATGTACCCGTTTCTTGAAATTAG3' is a 107 bp long fragment of DNA, specific for only the forest form of *Onchocerca volvulus* (Erttmann, 1987; Fischer *et al.*, 1996). On the other hand when the fragments of DNA are amplified by PCR using *O. volvulus* species specific primers S3 (5'ATCAATTTTGCAAATGCG3')

and S4 (5'ATAACTGATGACCTATG ACC3'), both the savannah and forest forms hybridize with *O. volvulus* specific oligonucleotide probes S9 (Meredith *et al.*, 1991) with sequence 5' (Di) AAATTGATTATTAACAGA TGACCTATGACATATAA3' and OvS2 (Zimmerman *et al.*, 1993) with the sequence 5' (Di) AATCTCAAAAAACGGGTACTATAC3'. This confirms that there is a gene difference between the forest and savannah strains of *O. volvulus* which has been found to correlate with the epidemiological pattern of blindness in West Africa where the forest and savannah strains coexist (Zimmerman *et al.*, 1992).

FIG. LIFE CYCLE OF *ONCHOCERCA VOLVULUS***Figure 1.** Life cycle of *Onchocerca volvulus* (WHO, 1995)

2.2 ONCHOCERCIASIS

The adult *O. volvulus* worms do not appear to cause disease except some minor discomfort (Walsh, 1984; WHO, 1985, 1995, 2000; Forgione, 2002). However, the microfilariae they produce can cause skin lesions characterised by itching and rashes, which may progress to focal depigmentation of the skin known as leopard skin ((Walsh, 1984; WHO, 1985, 1995, 2000; Forgione, 2002). These skin lesions can be extremely disturbing and vary in intensity from one individual to another. In some individuals the itching may be so severe that it can cause insomnia as well as affect the person's ability to work (Walsh, 1984). If the disease is not treated, the skin may be severely wrinkled or even lose its elasticity and hang in folds. Another skin manifestation of the disease occurs as a severe allergic response which affects only one limb so that the limb becomes dark and the lymph nodes draining it becomes swollen and painful (sowda). The lymphadenitis associated with these severe allergic forms together with the loss of elasticity of the skin in the groin often results in hanging groins and genital elephantiasis (WHO, 2000).

The larvae can also cause eye lesions as a result of permanent scarring of the cornea by the reaction of eye tissue against dead microfilariae within the eye (WHO, 1985, 1995, 2000). It is believed that most of the microfilariae enter the eye by passing beneath the bulbar conjunctiva and penetrate the globe at the limbus. From here they enter the cornea, aqueous humour and the iris. Other microfilariae enter the eye by passing along the sheaths of the anterior ciliary vessel to the peripheral parts of the choroid and retina (Knight, 1982). In the initial stages, there are sporadic opacities of the cornea (punctuate keratitis), which develops into more severe forms affecting both

the anterior and posterior segments of the eyes. The anterior segments may show inflammation of the iris and ciliary body in addition to an irreversible fibrotic reaction beginning at the periphery of the cornea near the limbus (sclerosing keratitis). The posterior segment may show chorioretinitis and optic atrophy. These lesions or any combinations of their different levels of severity are responsible for the blindness that onchocerciasis is well known for (Dadzie *et al.*, 1990).

In addition to skin and eye lesions, onchocerciasis can disturb the affected individual's immunity. It is known that only 25 to 30 per cent of individuals affected by severe savannah onchocerciasis show positive reactions to tuberculin tests as compared to 70 per cent of uninfected populations (Kurtak *et al.*, 1987). Also, uninfected individuals who are considered immunised, generally have stronger cell responses to onchocercal antigens than people with generalised onchocercal dermatitis (WHO, 1989). It has been reported that leprosy is more prevalent among onchocerciasis infected people than other populations (Plaisier *et al.*, 1990). However this remains to be proven as the theory of simple geographical coincidence was not ruled out in the study. There are also several reports indicating that cell mediated immune response to specific and nonspecific parasite antigens are also reduced among onchocerciasis patients (Berre, 1966; Barlet *et al.*, 1978; Barnett *et al.*, 1989; Hougard and Back, 1992; Boakye *et al.*, 1993; Brockhouse *et al.*, 1993; Le Tang *et al.*, 1995).

2.3 DIAGNOSIS OF ONCHOCERCIASIS

The presence of onchocercal nodules and/or prominent skin depigmentation in an individual living in an endemic area are significant indications of an onchocercal infection. The process of diagnosing onchocerciasis by this method is carried out either by visually looking for focal depigmentation of the skin (leopard skin) or physically palpating for subcutaneously placed nodules with the tip of the fingers. For the non palpable nodules which are not easy to detect by a physical examination, ultrasound technology can be used (OCRC, Hohoe; Forgiione *et al.*, 2002). The presence of an onchocercal nodule in an ultrasound scan is indicated by a homogeneous echogenic area containing echo-dense particles with a lateral acoustic shadow (Forgiione *et al.*, 2002). The limitations of this technology as a diagnostic tool is that a similar picture can be observed in scans of other foreign bodies which are capable of inducing fibrosis in the body. A more formal diagnosis of onchocerciasis for epidemiological studies, treatment follow-ups and research purposes require more sensitive and specific methods of which there are several of them ranging from simple skin biopsies to more sophisticated methods such as DNA polymerase chain reactions (PCR).

The most widely used method is the skin snip technique which is based on demonstrating the presence of skin microfilariae in a standardised skin biopsy. The microfilariae emerging from skin biopsies taken from the iliac crest are counted after 30 minutes of incubation in distilled water. Biopsies which fail to release microfilariae within 30 minutes, are re-incubated in physiological saline for 24 hours before they are discarded as negatives (Kurtak, *et al.*, 1987; Phillips *et al.*, 1985). The skin biopsies can also be fixed immediately in 10% formalin and processed by the paraffin method after

which thin sections can be examined microscopically for the presence of microfilariae. This technique is regarded as the most sensitive test for onchocerciasis since it allows for the detection of trapped microfilariae in the skin which could not emerge in physiological saline. A slit-lamp can also be used for a reliable diagnosis of onchocerciasis. A slit-lamp examination of the eyes of an infected person can reveal free floating intra ocular microfilariae in the aqueous of the anterior chamber of the eye and also as live or dead microfilariae on the cornea. This method has the advantage of being non-invasive but it requires the services of well-trained personnel who are not easily available.

A mazzotti test for onchocerciasis involves the administration of 50 mg of diethylcarbamazin (DEC) to individuals suspected of onchocerca infection but their skin snip findings are negative. DEC is known to provoke itching in areas of the skin where microfilariae are killed as well as induce general allergic response from the individual. This reaction is known as the mazzotti reaction (Forgione *et al.*, 2002; Killian, 1988). The reactions may occur within 15 minutes to 24 hours after the DEC administration and can be severe in both the skin and the eyes with some serious consequences including the precipitation of ocular damage in patients who have microfilariae in their eyes (WHO, 1995; Killian, 1988; Awadzi and Gilles 1980a). Because of the side effects associated with DEC administration, a mazzotti test must not be performed until other diagnostic methods have failed to confirm clinical diagnosis. A much safer DEC patch test is now being developed for use by the Onchocerciasis Control Programme of West Africa (OCP). The test involves the application of a mixture of 10% DEC in Nivea cream under an occlusive dressing so that the occurrence of a localised inflammatory

response indicates a positive test result. The accuracy of this test is however limited by the distribution of microfilariae in the skin since the microfilariae has to be killed at the site to induce the inflammatory response. The test is reported to be 30-80% reliable as a diagnostic tool (Forgione *et al.*, 2002; Killian, 1988).

Serological tests for onchocerciasis are available using blots of finger-prick blood collected on filter paper. This method relies on an enzyme linked immunosorbent assay (ELISA) to detect antibodies to *O. volvulus* antigens using multiple antigens in a single assay to enhance its sensitivity and specificity (WHO, 1985, 1987). The disadvantage of the method is its inability to reliably distinguish past infections from current infections probably due to the persistence of onchocerca antibodies in the host blood long after the death of the worm. *O. volvulus* microfilariae have also been found in urine and cerebrospinal fluid (CSF) of some infected individuals living in hyper endemic areas (Forgione *et al.*, 2002). To test for onchocerciasis in urine and CSF, it is suggested that pretreatment with a small dose of DEC mobilises microfilariae into the urine and CSF which can be observed on microscopic examination of centrifuged urine and CSF deposits. Because of the administration of DEC, the test carries the same risks associated with the mazzotti test and should only be used after other methods of diagnosis have failed to confirm clinical findings.

Assays based on the polymerase chain reaction are highly effective in diagnosing onchocerciasis, although the test is too expensive to be applied on routine bases (Fischer *et al.*, 1995). DNA is extracted from skin biopsies of suspected onchocerciasis infected individuals and PCR amplification performed using S3 and S4 primers. The

amplified DNA is denatured, neutralised and blotted onto a nylon membrane. It is then hybridized to non-radioactive DIG-labelled *O. volvulus* specific DNA oligonucleotide probes, S9 and OvS2 (Zimmerman *et al.*, 1993). The amplified DNA fragment of *O. volvulus* microfilariae in the skin biopsies hybridizes well with the *O. volvulus* species specific DNA probes S9 and OvS2. The S9 probe overlaps with the S3 and S4 primers used in the PCR amplification and therefore hybridizes with amplification products with lengths of 300 bp or longer. The OvS2 binds in the centre of 0-150 bp allowing for the detection of amplification products with 150 bp lengths (Fischer *et al.*, 1996). The technique determines the presence of *O. volvulus* DNA in the skin biopsies and can only be positive if there is onchocerca DNA in the specimen. The original expectations that the test could determine the presence of infection if the snip does not contain microfilariae, have not been proved. This is because *O. volvulus* DNA exist in the skin snip only if there is microfilariae present (Fischer *et al.*, 1995). The PCR-based assay on skin snips has therefore no clear advantage over the traditional skin snip technique which can be followed by collagenase digestion of the skin to retrieve microfilariae trapped within the snip (Fischer *et al.*, 1995). The reasons for the absence of microfilariae in the skin has been explained in several reports based on light and electron microscopy as well as immunohistochemical observations. These reveal that microfilariae disappear within three days after microfilaricidal treatment (Buttner *et al.*, 1991; Darge *et al.*, 1991) due to attacks by enzymes released by eosinophils (Wilddenburg *et al.*, 1994, 1995) and neutrophil (Gallin *et al.*, 1995) as well as phagocytic giant multi nuclear cells and macrophages (Buttner *et al.*, 1988).

2.4 EPIDEMIOLOGY AND SOCIOECONOMIC SIGNIFICANCE OF ONCHOCERCIASIS.

Onchocerciasis is the second most infectious cause of blindness in the world (WHO, 2000; Forgiione *et al.*, 2002). It is present in about 36 countries all over the world, and out of an estimated 120 million people who are at a risk world wide, approximately 18 million people are infected with the disease. This includes an estimated 6.5 million people who may be suffering from considerable loss of sight, severe itching and dermatitis and more than 270,000 people believed to be blinded by onchocerciasis (Walsh, 1983; Lariviere *et al.*, 1985; WHO, 1995, 2000). The intensity of infection varies from one area to another depending on the vector population and conducive weather conditions. In highly endemic areas up to 100% of the people in one community can be infected and more than 10% of them may be blind. The vast majority of people infected by the disease live in sub-Saharan Africa (WHO, 2000; Forgiione *et al.*, 2002). In Africa, the disease is found mainly in the tropics and limited to the area between latitudes 15°N and 14°S. Within this area the disease is more endemic in the northern parts of the equator than the southern parts (WHO, 1995). This area covers the West African subregion and some parts of central and east Africa where foci of infection are large areas covering the basins of most of the major rivers. Foci within these areas turn to share borders so that it is difficult to recognise the boundaries between individual foci. In West Africa the largest and the most severe focus is the Volta river basin which stretches across several countries including Burkina Faso, Togo, Ghana, Benin, Cote d'Ivoire and parts of Mali. This area covers about 1.3 million square kilometres (WHO, 1995; OCP, 1995) and before the introduction of control measures, it was estimated that several millions of people were infected including

approximately 35,000 people who were blinded by the disease. Large foci have also been detected in Nigeria, southern Chad and Cameroun which could increase the current estimates to higher figures. The foci of Central and East African parts consist of large foci in the Central Africa Republic, the northern parts of the Democratic Republic of Congo and parts of western Ethiopia. In the southern parts of the equator in tropical Africa, the foci are generally smaller, more isolated and dispersed. The largest focus in this area covers the southern parts of Democratic Republic of Congo and the northern parts of Angola. The rest are generally isolated and dispersed and can be found in Gabon, Congo, Equatorial Guinea, Malawi, Uganda, Tanzania, Burundi and Kenya (Lariviere *et al.*, 1985; Walsh, 1984; WHO, 1973). In the Eastern Mediterranean region, onchocerciasis is found in Sudan with the endemic zone extending through the south and eastern basins of the upper Nile, the blue Nile and some regions in the basins of Atbara and Setit rivers.

In the Arabian peninsula, onchocerciasis is endemic in the Ta'iz region of southern Yemen. The infestation appears to be limited to areas around large elevation ranging from 300 to 1,200 metres above sea level (Lariviere *et al.*, 1985; Walsh, 1984; WHO, 1973). The number of people estimated to be infected by onchocerciasis in Yemen are about 30,000. In Saudi Arabia, there are some indications that the disease may be transmitted in the southwestern regions of the country where a few cases have been reported (WHO, 1989). The second most affected area of onchocerciasis after Africa, is Latin America. In this region the foci of infection are either small or medium size as compared to those in Africa and are generally well defined (Lariviere *et al.*, 1985; Walsh, 1984; WHO, 1973). The countries affected in this region are Guatemala,



Mexico, Colombia, Brazil, Venezuela and Ecuador. In Mexico and Venezuela, the disease is limited to areas of mountainous and medium altitudes. In Brazil, there is a medium size focus along the northeastern border of the country and extends to the border region of Venezuela. It is estimated that the total number of people infected with the disease in Latin America are about 40,000 while more than 750 people are believed to be visually impaired or blinded by the disease.

Although onchocerciasis does not directly cause death, its social and economic consequences can be devastating. Disfigurement resulting from skin lesions and intense pruritus can cause psycho social consequences and isolation (WHO, 2000; Forgione *et al.*, 2002). Onchocerciasis is a chronic infection with clinical manifestations that develop years after initial infection which means that a sizable percentage of people infected are above 40 years (Lucas, 1992; Forgione *et al.*, 2002). The inability of the affected adults to cultivate the fertile lands in the river basins lead to a significant decline in the living standards of the people in these communities (WHO, 1995). This is one of the factors responsible for villagers leaving the fertile riversides land and migrating to urban areas. In the savannah areas in Africa, it is common for several square kilometres of major river valleys to be uninhabited because of the disease. Many of the blind people migrate to the urban areas and end up as beggars in the towns. For those who remain in the villages it is common to see chains of blind adults linked by walking sticks and being led by a young child (WHO, 1995). There is a general despondency among the people, a slow down of economic and social activities and an unacceptable level of illiteracy due to the withdrawal of children from schools to lead the blind adults. In some communities the people turn to their special gods for

assistance to combat the disease. This often involve sacrifices which may compound their economic problems.

2.5 CONTROL AND TREATMENT OF ONCHOCERCIASIS

There are considerable losses during the life cycle of *O. volvulus*, especially where a large percentage of microfilariae ingested by the vector during a blood meal are digested and therefore fail to develop into infective forms. Despite these losses, infections in endemic areas are epidemiologically stable and difficult to eradicate. The parasite-host-vector relationship is well balanced in these areas so that the overall microfilarial density in the host population is sufficient to guarantee a stable transmission (Shultz-Key, 1990). Control measures that aim at reducing transmission of the disease by disrupting the well-balanced parasite-host-vector relationships have been put in place. These disruptions are based on the reduction of vector population and skin microfilarial densities (WHO, 2000). The vector control measures involve aerial larviciding with biodegradable insecticides which destroy the aquatic larval stages of *S. damnosum*. The basic strategy here is to drastically reduce the vector population to a level that will achieve a recognisable interruption of their ability to transmit microfilariae in the community (WHO, 2000). This level must be maintained throughout the live span of the adult worms which may take as long as 9 to 14 years. The advent of ivermectin, suited for community distribution added a new dimension to onchocerciasis control activities. A single dose of ivermectin reduces the skin microfilarial density to a very low level for at least six months (Farthing and Rolston, 1992). Such low levels of skin microfilariae can achieve a recognisable interruption of the transmission of onchocerciasis. Like the vector control measures, the skin

microfilarial levels should be maintained at low levels as long as the adult worm is alive which requires a yearly administration of ivermectin for as long as 9 to 14 years. Studies on the effects of ivermectin on the uptake and development of *O. volvulus* in *S. damnosum* show that soon after treatment with ivermectin, the uptake of microfilariae by *S. damnosum* is reduced almost to zero and rises again over the next six months. However, the average recovery of microfilariae from flies fed on volunteers after successive six monthly treatments are generally reduced (Chavasse and Davies, 1990). This shows that multiple treatment with ivermectin may have a cumulative effect in reducing the rate of transmission in an endemic area. It is anticipated that with the disruption of parasite-host-vector balance in affected areas using the combination of larvicides and ivermectin, the transmission of onchocerciasis will reduce drastically and for that matter effectively reduces the pathology associated with the disease (WHO, 1995). Computer simulations on the long term effects of the combined effect of vector control and drug treatment regimes on onchocerciasis indicate that such combinations can reduce the length of time anticipated for the total eradication of Onchocerciasis (Davies, 1990).

Surgical removal of nodules can be used to reduce the adult worm population which will reduce the population of microfilariae. However, in endemic areas it is not possible to locate all the nodules in an individual for removal since some of the nodules may not be subcutaneously placed. The use of surgery for the control and treatment may also not be feasible since the facilities for nodulectomy may not be available in the endemic areas which are mostly rural. In addition, persistent reinfection may require repeated surgery which may not be sustainable (Guderian, 1988; Alley *et al.* 2001).

The chemotherapy of onchocerciasis has not been a complete success. The ideal drug must be safe and effective as a single oral or parenteral dose or administered in small number of doses. By the nature of the disease the drug must also allow distribution in the affected communities by medical staff with minimum supervision. A successful treatment of onchocerciasis will require the administration of a safe macrofilaricide with or without an equally safe microfilaricide (Forgone, 2002; Alley *et al.*, 2001).

So far, drugs that are available for the treatment of onchocerciasis or are in use for other diseases but considered to have some level of activity against *O. volvulus* are; ivermectin, suramin, diethylcarbamazin (DEC), metrifonate and the benzimidazole carbamates (Awadzi *et al.*, 1994). Ivermectin is a micro cyclic lactone which has a rapid effect against skin microfilariae. It has no intrinsic toxicity in single doses of up to 800 $\mu\text{g}/\text{Kg}$ of body weight or two doses of 800 $\mu\text{g}/\text{Kg}$ given approximately two weeks apart (OCRC, Hohoe). Patient reaction to treatment is usually mild and ocular reactions are minimal even after high doses. There is a massive reduction in skin microfilarial density during the first few days after administration. Ivermectin does not kill the adult *O. volvulus* and does not interfere with embryogenesis or spermatogenesis. It does however, interfere with the release of microfilariae by the adult female *O. volvulus* resulting in intrauterine degeneration of microfilariae (Awadzi *et al.*, 1994).

Suramin is a highly complex compound, which is a macrofilaricide as well as a microfilaricide for *O. volvulus*. The use of suramin for the treatment of onchocerciasis has a wide range of drawbacks which can restrict its application on a regular basis

(Awadzi *et al.*, 1995). It has an inherent toxicity which can cause death from a rare idiosyncratic reaction due to the cumulative effect of suramin. Suramin can also affect the general state of well being of the patient and aggravate existing ocular involvement or precipitation of new lesions (Dawood, 1978; Rougemont *et al.*, 1980, 1984). Attempts to manipulate the dosage and treatment regimes to achieve some reasonable margin of safety failed to come out with a treatment schedule that does not carry any significant risk of side effects (Awadzi *et al.*, 1995). The treatment of onchocerciasis with suramin is therefore recommended for patients with sight threatening disease, severe hyperactive onchodermatitis or high skin microfilarial loads where symptoms are not adequately controlled by repeated treatment with ivermectin (WHO, 1987; Awadzi *et al.*, 1995; Forgiione, 2002).

DEC is, 1-diethylcarbamoyl-4-methyl peperazin dihydrogen citrate. It is a microfilaricide which is capable of destroying 99% of microfilariae after a two-week treatment (Awadzi and Gilles, 1980a). It produces severe systemic reactions and aggravate existing ocular lesions or precipitate new ones which may result in ocular deficiency (Awadzi and Gilles, 1980a). These effects are prominent in individuals who have high skin microfilarial loads and for that matter needs therapy most. Even in the absence of severe reactions, the intense itching associated with repeated administration of DEC makes it unpopular among infected populations and therefore unsustainable.

Metrifonate is 0,0-dimethyl-(1-hydroxy-2,2,2-trichloroethyl)-phosphate. It is an organophosphorous compound which is transformed *in-vivo* into an anti acetylcholine

esterase agent, dichlorvos, 2,2-dichlorovinyl dimethyl phosphate (DDVP). The effects of this transformation are demonstrated by severe abdominal pain, vomiting and diarrhoea in most patients. Its effects sometimes persist well beyond the completion of treatment and without any correlation with the duration of therapy (Awadzi and Gilles, 1980b). Studies on the microfilaricidal effect of metrifonate show that there is only an 83% reduction of skin microfilarial density after a six-day treatment regime which makes it a less efficient microfilaricide than DEC and ivermectin (Awadzi *et al.*, 1980 ; Awadzi, 1980; Awadzi and Gilles, 1980b).

The benzimidazole carbamates include mebendazole, flubendazole and albendazole. They are poorly absorbed and their absorption is usually aided by co-administration with fatty meals. (Awadzi *et al.*, 1990). Mebendazole has to be administered at very high doses of 28.0-35.0 g alone or in combination with levamisole before it is effective against *O. volvulus* (Duke, 1974; Martens and Wery, 1975; Kale, 1978, 1982; Taylor, 1985). Flubendazole on the other hand is only active when administered parenterally and often produce sterile abscesses at the site of injection (Dominquez-Vasquez *et al.*, 1981). The most effective of the benzimidazole carbamides is albendazole when administered at 2.4-6.0 g (Morris *et al.*, 1983; Awadzi *et al.*, 1991). A combination of all three given sequentially over a period of several weeks produces a marked degeneration of the early embryonic stages in the adult female *O. volvulus* (Awadzi *et al.*, 1982). Although the drug effect may last for only a few months, there is also a prolonged suppression of skin microfilarial density (Rivas-Alcala *et al.*, 1981a, 1981b; Awadzi *et al.*, 1982). This mode of administration of the benzimidazole carbamides is time consuming and unsustainable in rural communities.



Ivermectin (Mectizan) is the safest and most effective microfilaricide and since it does not affect the adult worms, the pathology of onchocerciasis can only be avoided when it is administered once a year throughout the life span of the adult worm ((Farthing and Rolston, 1992; WHO, 1995, 2000). This may create a possible *O. volvulus* resistance to ivermectin due to repeated administration. Suramin on the other hand, is the most effective macrofilaricide, but its complex administration and many side effects does not encourage its use for the treatment of onchocerciasis on routine basis (Awadzi *et al.*, 1995; Thylefors and Rolland, 1979). Large scale nodulectomy which has been attempted in Latin America, has been shown to be impractical and incapable of eliminating all adult worms (Guderian, 1988; Guderian *et al.*, 1997).

The search for a safe and effective macrofilaricide against *O. volvulus* is therefore still a high priority to the World Health Organisation (Alley *et al.*, 2001; WHO, 1987, 1995; Buttner *et al.*, 1988). Macrofilaricides have a substantially higher potential of achieving a total elimination of onchocerciasis than ivermectin (Alley *et al.*, 2001). To develop a macrofilaricide, promising compounds are selected as a result of their activity against other filarial worms in rodents and animals or their direct activity against *O. volvulus* *in vitro*. The selected compounds are tested on human volunteers since the adult *O. volvulus* has no other suitable animal reservoir (Buttner *et al.*, 1988). It is known that *O. volvulus* has been successfully transmitted to the chimpanzee on experimental basis (Troare and Hebrard, 1983) however, such artificial transmissions are not suitable for mass drug investigations. In clinical trials therefore, economic and ethical reasons require that information should be gained from very few volunteers to reduce the risk involved in experimenting new compounds directly on humans. This is the main reason

why there is a need for a reliable method of assessing the viability of adult *onchocerca volvulus* during drug trials.

2.6 SKIN SNIP METHOD OF ASSESSING THE VIABILITY OF ADULT

ONCHOCERCA VOLVULUS.

A skin snip is a bloodless skin biopsy taken down to the level of the dermal papillae so that microfilariae obtained from the snips are used to assess the severity of onchocerciasis (Auer *et al.*, 1984). The method can be used effectively as a diagnostic tool as well as for the development of filaricides. For the development of filaricides, the traditional skin snip technique which demonstrates the presence or absence of microfilariae in the skin is modified to enhance its accuracy and sensitivity. This is done by increasing the number of snip sites to six or seven instead of the usual one or two sites to avoid false negatives. In addition, the individual snips are weighed and transferred into identifiable wells of a micro titre plate containing normal saline. The snips are incubated in the saline for 18 to 24 hours at room temperature to allow most of the microfilariae to emerge (OCRC, Hohoe) to maximise the number of microfilariae that will emerge from the snip. It is estimated that more than 80% of microfilariae in a skin snip will emerge from a snip within 24 hours when it is immersed in physiological saline (Schultz-Key, 1978). The microfilariae that emerge from the snips are counted and this allows the skin microfilarial density to be quantified and expressed as the average number of microfilariae per mg of skin. It also allows for the calculation of the total skin microfilarial load of an infected individual taking other factors into consideration (Schultz-Key, 1978). By this modifications, the method can be used to detect minor changes in skin parasite densities caused by any drug under investigation.

The skin snip technique is useful when anti filarial drugs which do not kill the adult worms but interfere temporarily with the development of microfilariae or destroy the

matured microfilariae in the skin are being studied (Buttner *et al.*, 1988). In studies concerning these drugs, the technique is employed directly to determine the effects of the drugs by snipping the patient at regular intervals and observing the differences between the initial skin microfilarial density and the subsequent snips (Duke, 1968; Buttner *et al.*, 1988). The skin snip technique can also be used indirectly to assess the effect of drugs against the adult worms (macrofilaricides) by studying the pattern of reduction and reconstitution of skin microfilariae during investigations (Duke, 1968). The patterns of skin microfilarial densities obtained from skin snips during drug trials allow investigators to draw reliable conclusions regarding the effects of macrofilaricides. Apart from the well publicised observation that skin microfilarial densities fall drastically after the administration of ivermectin and rises after a few months which confirms its microfilaricidal status, it is known that a combination of levamisole and mebendazole affects the embryonated stages of the development of microfilariae (Schultz-Key, 1980). For these drugs it has been observed that during the three weeks that they are administered, the skin microfilarial density falls gradually. At the end of the treatment, there is a significant reinvasion of the skin with microfilariae. It is understood that the reinvasion of the skin by the microfilariae comes from the stock of unaffected stretched microfilariae present in the female worms, followed by new productions as the adult worms recover. This phenomenon is confirmed by the collagenase digestion technique using embryogramme and spermatogramme methods of testing worm viability (Schultz-Key, 1980). Similarly, reports on the reconstitution of skin microfilariae after the administration of metrifonate, mebendazole and diethylcarbamazin show that there are different patterns of reduction and reconstitution of skin microfilarial densities for each drug during and after the cessation of therapy

(Awadzi and Gilles, 1980a; Awadzi and Gilles, 1980b; Schultz-Key, 1980).

2.7 DISSECTION AND COLLAGENASE DIGESTION METHOD OF ASSESSING THE VIABILITY OF ADULT *ONCHOCERCA VOLVULUS*. (IN-VITRO)

Unlike the skin snip method, dissection and collagenase digestion methods provide the opportunity to examine the adult *Onchocerca volvulus* directly. The worms are isolated from nodular tissue either by dissecting the nodules (Friedhein, 1961; Lartigue, 1964) or after collagenase digestion of the nodular tissue (Shultz-Key *et al.*, 1980, 1988). Generally, both male and female worms can be isolated using either dissection or collagenase digestion techniques. However, the nature of their sizes allow for a convenient isolation of male worms by dissection and the female worms by collagenase digestion (Shultz-Key *et al.*, 1980, 1988).

To isolate adult male *O. volvulus*, the nodule is held firmly in focus under a dissecting microscope with a pair of sharp-toothed forceps so that it can be cut lengthwise across its smallest diameter. The cut ends (lips) of the nodule are held with two pairs of toothed forceps and pulled gently apart to tear along the line of incision while observing for the emergence of any part of a male worm (Friedhein, 1961; Lartigue, 1964; OCRC, Hohoe). The diameter of the male worm is fairly small and uniform, more like the posterior parts of their female counterparts and are therefore easy to identify. When they are spotted, they are gently pulled out using a fine pointed pair of forceps and transferred into a culture medium. The tearing process is continued until all the male worms are retrieved from the nodule (OCRC, Hohoe).

The female worms which are extensively entangled with the nodular tissue are isolated by collagenase digestion (Shultz-Key *et al.*, 1988). The nodules are submerged in 0.5% collagenase solution and incubated at 37°C in a water bath for a minimum of 24 hours. After the incubation, the worms are isolated from the debris of digested nodular tissue by washing them thoroughly with sterile saline and transferred into a culture medium (Shultz-Key *et al.*, 1980; Shultz-Key *et al.*, 1988; Lager, 1988; Buttner *et al.*, 1988; OCRC, Hohoe). The isolated adult worms are assessed for their viabilities based on physical, biochemical and histological techniques. These are;

- (a) Test for motility.
- (b) Test for Lactate.
- c) Tetrazolium reduction tests (XTT and MTT test).
- (d) Embryogramme and spermiogramme (Strote, 1993, 1998; Shultz-Key *et al.*, 1980, Shultz-Key *et al.*, 1988; OCRC, Hohoe).

2.7.1 TEST FOR MOTILITY

One of the basic characteristic of most living organisms is movement. Accordingly, when living adult *O. volvulus* is placed in a culture medium, part of it or the entire worm exhibit movements. Such movements can be observed visually using an ordinary light microscope or a computer assisted micromotility metre. The movements of the adult female worms are conveniently observed by the visual method using an inverted light microscope (OCRC, Hohoe). The worms are placed in a culture medium and then observed under the microscope for movements within and outside the worm. Adult *O. volvulus* exhibit involuntary muscle movements when they are alive. Such movements, in addition to their total body movements are scored on a scale of zero to three. A zero

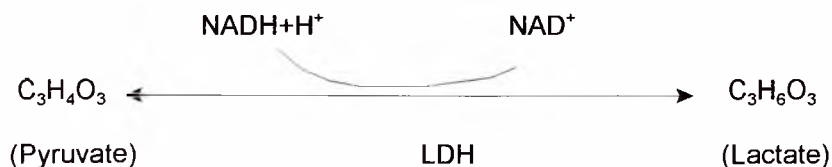
score represents no movement at all while the highest score of three is awarded for movements of the entire worm body plus muscle movements, (Strote, 1993, 1998; OCRC, Hohoe).

The movement of male worms and severed anterior ends of the female worms are conveniently measured using a computer assisted micromotility metre (Bennett, 1987). A micromotility metre is a product of PB Instruments Co., Lansing Mi. USA which is designed to measure the activity of small worms of the size of whole adult *O. volvulus* male worms and severed anterior ends of the female worms since the female worms are too large for the motility metre (Strote *et al.*, 1993; Strote, 1998). The equipment is made up of a built-in housing device able to hold a small precipitin tube of about 5 mm volume, a photodiode, an amplifier, a digital AC-DC converter and a microprocessor. When a motility metre is switched on, light from a pin lamp passes through a plastic pipe to strike the bottom of the motility tube containing a male worm or the anterior end of a female worm in a culture medium. On reaching the meniscus, part of the light is reflected to the photodiode which sends a signal to the microprocessor through the amplifier and digital converter. The digital converter then samples the signal at sixty units per second and the microprocessor calculates the average deviation of the voltage from the mean voltage being produced by the light reaching the photodiode. This deviation is expressed in arbitrary units which is designated motility index (excepts from micromotility metre, product literature). When a blank medium alone or a dead worm is put in a precipitin tube and placed in the housing device of the motility metre, little or no light is reflected and the light reaching the photodiode remains constant. Hence, the motility index is either near zero or at zero. The presence of a living worm

and its movement in the medium causes the fluctuation of light reaching the photodiode, thereby producing an average deviation in the signal ten to a hundred times greater than that produced by the medium without a worm or with a dead worm (immotile worm). The motility indexes of the life worms are compared with that of a controlled heat-killed adult male worm. The difference between the motility indexes of the life worms and that of the heat-killed worms are recorded as the level of activity of the life worms and for that matter their viabilities.

2.7.2 TEST FOR LACTATE

The process of obtaining energy from carbohydrates in most tissues begins with glycolysis. This involves splitting single molecules of glucose into two molecules of pyruvate in the presence of enzymes within the cell (Darnell *et al.*, 1990). Under anaerobic conditions the reduced form of nicotinamide adenine dinucleotide (NADH) reacts with pyruvate under the influence of LDH to yield lactate (Darnell *et al.*, 1990). This process occurs in mammals as well as life filarial worms.



In *Onchocerca volvulus* the lactate produced is secreted into the culture medium in which they are incubated. The amount of lactate produced by a worm indicates the activity of the worm and for that matter its viability (Strote *et al.*, 1993; Strote, 1998). The production of lactate from pyruvate is a reversible process so that when NAD is added to a sample containing lactate in the presence of LDH, the lactate is converted to

pyruvate and NADH (Darnell *et al.*, 1990). In this process the rate of NADH production is directly proportional to the enzymatic activity of LDH. The quantification of NADH produced from the conversion of lactate to pyruvate is used to determine the amount of lactate produced by the living worm.

The measurement of lactate as a viability method involves sampling the spent culture medium in which an isolated adult *O. volvulus* is incubated. The sample is mixed with distilled water, glycine-hydrazine buffer and NAD and incubated at room temperature. The optical density of the mixture is measured at 340 nm as the first extinction value (blank). An amount of lactate dehydrogenase is then added and incubated at 37°C before measuring the optical density (OD) at 340 nm to obtain the second extinction value (test). The amount of lactate produced by the worm and expressed as amount of lactate per mg of worm tissue is calculated from the results using the following formula. $C = \frac{AE \times V \times 100}{E \times d \times v \times w}$

$$C = \frac{AE \times V \times 100}{E \times d \times v \times w}$$

Where C = concentration of lactate per mg of worm tissue, AE = difference between the ODs of spent medium (test and blank). V = volume of probe (total dilution), E = extinction coefficient of NAD at 340 nm (6.22 cm²/umol), v = volume of spent medium (test volume), d = diameter of the well of micro titre plate and w = weight of the worm that produced the lactate.

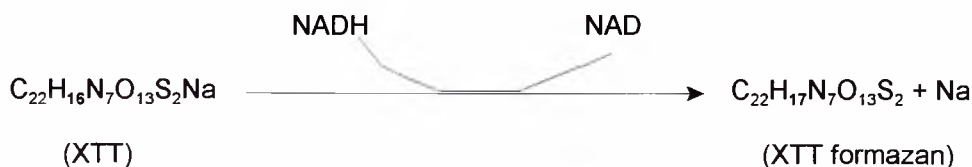
The test allows for multiple testing without affecting the worms. It is therefore used daily in evaluating the viability of adult female worms because of their sizes. The amount of lactate produced daily by the relatively smaller male worms is too small and therefore

cannot be measured.

2.7.3 TETRAZOLIUM COMPOUND REDUCTION TESTS (XTT AND MTT)

Tetrazolium reduction tests are used in testing the viabilities of adult *O. volvulus* based on their ability to absorb and biologically reduce the compound to formazan when they are alive. The reduction of tetrazolium compounds depends on the ability of the worm to release hydrogen ions from a suitable substrate which can be accepted by the tetrazolium compound through the coenzyme NAD. The amount of reduced tetrazolium (formazan) is measured colorimetrically to determine the activity of the worm.

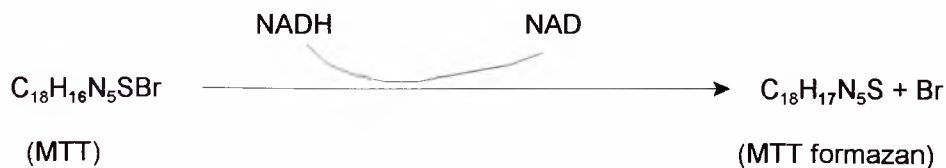
XTT is 2,3-bis (2-methoxy-4-nitrosul-phenyl)-5-(phenylamino)-carbonyl-2H-tetrazolium hydroxide ($C_{22}H_{16}N_7O_{13}S_2Na$) which is a yellow compound when it is dissolved in phenazine methosulphate/phosphate buffered saline. A living adult *O. volvulus* placed in this solution, absorbs and reduces the XTT to an orange coloured XTT formazan ($C_{22}H_{17}N_7O_{13}S_2$).



The XTT formazan which is produced from this reaction is water soluble so that when it is formed in the worm tissues it can dissolve back into the solution which changes colour from yellow to orange (Strote *et al.*, 1993; Strote, 1998; OCRC, Hohoe). The intensity of the orange colour depends on the level of activity of the worm.

To measure the viability of adult *Onchocerca volvulus* by this method, single isolated worms are transferred from their culture media into individual wells of a microtitre plate containing XTT solutions. The worms are incubated in the solution for 1 hour at 37°C after which they are removed and returned to their culture media. The amount of XTT formazan produced by the worms are measured colorimetrically at 450 nm using an ELISA reader (OCRC). The optical densities of the formazan produced by each worm is compared with the optical densities obtained from XTT solutions in which controlled heat-killed adult *O. volvulus* worms are incubated for 1 hour (heat-killed worms are incapable of reducing XTT to formazan).

MTT is 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide ($C_{18}H_{16}N_5SBr$) which is a pale yellow compound when in solution. The use of MTT reduction as a viability test on adult *Onchocerca volvulus* is based on the same principle as that of the XTT test. The difference between the two compounds is that, the MTT compound produces blue crystallised MTT formazan ($C_{18}H_{17}N_5S$) which is not soluble in water but soluble in dimethylsulphuroxide (DMSO).



To measure the viability of adult *O. volvulus* with the MTT compound, isolated adult worms are transferred from their culture media into a 48 well plate containing 0.5 ml of 0.5% MTT in phosphate buffered saline in each well. The loaded plate is incubated at

37°C for 30 minutes after which the worms are removed from the MTT solution and transferred into individual wells of a 96 well microtitre plate containing 0.2 ml dimethylsulphuroxide (DMSO). The 96 well plate is incubated in the dark at room temperature for an hour. The worms are then removed from the dimethylsulphuroxide after the crystallised formazan has dissolved in it, so that the optical densities can be measured at 510 nm using an ELISA reader. The optical densities are compared with the optical densities of DMSO in which controlled heat-killed adult *O. volvulus* are incubated (heat-killed adult *O. volvulus* are incapable of reducing MTT compound to formazan)

In practice, the water soluble XTT formazan, does not kill the worms while the extraction of MTT formazan with DMSO results in the death of the worms. The XTT test can therefore be performed on the same worm several times which allows it to be performed on a daily basis along with other viability assessments. The XTT test is therefore used conveniently to assess the viabilities of adult female *O. volvulus* because of their sizes and ability to produce enough formazan to be measured on daily basis. The death of the worms after the use of DMSO in extracting the crystallised MTT formazan does not allow further viability testing on the worms. For this reason the MTT test is applied as the last test on the worms to confirm the results of other viability tests on isolated adult *O. volvulus* worms. It is also reserved for the male worms because it is the only other test on the male worms after the motility test since the others described above are mainly applied to the female worms.

2.7.4 EXAMINATION OF EMBRYOS (EMBRYOGRAMME) AND SPERMATOGENIC STAGES (SPERMATOGRAMME)

Drugs may not necessarily kill the adult worms but may be able to permanently or temporarily sterilise them to control the level of microfilarial densities in the skin.

Whereas the skin snip method is suitable for these studies, isolated worms can be studied for abnormalities in embryogenesis and spermatogenesis caused by the drugs.

The examination of embryos (embryogramme) and spermatogenic stages (spermatogramme) are therefore used essentially to detect the effects of drugs on the development of microfilariae in the adult worms.

Embryogramme is a quantitative analysis of the various intra uterine developmental stages of the worm. Isolated female worms are chopped into short fragments of about 1 mm lengths and placed in a drop of saline or phosphate buffered saline in a plastic mortar. The uterine content of the worm is then extracted by placing a pestle on the fragments and pressing gently while rotating around its axis. This is done until all embryos are squeezed out of the uterine fragments. The embryonic suspension is diluted with saline or phosphate buffered saline so that the various developmental stages are counted using an improved Neubauer's counting chamber. The method allows the number of each embryonic stage (normal or abnormal), to be compared with results from other worms from untreated onchocerciasis infected individuals. The number of abnormal embryonic stages in worms from treated patients is compared with those from untreated individuals and the difference is attributed to the effects of the drug under investigation.



Unlike the embryos, it is difficult to conduct a comprehensive quantitative analysis on the developments of spermatozoa in male *O. volvulus*. Suspended *O. volvulus* spermatozoa transform spontaneously from elongated form to a roundish form which makes it difficult to distinguish them from spermatids unless they are stained (Schultz - Key *et al.*, 1980). To perform a spermatogramme, isolated male adult *O. volvulus* are transferred from phosphate buffered saline into dilute Giemsa stain for 15 minutes. Individual worm are stretched on a microscope slides in a drop of phosphate buffered saline and cut into small fragments. Cover slips are placed on each fragmented worm and light pressure applied to squeeze out the contents of the fragments. The various stages of the spermatogenic processes are identified and counted differentially. A differential count of the various spermatogenic stages including abnormal ones will be used to determine abnormal spermatogenesis as the effect of chemotherapy.

A method of examining isolated *O. volvulus* which allows for a subsequent histological examination of the worms was introduced in 1990. This method was introduced to combine the advantages of collagenase extraction of worms and histological examination to obtain the maximum information on each worm extracted from a nodule (Duke, 1990). Nodules containing adult worms are fixed in alcohol-glycerol mixture after removal from treated onchocerciasis patients. The fixed nodules are washed in several changes of tap water over a period of 24 to 48 hours and then placed in test tubes containing collagenase solution. They are incubated at 37°C for a minimum of 24 hours and transferred into petri dishes containing a culture medium. The exposed worm is held steadily with a fine forceps while the digested host tissue is washed away by gentle jetting of physiological saline from a pasteur pipette.

The isolated adult *O. volvulus* worms are transferred into fresh petri dishes containing Mayer's haematoxylin at room temperature for 15 to 20 minutes and blued in tap water. The worms are brought up to glycerol through changes from low concentrations of glycerol in alcohol, to pure glycerol. With the viscous glycerol providing sufficient support to prevent the worms from breaking, the stained and cleared worms are unravelled and for the female worms, cut sequentially into fragments of 4 - 6 cm lengths. The fragments are stretched and mounted on microscope slides under cover slips as series of consecutive lengths. A single female worm can be cut into several fragments and placed 2 or 3 fragments side by side on a slide until the whole worm is stretched serially on a number of slides. The male worms are small enough to be stretched without cutting them so that two or three males can be examined on one slide. Having fully stretched out all the fragments of a whole worm, the lengths of the mounted fragments can be measured and the positions of various organs and reproductive stages along the body can be observed through the cleared cuticle (Duke, 1990).

After the initial examination, the fragments are further cut into smaller pieces while maintaining the sequential order and implanted into a block of brain tissue for histological processing. The brain blocks are prepared from the basal and mid portions of a calf's brain fixed in 10% buffered formalin. These blocks of brain tissue are dehydrated in alcohol and cleared in glycerol, after which parallel slits are made on the flat faces of the blocks to the depth of 3 - 4 mm. The worm fragments are placed into these slits so that one block can contain a whole female worm. With the worms firmly positioned in the slits, the blocks are then transferred into several changes of absolute ethanol to remove the glycerol which may take as long as 3 to 4 days. The blocks are

then processed histologically and embedded in paraffin wax where sections are taken at approximately five centimetres intervals along the worm segments to the end. A single female worm can produce over 40 sections. The sections are stained with the haematoxylin and eosin method and with good orientation of the block, most of the sections will be transverse allowing a cross sectional observation of the worm at various stages along its length (Duke, 1990).

The method appears to be relatively simple but time consuming. Even though it does not allow the performance of other viability assessments, it is the only technique that allows a good longitudinal view of a worm especially its reproductive system.

Histological examination of transverse sections of the worms in an orderly manner along the whole length allows studies of the stages of development from oocytes to microfilariae or spermatogonia to spermatozoa as well as changes in the reproductive activity that occurs along the length of the genital tract.

2. 8 HISTOLOGICAL EXAMINATION OF FIXED *ONCHOCERCA VOLVULUS* NODULE SECTIONS FOR ASSESSING THE VIABILITY OF ADULT *ONCHOCERCA VOLVULUS*

Histological examination of sections from fixed *Onchocerca volvulus* nodules started as far back as 1908 (Fulleborn, 1908; Hoffmann, 1909; Shafie Mohammed, 1931; Ochoterena, 1931 and Martinez Baez, 1935, 1949) through to the early stages of evaluating promising compounds for the treatment of onchocerciasis (Barter *et al.*, 1948, Wanson, 1969, Hawkins, 1952; Martinez Baez, 1952; Gonzalez *et al.*, 1969 and Salazar Mallen *et al.*, 1971). Currently, histology is still the most widely used method in the assessments of adult *O. volvulus* for the evaluation of macrofilaricides. The method allows for a quantitative assessment of the adult *O. volvulus* after the administration of drugs suspected to have some macrofilaricidal activities.

O. volvulus nodules are removed from onchocerciasis infected individuals a few months after they have been administered with a promising drug. They are dehydrated in increasing concentrations of alcohol up to absolute alcohol, cleared in a clearing agent (methyl benzoate) and embedded in paraffin wax. The processed nodules are cut into thin sections and mounted on light microscope slides. The sections are stained with the haematoxylin and eosin technique and histologically examined. A well stained *O. volvulus* nodule section may reveal cross sections, oblique and short longitudinal sections of the adult worms surrounded by nodular tissue. The sections may show variable presence of chronic inflammatory cell infiltrate and eosinophils. There may be neutrophils in sections from nodules in which there are dead worms. The nodular tissue is extensively fibrotic and may contain mature microfilariae depending on the

reproductive stage of the adult female worms. In large nodules certain areas are extensively fibrotic without adult worm sections. These areas are believed to be areas previously occupied by old and dead worms whose tissues have been resorbed by nodular tissue. Some worm sections may also show calcification.

To assess the viability of worms in histologically prepared nodule sections, it is important to understand the anatomy of the *O. volvulus* sections and differentiate between adult female sections and male sections in order to determine their numbers in a nodule. Generally, both male and female sections have an outer lining that gives the worm its cylindrical structure known as the cuticle. Beneath the cuticle is a thin lining of connective tissue which forms the hypodermis. The hypodermis is closely attached to the muscles of the worm which is boarded internally by the pseudo colonic cavity containing fluids. In histological sections the cavity is usually empty due to the paraffin processing. The central portion of the sections contain the genital organ and the intestines. In large nodules, about 95% of sections may belong to the female worms which are much larger and more extensively entangled with the nodular tissue (Duke *et al.*, 1988). It also has sections of the two genital tracts which extends from their anterior to the posterior ends only to merge before they exit at the posterior end of the worm, so that sections from most parts of the worm show the twin tracts of the genital organ (Nealie, 1972; Striebel, 1988; Franz, 1988; Schultz-Key, 1988). The genital tracts of the female may contain various developmental stages of microfilariae or empty depending on the reproductive stage of the worm. For female sections which are cut obliquely or longitudinally, it is possible to observe the undulating cuticle as against the serrated appearance of the male cuticle (Buttner, 1988). Male worm sections are

smaller and has a single genital tract which extends from the anterior to the posterior end of the worm so that every section of the worm also contain a section of the testis (Nealie, 1972; Striebel, 1988; Franz, 1988; Schultz-Key, 1988). The testis may contain the various developmental stages of spermatogenesis depending on the reproductive stage of the male worm.

The determination of the number of worms in a nodule is the most contentious area in nodule examination where the differences between individuals reporting on the number of worms in a nodule is significant (Duke, 1996). For very small nodules which contain only one worm or two worms, of which one is a male and the other a female, there is no significant disagreements between individuals in the number of worms per nodule (Albiez *et al.*, 1988). Large nodules containing multiple worms provide significant variations between individuals reporting on the number of worms (Duke, 1996). To narrow the differences in the number of worms declared by individuals, certain conventions are adopted. Ideally, a nodule must be sectioned serially from one surface to the opposite surface and all sections mounted on slides and examined. This will allow all the worms in a nodule to be sectioned including the worms lying in the periphery of the nodule especially the male ones. By this technique a tiny nodule of just 1 mm diameter which is cut at 4 μ per section can produce over 200 slides for examination. In practice, the number of slides is cut down to save time used in examining onchocerca nodules (Buttner *et al.*, 1988). Sections are taken from the first third of the nodule, followed by the same number of sections from the mid-portion and the last third of the nodule (Duke, 1996). The number of sections taken from each portion varies from one individual to another, and this may cause some differences in

the number of worms declared by the individuals.

Having decided on the number of sections to be examined, the number of worms in a nodule can also be determined by looking for posterior and anterior ends of the worms. The anterior ends are smaller in diameter than the rest of their bodies and since every worm has one anterior end, the number of anterior ends detected could be the number of worms in the nodule (Buttner *et al.*, 1988). Depending on the number of sections taken from the different portions of the nodule, the anterior ends of some worms may not be present in any of the sections for examination. In studies that involve the examination of over 100 nodules these situations are very common and other methods of determining the number of worms in a nodule are used. Sections lying in close proximity with each other and separated by nodular tissue may be regarded as sections from the same worm. In other situations where sections are mixed with no demarcations, the staining pattern of the sections may allow the observer to determine the sections belonging to one worm. This observation is used in conjunction with the contents of the genital organs so that sections containing the same types of embryos may belong to the same worm. Lastly old worms may have some peculiarities such as highly pigmented guts or be prone to fixation artifacts such as collapsed genital tracts as against the less pigmented guts and more stable genital tracts of the younger worms.

Because of the fixation and processing of nodules, all worms examined by the histological technique are dead even before the examination, for this reason the terms "live" and "dead" worms in histological assessments refers to the state of the worms at

the time of nodulectomy. The distinction between live and dead worms is very important for studies based on the evaluation of compounds for macrofilaricidal purposes. Groups of sections belonging to one worm are examined for early or late signs of disintegration. For most dead worms there are definite signs of death which are very easy to detect. These are disintegration of organ structures, wide spaces between hypodermis and cuticle or between cells, completely calcified worm sections and the presence of host cells in the pseudo colonic cavity. Host cells present in the tissue may also show some indication of death of the worms. Live worms only show very few inflammatory cells attached to their cuticles so the attachment of large cells to the cuticle of worm sections may indicate death. The level of disintegration of worm organ structures are usually as a rule scored and compared with laid down criteria for the distinction between worms which are dead and those which are not dead.

2.9 PROJECT BASE

Three methods of testing the viability of isolated adult *O. volvulus* described above, are based on the activity of LDH in the worms. These are: the lactate test and the tetrazolium compound reduction tests (MTT and XTT tests). LDH is an enzyme responsible for the conversion of pyruvate to lactate under anaerobic conditions which is one of the sources of energy for the adult *O. volvulus* (Strote *et al.*, 1993; Strote, 1998). The test for lactate as a viability test on isolated adult *O. volvulus* is therefore an indirect method of testing for the presence of LDH. The tetrazolium compound reduction tests (MTT and XTT) also rely on LDH to release hydrogen from substrates which is transferred along the oxidative pathway. The released hydrogen is accepted by the coenzyme NAD which transfers it to a tetrazolium compound resulting in the

reduction of the tetrazolium compound to formazan. The production of formazan by a worm therefore indicates the presence of LDH activity in the worm. In addition, unpublished data from OCRC shows that there is some relation between the motility of adult female *O. volvulus* and its production of lactate. A group of female worms were studied for a period of 5 days in which the motilities and production of lactate by the worms were measured every 4 hours. The test results showed that for every period that there was a general increase in activity of the worms there was a corresponding increase in the production of lactate (figure 2). These are overwhelming evidences that LDH, is an important enzyme without which the worm may not survive. It is our view that the determination of LDH activity in worm tissues can be a better criterion for determining whether the worm is dead or alive than the other methods described above .



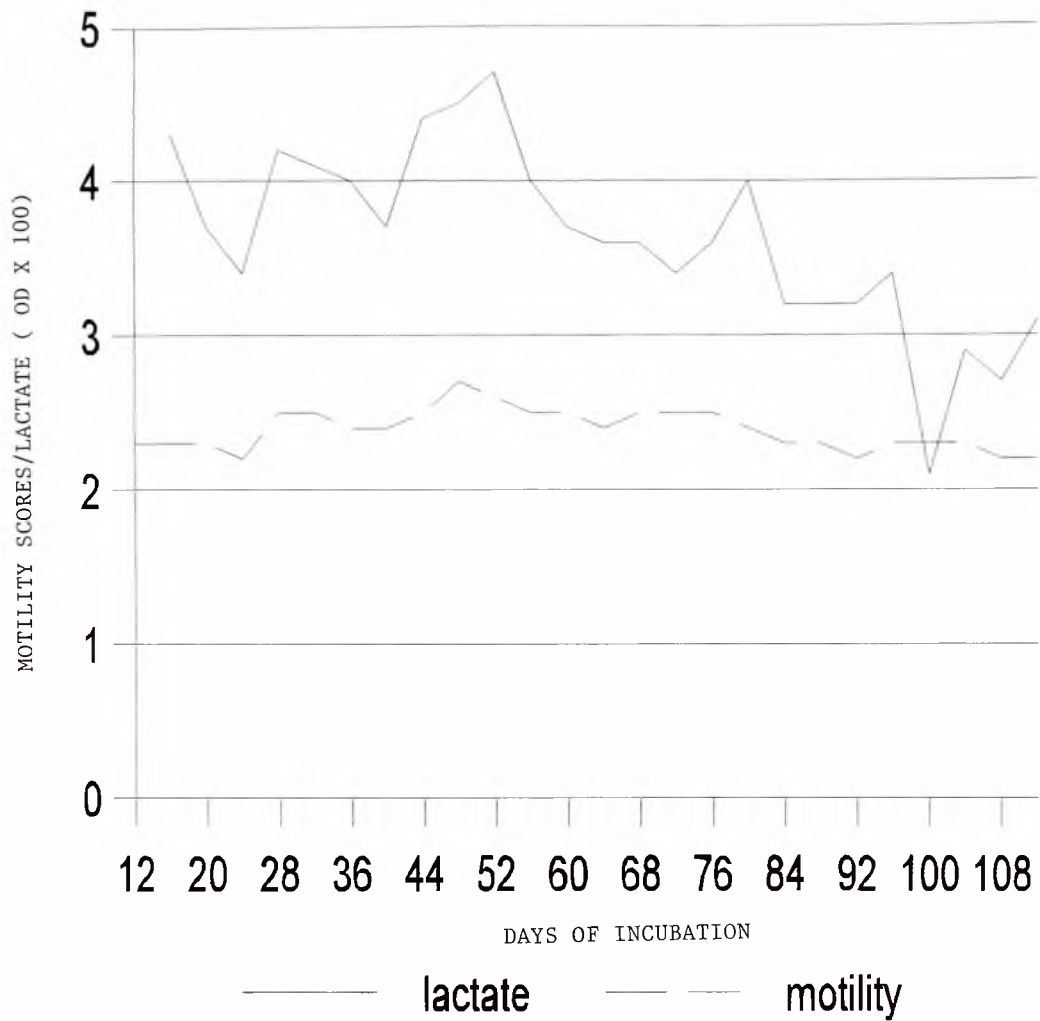


FIGURE 2. A graph showing the relationship between the average production of lactate by a group of adult female *O. volvulus* and their average motilities (OCRC, unpublished)

CHAPTER THREE:

3. MATERIALS AND METHODS

3.1 RECRUITMENT OF VOLUNTEERS AND COLLECTION OF NODULES

Ten onchocerciasis infected volunteers living in Honuta Gbogame a village in an ongoing onchocerciasis transmission area in the south-eastern parts of the Volta region of Ghana and one volunteer from the Alavanyo area in the Hohoe district of the Volta region of Ghana were recruited to donate nodules for the project. All the volunteers were briefed on the aims and objectives of the project and their roles regarding the procedures involving their participation. Each of them signed a consent form to attest that they fully understood the contents of an information sheet which explained the objectives of the project and an invitation to donate nodules (appendix I). The eleven volunteers were transported from their villages to the OCRC in Hohoe. On arrival, each of them was given a serial number (01-11) and nodules extirpated from them in an operating theatre under sterile conditions and local anaesthetic injection.

A total of sixty-one nodules were harvested from the eleven volunteers which was divided into two groups (Groups 1 and 2). Each volunteer's nodules were randomly divided among the 2 groups so that each group finally had a fair representation of nodules from each volunteer. At the end of nodulectomy, there were 29 nodules in Group 1 and 32 nodules in Group 2. Two nodules from each group were selected randomly and one nodule each out of these two in each group were "heat-killed" in distilled water at 60°C for 2 hours. The other two nodules were "heat-killed" at 60°C for 4 hours. All the nodules in Group 1 were fixed in 10 % phosphate buffered formalin (appendix V, reagent 1) and each volunteer's nodules were fixed in one container and

labelled with the patients serial number. The heat-killed nodules in group one were fixed in 10% phosphate buffered formalin in separate containers and labelled HK-2 and HK-4 (heat-killed-2 hours and heat-killed-4 hours respectively). The nodules were fixed for 3 days during which the fixative was changed daily.

The Group 2 nodules were preserved in liquid nitrogen by placing each nodule in a pre-labelled aluminium foil cup designed for this purpose and covered with tissue tek freezing gel. The contents of the cup was pre-frozen in chilled isopentane until the freezing gel froze around the nodule. Each cup was wrapped to seal the nodule in it and all the nodules harvested from each volunteer were wrapped in a larger aluminium foil and then immersed in liquid nitrogen. The heat-killed nodules in this group were labelled and preserved by the same technique.

3.2 PARAFFIN PROCESSING OF GROUP 1 NODULES

The Group 1 nodules were put in labelled tissue processing cassettes (1 nodule per cassette) so that each cassette is labelled with the patient's serial number followed by the nodule number (e.g. 01/1, 01/2 for the first two nodules respectively from the first patient). They were washed in tap water for 24 hours and processed using the following protocol.

Dehydration

The nodules were dehydrated by passing the cassettes containing them through increasing concentrations of ethyl alcohol (ethanol) as follows:

- a. 30%, 40%, 50% and 60% aqueous ethanol for 2 hours each.
- b. Two changes of 70% aqueous ethanol for 12 hours each.

- c. 80% aqueous ethanol for 2 hours.
- d. Two changes of 96% aqueous ethanol for 12 hours each.
- e. Three changes of absolute alcohol for 1 hour each.

Clearing

The cassettes were passed through 5 changes of methyl benzoate for 12 hours in each change.

Impregnation

The nodules were impregnated in molten paraffin wax (Leica histowax, a product of Leica microsystems Nussloch GmbH, Germany) with melting point 57-58°C. The process was achieved by passing the nodules through changes of the molten paraffin wax at 61°C until the smell of methyl benzoate was no longer detectable.

Embedding

Shallow metal embedding forms were pre-heated to 61°C and filled with molten paraffin wax. The nodules were removed from the tissue processing cassettes and placed in the metal embedding forms containing the molten wax (1 nodule per form). The empty labelled tissue processing cassettes were placed in the embedding forms and on top of the nodules (each cassette on its corresponding nodule) and the wax allowed to solidify by cooling at room temperature. After further cooling in a freezer, the nodules were removed as blocks from the embedding forms by lifting the processing cassette.

Microtomy or sectioning

Two sections of 4 μ thicknesses each were cut from the central portions of the nodules by using a Reichert Jung microtome after adequately cooling the nodule blocks in a freezer and mounted on microscope slides.

Haematoxylin and eosin staining of paraffin processed sections

The sections were dewaxed at 61°C in an oven and 2 changes in xylene for 15 minutes each. They were rehydrated by passing through 3 changes of absolute ethanol, 2 changes each of 96%, 80% and 70% aqueous alcohol for 5 minutes each before passing through tap water. The sections were stained in Mayer's haematoxylin (appendix v, reagent 2) for 20 minutes, blued in tap water before staining in alcoholic eosin (appendix v, reagent 4). The stained sections were dehydrated in increasing concentrations of ethanol, cleared in xylene and mounted in DPX..

3.3 CRYOSTAT SECTIONING AND ENZYMEHISTOCHEMICAL STAINING OF GROUP TWO NODULES

Two unfixed cryostat sections of 7 μ thicknesses each were cut from the Group 2 nodules using a cryotome and mounted on light microscope slides. The slides were then labelled with the patient's serial number followed by the nodule number as described for Group 1 nodules. Sections were taken from the first third portions of the nodules and the rest transferred into 10% phosphate buffered formalin in labelled containers. The sections were immediately immersed in an incubating solution pre-heated to 37°C in a water bath for 60 minutes. The reaction in this solution has the same principle as described under the tetrazolium reduction tests earlier except that the tetrazolium compound used in this solution is nitro blue tetrazolium (NBT) which is reduced to a purple insoluble formazan (appendix iv, reagent C). The sections were transferred into 15% formal saline (appendix iv, solution 4) for 15 minutes and washed in distilled water. They were then stained for 5 minutes in 2% methyl green (appendix iv, solution 5) and washed again in distilled water. The sections were dehydrated in

ethanol, cleared in xylene and mounted in DPX.

The nodules which were transferred into formalin after the cryostat sections were fixed in 10% phosphate buffered formalin and paraffin processed using the same protocol as for the Group 1 nodules.

3.4. EXAMINATION OF SECTIONS

For the purpose of this study, the paraffin processed sections from Group 1 nodules were labelled “H1” so that each nodule now had H1 followed by their nodule numbers (e.g. H1/01/1 for the first nodule of the first volunteer). The other paraffin processed sections from Group 2 nodules were labelled with “H2” before their numbers (i.e. H2/01/1) and the cryostat sections from the Group 2 nodules stained by the enzyme histochemical technique were labelled with “EH” preceding their numbers. (i.e. EH/01/1).

Examination of H1 and H2 sections

Two haematoxylin and eosin stained sections from each nodule were examined. The number of worms per nodule were determined using the conventional methods described earlier on page 54. To determine live and dead worms, the various anatomic areas such as the cuticles, hypodermis, muscles, intestines and genital tract walls of sections belonging to individual worms were examined for disintegration and vacuolation. Sections belonging to live worms were anatomically well-preserved as against those belonging to dead worms which showed various levels of disintegration, large spaces between the hypodermis and cuticle or large spaces between individual cells of the same anatomical structure. The cuticle is usually the last portion of the

worm to disintegrate however, when large giant cells are attached to cuticles of worm sections, they are recorded as indications of dead worms. The genital tract content were examined for embryos and spermatozoa in females and male worms respectively. The presence of various developmental stages of the embryos and spermatozoa in sections belonging to individual worms were recorded as signs of their viability. Sections with empty genital tracts were not necessarily considered as sections belonging to dead worms but rather observed for other changes before they were declared dead or alive. The cavities of sections belonging to live worms were all empty while some dead worms had host cells or debris in their cavities and were noted. Calcification of adult *O. volvulus* occurs regularly (Albiez, 1985) especially, among very old worms. To this effect, calcification of small portions of worm organs were not considered as signs of death but when large or complete worm sections were calcified, they were recorded and classified as sections from dead worms. Histological assessment forms (appendix II) were designed to record these observations on each worm and those which were dead were differentiated from live worms using the criteria set down in Table 1. Figure 3 shows sections of a live female worm demonstrating well-preserved anatomical structures. The cuticles (cu) are fine and clear of host cells, the uteri (ut) are well preserved but empty, the hypodermis (hyp) is not vacuolated and the cavity (ca) is clear. On the other hand, Figure 4 shows sections of a dead female worm whose cavity (ca) contain debris, the cuticle (cu) has some cells attached to them and the walls of some uteri (ut) appears to be disintegrating.

Table 1. Criteria for the distinction between worms which were probably alive and those which were dead at the time of nodulectomy

'LIVE' WORMS	'DEAD' WORMS
Morphology of worm organs well preserved	Worm organ structures disintergerated or vacuoles between cuticle and hypodermis
Morphology of nuclei well preserved	Nuclei with altered morphology
Worm not or hardly calcified	Worms much or completely calcified
Cavity nearly empty	Cavity containing embryos or host cells
No giant cells attached to cuticle or small ones attached to surface	Large giant cells attached to cuticle

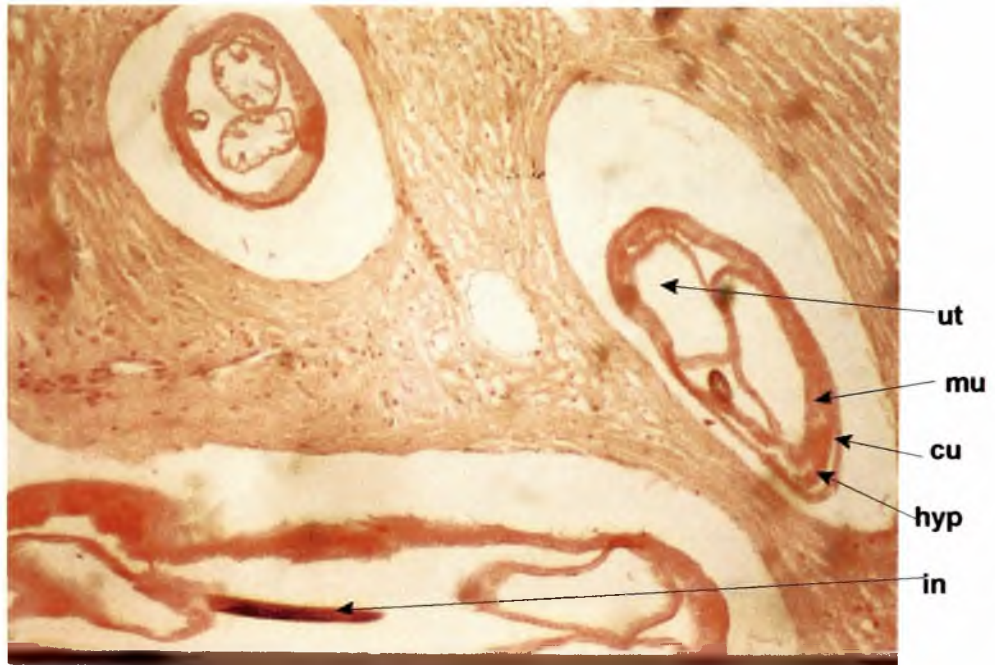


Figure 3. A photomicrograph of a nodule showing morphologically well preserved sections of a live *Onchocerca volvulus*. ca = cavities are empty, cu = cuticles are well defined, hyp = hypodermis, mu = muscles are well-preserved, in = intestines are well-preserved and ut = uterus empty but well-preserved (H & E X200).

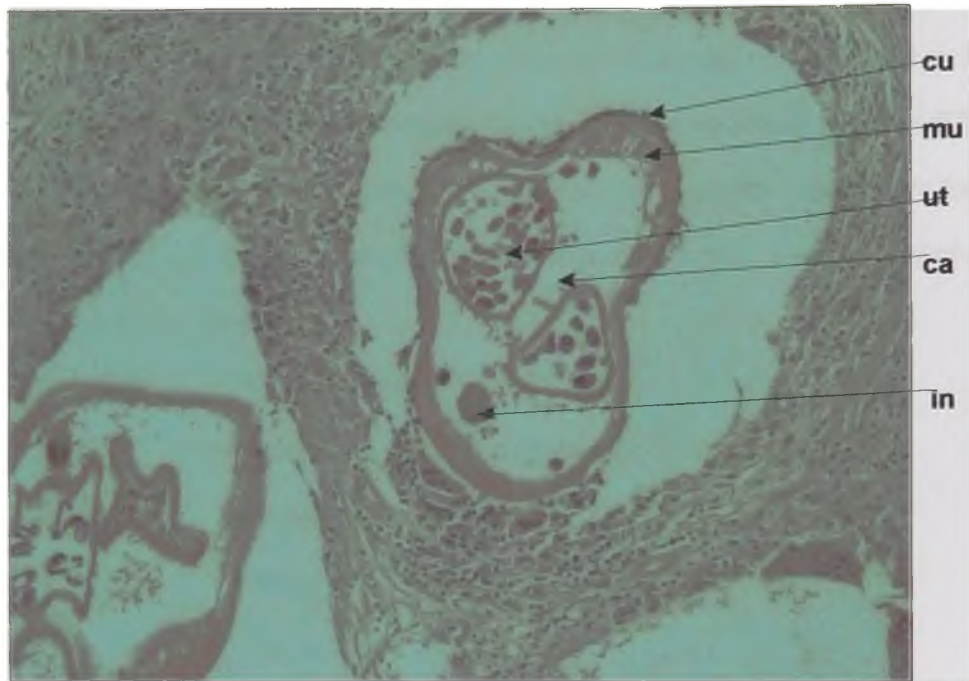


Figure 4. A photomicrograph of a nodule showing sections of a dead female *Onchocerca volvulus*. ca = cavity has debris, cu = cuticle has giant cells attached, hyp = hypodermis, mu = muscle vacuolated, in = intestine and ut = uterus (H & E X200).

Examination of EH sections

The number of worms in the sections stained by the enzyme histochemical method was determined using the same technique described for paraffin processed haematoxylin and eosin stained sections. The determination of live and dead worms was based on light microscopic determination of the presence or absence of enzyme activity (purple NBT formazan) in the various anatomical areas of individual worm sections respectively (i.e. cuticle, hypodermis, muscles, intestines, genital tract and the cavity). The intensity of the reactions were scored as follows:

<u>Score</u>		<u>Interpretation</u>
0	-	No purple colour (no reaction)
1	-	Mild purple colour
2	-	Moderate purple colour
3	-	Deep purple colour

Sections belonging to an individual worm which showed any shade of purple coloured NBT formazan at any anatomical site was regarded as sections of live worms (figure 5) and recorded in an assessment form designed to record these findings (appendix III).

On the other hand, worm sections which did not show any purple coloured NBT formazan were collectively assessed and scored zero in the form at all the anatomical areas of the worm and regarded as sections belonging to dead worms (Figure 6).

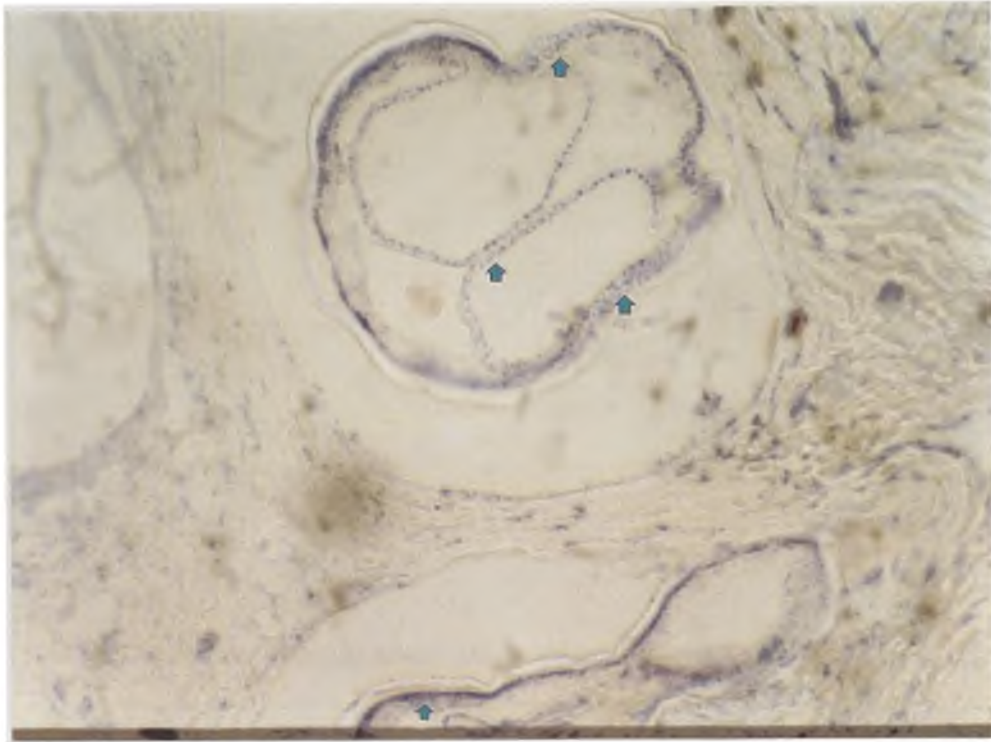


Figure 5. A photomicrograph of a nodule showing sections of a live female *O. volvulus* stained by the enzyme histochemical method. The enzyme activity areas are the purple stained granules in the worm sections (EH X200).



Figure 6. A photomicrograph of a nodule showing sections of a dead female *O. volvulus* stained by the enzyme histochemical method. There are no purple stained enzyme activity areas (EH X200). This is the same worm stained by the H and E stain in Figure 8

CHAPTER FOUR:

4. RESULTS

The eleven onchocerciasis infected individuals who volunteered to donate nodules for this study composed of ten females and one male with all the females coming from Honuta Gbogame in the Ho district of the Volta region. The females were between the ages of 22 and 56 years and appeared to be in good health on arrival for nodulectomy except for the signs and symptoms of onchocerciasis (their body temperatures were between 36.0°C and 37.0°C and they weighed between 40.5 and 50.0 Kg). The eleventh volunteer was a male, 40 years of age from Alavanyo Wudidi in the Hohoe district of the Volta region. He was also in apparent good health on arrival, weighing 60.0 Kg and had a body temperature of 36.5°C.

Out of the 27 nodules from Group 1, a total of 45 worms were detected of which 32 were female worms and the remaining 13 were males (Table 2). The paraffin processed nodules from Group 2 had 42 worms detected from the 30 nodules examined and out of these worms, 34 were females (Table 3). An additional two nodules from each group were heat treated.

Histologically, all the male worms detected in the paraffin processed nodules sections from Group 1 and 2 were alive and about 94% of the females worms detected in the Group1 nodules were alive (Table 2). The results of the paraffin processed nodules from Group 2 showed that about 88.2% of the female worms were alive (Table 3).

Table 2. RESULTS OF PARAFFIN PROCESSED SECTIONS - GROUP 1 NODULES

PT. NO	NN	NW	FE W	LF	DF	MW	LM	DM
1	1	1	1	1	0	0	0	0
2	2	3	2	2	0	1	1	0
3	2	3	2	2	0	1	1	0
4	5	8	6	5	1	2	2	0
5	3	3	3	3	0	0	0	0
6	2	4	3	3	0	1	1	0
7	1	3	2	2	0	1	1	0
8	3	4	2	2	0	2	2	0
9	1	2	1	1	0	1	1	0
10	4	9	7	7	0	2	2	0
11	3	5	3	2	1	2	2	0
TOT	27	45	32	30(94%)	2(6%)	13	13(100%)	0(0%)
HK-2	1	1	1	1	0	0	0	0
HK-4	1	1	1	0	1	0	0	0

KEY

NN Number of nodules

NW Total number of worms

FEW Number of female worms

LF Number of live female worms

DF Number of dead female worms

MW Number of male worms

LM Number of live male worms

DM Number of dead male

HK-2 Nodules heat-killed for two hours HK-4 Nodules heat-killed for four hours

Table 3. RESULTS OF PARAFFIN PROCESSED SECTIONS - GROUP 2 NODULES

PT. NO	NN	NW	FE W	LF	DF	M W	LM	DM
1	1	1	1	1	0	0	0	0
2	2	2	2	2	0	0	0	0
3	3	4	3	2	1	1	1	0
4	4	8	6	4	2	2	2	0
5	3	3	3	3	0	0	0	0
6	3	6	5	4	1	1	1	0
7	1	1	1	1	0	0	0	0
8	4	4	3	3	0	1	1	0
9	1	1	1	1	0	0	0	0
10	5	8	5	5	0	3	3	0
11	3	4	4	4	0	0	0	0
TOT	30	42	34	30(88.2%)	4(11.8%)	8	8(100%)	0(0%)
HK-2	1	2	1	1	0	1	1	0
HK-4	1	1	1	1	0	0	0	0

KEY

NN Number of nodules

NW Total number of worms

FEW Number of female worms

LF Number of live female worms

DF Number of dead female worms

MW Number of male worms

LM Number of live male worms

DM Number of dead male

HK-2 Nodules heat-killed for two hours

HK-4 Nodules heat-killed for four hours

One heat-killed worm in Group 1 showed signs of degeneration after 4 hours in 60°C distilled water. The degeneration was in the advance stage of necrosis which indicates that the worm was not necessarily killed by the heat but rather, it was probably dead before the heat-killing process (Figure 7). The other heat-killed worms in the two groups were relatively well preserved after two and four hours incubation at 60°C (figures 8 and 9). In both sections the worm sections were well preserved with viable embryos (emb) in the uteri of the sections.

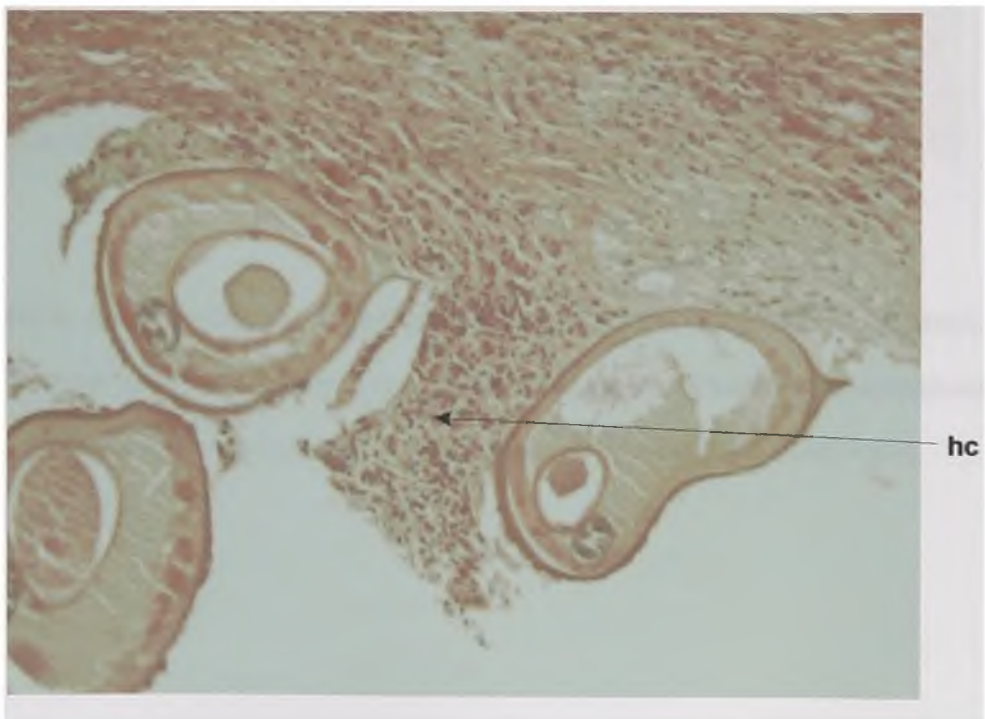


Figure 7. A photomicrograph of a nodule showing sections of a female worm which was heat-killed for four hours but showed advanced stages of worm death with host cells (hc) aggregating around the cuticle (H&E X200)

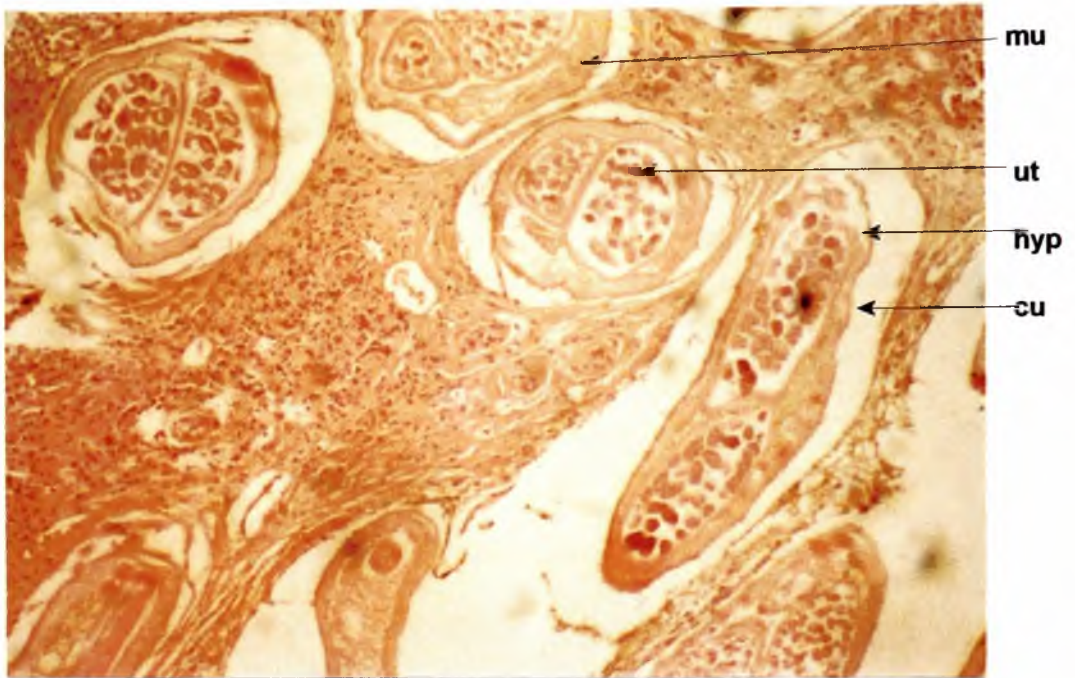


FIGURE 8. A photomicrograph of a nodule showing sections of a female worm which was heat-killed for two hours. The sections appear morphologically well preserved especially the muscles (mu), cuticle (cu), uterus (ut) and the hypodermis (hyp) (H&E X200).

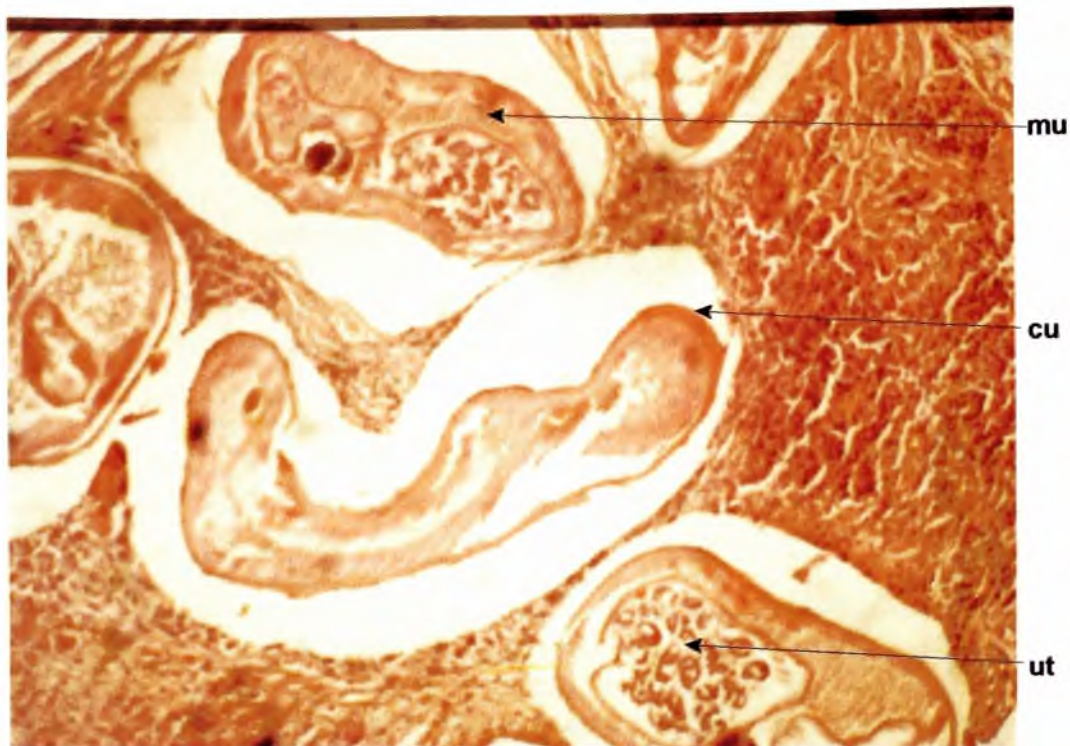


Figure 9. A photomicrograph of a nodule showing sections of a female worm which was heat-killed for four hours. The sections appear morphologically well preserved in this haematoxylin and eosin stained section especially the muscles (mu), cuticle (cu) and uterus (ut) (H&E X200).

A total of 36 worms were detected out of the 30 Group 2 nodules stained by the enzyme histochemical method. Thirty-four were females while the other two were males. All the males worms as well as 88.2% of the female worms were alive (table 4).

Table 4. RESULTS OF ENZYME HISTOCHEMICAL STAINED SECTIONS OF GROUP 2 NODULES.

PT. NO	NN	N W	FEW	LF	DF	MW	LM	DM
1	1	2	2	2	0	0	0	0
2	2	2	2	2	0	0	0	0
3	3	5	3	2	1	2	2	0
4	4	5	5	4	1	0	0	0
5	3	3	3	3	0	0	0	0
6	3	4	4	3	1	0	0	0
7	1	1	1	1	0	0	0	0
8	4	3	3	3	0	0	0	0
9	1	1	1	1	0	0	0	0
10	5	6	6	6	0	0	0	0
11	3	4	4	3	1	0	0	0
TOT	30	36	34	30(88.2%)	4(11.8%)	2	2(100%)	0
HK-2	1	2	1	0	1	1	0	1
HK-4	1	1	1	0	1	0	0	0

KEY

NN Number of nodules

NW Total number of worms

FEW Number of female worms

LF..... Number of live female worms

DF Number of dead female worms

MW Number of male worms

LM Number of live male worms

DM Number of dead male

HK-2 ..Nodules heat-killed for two hours HK-4 Nodules heat-killed for four hours

The purple NBT formazan deposits which appeared granular under light microscopy were found mainly in the muscles (mu) of the worm and very close to the hypodermis (hyp). It was also found in small amounts and in the same granular appearance in the muscles of the genital tracts walls (ut) while the cuticle (cu), hypodermis, cavities (ca) and intestines (in) did not show any enzyme activity (figure 10).

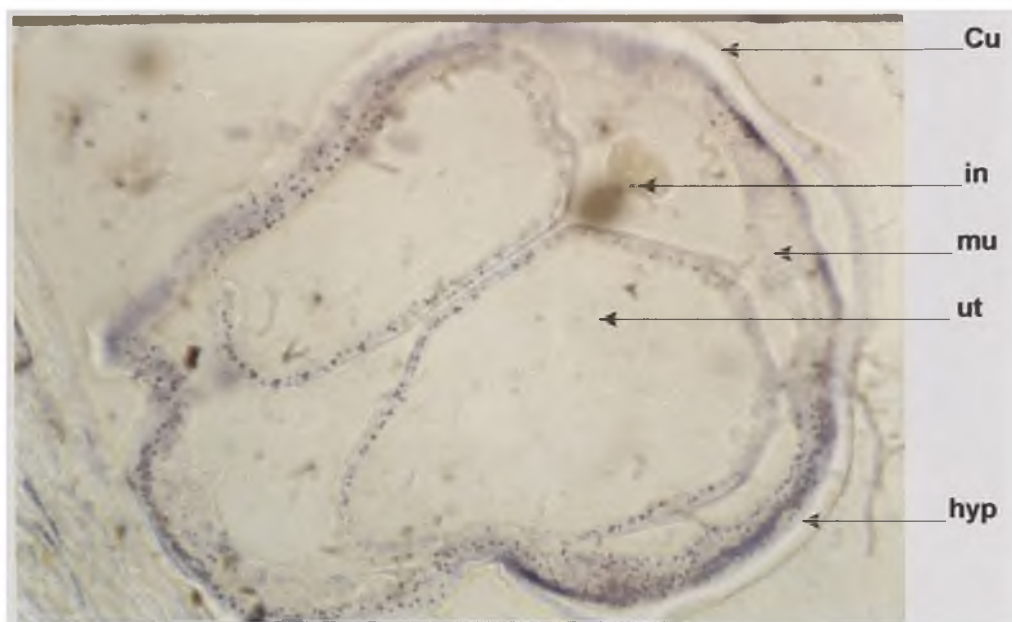


Figure 10. A photomicrograph of a nodule showing a section of the same worm in figure 5 magnified to show the anatomical areas of enzyme activity. mu = muscle, ut = uterus, cu = cuticle, in = intestine, hyp = hypodermis. The enzyme is located at the base of the muscle cells close to the hypodermis and the muscle cells lining the walls of the genital tracts (EH X400).

In some nodules, microfilariae in the nodular tissue as well as stretched intra-uterine microfilariae showed enzyme activity indicating the presence of LDH in matured live *O.*

Volvulus microfilariae (figure 11 and 12). All the worms detected in the nodules which were heat-killed at 60°C for 2 and 4 hours did not show any enzyme activity (figures 13 and 6).



Figure 11. A photomicrograph of a nodule section showing enzyme activity in *O. volvulus* microfilariae (mf) in nodular tissue (EH X200)

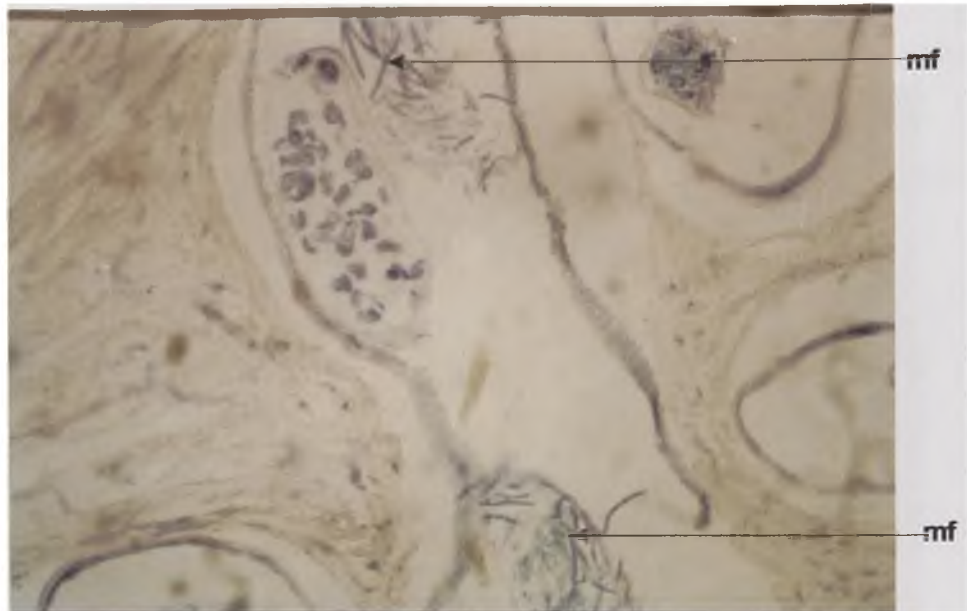


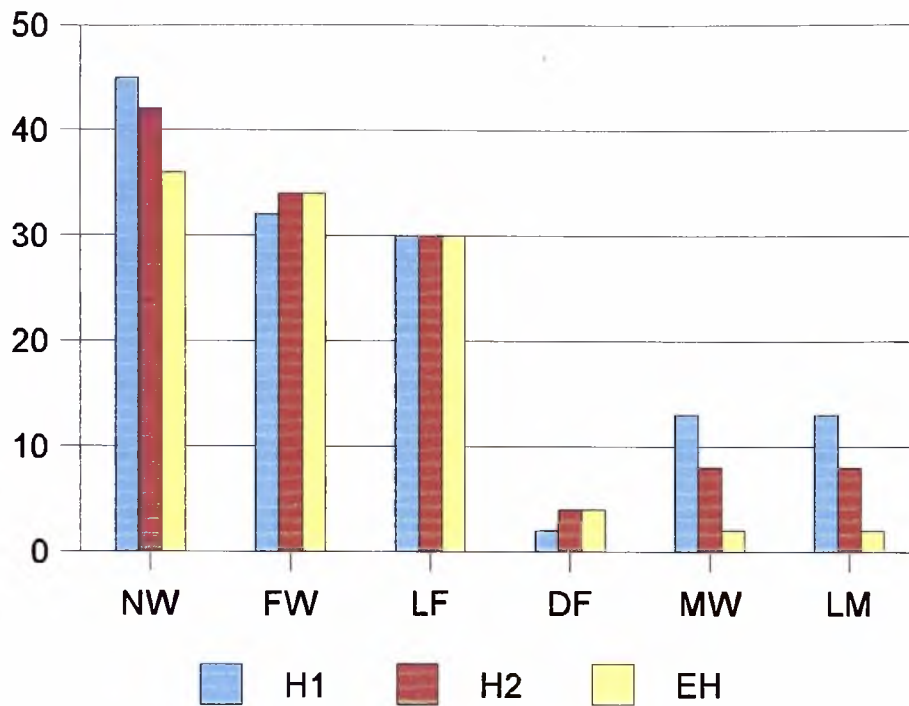
Figure 12. A photomicrograph of a nodule showing enzyme activity in intra-uterine microfilariae (mf) of *Onchocerca volvulus* (EH X200).



Figure 13. A photomicrograph of the same worm as in figure 8 which was heat-killed for two hours but stained by the enzyme histochemical method. The sections show no enzyme activity (EH X200)

Figure 14 is a summary of the results obtained from the enzyme histochemical examination of Group 2 nodules and the results of two sets of paraffin processed nodules (H1 and H2). The number of female worms (FEW) declared from the same nodules by both enzyme histochemistry and histology (EH and H2) were the same (34). The total number of worms (NW) declared by both methods on the same nodules showed that the enzyme histochemical technique detected 36 worms (an average of 1.2 worms per nodule) while the histological technique detected 42 worms (an average of 1.4 worms per nodule).

Figure 14. A graph comparing the number of live and dead worms declared after the examination of H1, H2 and EH slides



NW----- Total number of worms declared FW----- Total number of female of worms

LF----- Live female worms

DF----- Dead female worms

MW----- Total number of male worm

LM----- Live male worms

CHAPTER FIVE:

5.1 DISCUSSIONS

The current procedures of evaluating macrofilaricides depend on methods which can determine the number of worms in the nodules as well as detect the number of live and dead ones among them. The number of dead worms compared to the total number of worms in a study can be used to determine the effects of a drug on the adult worm population. In this study, a comparison between the results obtained from enzyme histochemical examination and paraffin processed sections on the same nodules did not show any statistical difference (Fisher exact 1-tail $P = 0.073$; appendix vi). However, there is a small difference between the two methods when the total number of worms detected by each of them is observed (the histology declared 42 worms as against 36 worms declared by the enzyme method). This difference was found to be limited to the detection of male worms only. The difference between the two methods in the detection of the number of male worms (MW) was due to the areas of the nodules from which sections were obtained for examination. The enzyme histochemical staining technique was performed on sections taken from the first or upper one-third portions of the nodules after which the rest of the nodules were processed by the paraffin method so that sections could be taken from their central portions for histological examination. This was done for the convenience of this study to allow both methods to be performed on the same nodules for comparison. It is known that the areas from which sections are taken from a nodule can affect the number of worms detected from it. Large worms such as the female worms can occupy areas in the nodules that transverse the whole nodule so that it can be detected in sections taken from many portions of the nodule. The smaller worms (males worms) are often limited to the parts of the nodule where they are found and are not often detected at other areas of the nodule. The following observations

from the results of some of the nodules may clarify the above assertion. In nodule numbers H2/04/1, H2/04/2, H2/06/3, H2/08/3, H2/10/2, H2/10/3 and H2/10/5, male worms were detected without their corresponding presence in sections of the same nodules for enzyme histochemistry. Also, a male worm was detected in nodule number EH/03/3 without a corresponding presence in its histological counterpart. This means that the detection of male worms in nodules depends very much on where the worm is located in the nodules at the time the nodule was processed and where the sections are taken.

The enzyme histochemical method adopted the procedures employed by histological techniques to detect the number of worms in the nodules thus, allowing it to benefit from the advantages of histology in the detection of worms over the collagenase digestion techniques. Both enzyme histochemistry and histological methods detected the same number of live and dead female worms (LF and DF) from the same nodules. In almost all the nodules in Group 2, most of the worms which were well preserved in the histological preparations were found to have LDH activity in them and the worms which showed gross and significant morphological distortions did not show any enzyme activity. However certain observations are worth recounting. In nodule number 03/2 of Group 2, both enzyme histochemistry and histological methods detected that the only worm in that nodule was dead based on the absence of LDH and morphological changes respectively. Again, nodule number 04/1 had two female worms whose histological examination showed that one of them was dead based on the level of morphological changes in its sections and the other was alive (well preserved) while the enzyme histochemical method showed that sections belonging to one of the worms indicated enzyme activity (alive) and sections belonging to the other worm did not show any enzyme activity (dead). Nodule number 11/2 was extirpated from the only volunteer from a long term

onchocerciasis control area where transmission had stopped for several years. Naturally, nodules from such areas have very old worms or dead and calcified worms. Histological examination of the two worms in that nodule declared that both worms were alive but the enzyme histochemical techniques showed that sections from one worm had no enzyme activity (dead) and the other had a weakly reactive enzyme activity (alive but less active). This was the only nodule in which the two methods did not agree with each other showing that the morphological changes used by histology in determining dead worms occur gradually and that there is no sharp distinction between dead and live worms in the histological process as occurs in enzyme histochemistry.

All the worms in the nodules which were heat-killed did not show any enzyme activity and were declared dead by the enzyme histochemical method. On the other hand the same worms in paraffin processed sections were declared alive and productive by the paraffin processed histological method. Heat-killed worms are used regularly as negative controls in *in-vitro* evaluations of isolated adult *O. volvulus* during drug trials (Strote *et al.*, 1993; Strote, 1998; OCRC, Hohoe). In these evaluations the heat-killed worms do not show motility, they do not produce lactate and are unable to reduce tetrazolium compounds to formazan. As far as *in-vitro* practices are concerned, these are positive indications of dead worms. The exact causes of deaths of these worms which are heated at 60°C for more than an hour is not clearly understood. However, the declaration of death in these worms in *in-vitro* practices are based on their inability to produce lactate as well as reduce a tetrazolium salt to formazan (Strote *et al.*, 1993; Strote, 1998; OCRC, Hohoe). These are indirect measurements of the presence of LDH activity in the worms. The *O. volvulus* is a strict human parasite and in man, there are five LDH isoenzymes designated LDH₁, LDH₂, LDH₃, LDH₄ and LDH₅. The LDH₁,

LDH₂ are known cardiac isoenzymes while the others are either liver or skeletal muscles isoenzymes. The cardiac isoenzymes are heat stable at 60-65°C for only 30 minutes while the others are relatively heat labile and will be inactivated at temperatures below 60°C (Zimmerman and Henry 1979). It is not clear whether the adult *O. volvulus* uses the same isoenzymes as their host but it may be right to be believed that its LDH enzyme may have the same response to heat as those of its host since the enzymes are all protein in nature. Under these circumstances the negative reactions of the heat-killed worms to the enzyme histochemical staining technique could be a simple inactivation of the LDH enzymes in the worms.

The enzyme histochemical staining technique adopted the tetrazolium reduction technique in determining live and dead worms which is used in *in-vitro* practices on isolated adult *O. volvulus* to confirm their viability (Strote *et al.*, 1993; Strote, 1998; OCRC, Hohoe). The method therefore benefits from the advantages of the *in-vitro* technique of determining dead worms based on tetrazolium reduction which is the main attraction to the *in-vitro* technique over the histological method during drug evaluations.

The first two objectives of this study were to demonstrate the presence of LDH and its anatomical location in adult *O. volvulus* tissues. The enzyme histochemical staining technique demonstrates the presence of enzymes in tissues using substrates which are specific for the targeted enzymes. An incubating solution containing sodium DL-lactate, NAD, magnesium chloride and nitro blue tetrazolium (NBT) compound was used as a specific substrate for LDH. With the exclusion of LDH in the incubating solution, the purple colour in the worm tissue sections after the incubation confirms that this reaction had taken place and

therefore indicate the presence of LDH in the worm. Also, by the incubation of sections in the incubating solution, it was expected that the reactions will take place only at the sites where the enzyme is stored or produced in the worm. The observation that the enzyme activity can be located at the areas of the worm body represented by its muscles adjacent to the hypodermis and the cells lining the walls of genital tracts shows that the enzyme is located in these areas. Stretched intrauterine microfilariae and those in the nodular tissue also showed enzyme activity indicating the presence of LDH in matured actively motile *O. volvulus* larvae.

As a strict human parasite, *O. volvulus* is not readily available for studies in the laboratory without involving the infected human hosts which raises ethical issues. Most judgements on their possible reaction to drugs are obtained from studies on other filariae species and some animal onchocerca species such as *O. gibsoni*, *O. lienalis* and *O. gutturosa* which are more available in the laboratory but often have different metabolic and drug responses from *O. volvulus* (Ginger, 1990). Studies on the other filariae species show that filariae worms have a wide range of metabolic requirements with obligate aerobes such as *Litomosoides carinii* at one end of the spectrum and the *Brugia* species which has been found to survive for long periods under anaerobic conditions at the other end (Ginger, 1990) The provision of energy from glucose in filariae worms is therefore believed to comprise a complex series of interrelated pathways resulting in several alternative end products, each of which may be able to generate energy for the worms (Kohler, 1986). In some filariae species it appears that their energy generation pathways are related to their muscle activity which relates to their overall maintenance of viability (Ginger, 1990). *O. volvulus* probably belongs to this group. In view of the unique properties of glucose metabolism in filariae worms, the Wellcome foundation proposed the inhibition of glucose uptake as a target for drug developments in

onchocerciasis (Gutteridge, 1990). Earlier, studies on the animal species of onchocerca suggested that the inhibition of rate limiting glycolytic enzymes such as hexokinase, phosphofructokinase and pyruvate kinase could affect *O. volvulus* (Dunn, 1988). However attempts to obtain these enzymes in their active forms from adult *O. volvulus* isolated from patients in the field and transported to testing centre in the United States of America were faced with technical problems at that time (Ginger, 1990).

In the early 1980s, some meetings were held under the auspices of the WHO in which researchers who were testing potential drugs on animals met chemists and biochemists to suggest or identify biochemical targets in *O. volvulus* and propose their potential inhibiting drugs. Five targets were initially suggested of which glucose catabolism was the main target to be inhibited in several different ways. Later, the range of targets was increased to cover other aspects of worm survival including structural elements (Ginger, 1990). Below are the targets suggested in 1980, 1983 and 1985..

Biochemical targets identified in 1980 and their suggested drugs (Ginger, 1990)

<u>Target</u>	<u>Drug</u>
1. Folate metabolism	Methotrexate, Menoctone and 2,4-diaminoquinazolines
2. Glucose catabolism	
a. Glucose uptake	levamisole, benzimidazole
b. Glycogen synthesis	levamisole, benzimidazole
c. Glyceraldehyde-3-phosphate Dehydrogenase	Suramin

d. Lactate dehydrogenase	Suramin
e. Malic dehydrogenase	Suramin
f. Malic enzyme	Suramin
g. Phosphofructokinase	Antimonials
h. Fumarate reductases	benzimidazole
3. Protein kinase	Suramin
4. Tubulin polymerization	benzimidazole
5. Neurotransmission and receptor function	diethylcarbamazin
a. acetylcholine esterases	Metrifonate

Biochemical targets identified in 1983 (Ginger, 1990)

1. Developmental hormones
2. Active transport/drug uptake
3. Structural elements
 - a. cuticle
 - b. collagen
 - c. chitin
4. Lipid metabolism

Biochemical targets identified in 1985 (Ginger, 1990)

1. polyisoprenoid synthesis
2. Retinol binding proteins
3. Pyrimidine metabolism/Thymidylate synthetase
4. Cyclic AMP function
5. Aminoacyl-tRNA synthetases.

The targets suggested in 1980 included five targets to be inhibited by suramin and four of these targets were identified from glucose catabolism including LDH. Incidentally, Suramin, is the only drug that is currently known to have some recognisable effect on the adult *O. volvulus*. The only disadvantage that discourages the use of suramin as a macrofilaricide is its extreme toxicity (WHO, 1987; Awadzi *et al.*, 1995; Forgione, 2002). In view of the above observations and the emphasis now placed on LDH activity as the measurement of the viability of adult *O. volvulus* in *in-vitro* practices, this study could be of immense help as it was able to detect the presence and anatomical location of LDH. Its contribution to any efforts that would revive the inhibition of enzymes involved in glucose catabolism including LDH in adult *O. volvulus* using less toxic drugs will be immense.

Onchocercal infections follow the normal epidemiologic procedures of transmission associated with parasites who have insects as their vectors. The intensity and regularity of its transmission depends on both the vector population and the distribution of the transmissible larvae by the infected individuals. This means that the number of adult worms and their ages within an individual varies according to the regularity of the incidence of infections and reinfections. The number of worms in individual nodules of one infected person varies and this variation increases between separate individuals and societies. To this effect, comparing the percentage of dead worms in a treated population with that of an entirely different group of individuals who have not been treated but come from the same area as those who have taken the drug appears inappropriate. The Group 1 nodules were included in this project to test the significance of comparing one group of nodules with another despite the varied sizes and number of worms in the nodules. The results of Group 1 nodules compare favourably with the results of the paraffin processed sections of the Group 2 nodules whose sections

were both taken from the central portions of their respective nodules. The Group 1 nodules which was paraffin processed had an average of 1.6 worms per nodule as against 1.4 worms per nodule in the Group 2 paraffin processed sections. Even though the number of nodules in each case was different and the nodules themselves were completely different there was no significant difference between the number of worms declared from both results ($P=0.284$; appendix vii). The similarities between the results of H1 and H2 sections show that despite the wide variations between the contents of individual nodules, statistical analysis to compare one group of nodules with another is acceptable.

5.2 CONCLUSION

The main aim of this project is to introduce a new method of assessing the viability of adult *O. volvulus* for drug evaluations. In the determination of the number of worms in the nodules the method compares favourably with the histological technique which is by far the best method of determining the number of worms in a nodule. For the detection of live and dead worms, the enzyme histochemical method employed the tetrazolium reduction technique which does not rely on tissue degeneration to indicate death. This gives the method an added advantage over the histological technique of determining dead and live worms in that, recently dead worms showing no degenerative changes can be identified by the absence of LDH activity.

In addition to its ability to determine the number of worms as well as effectively distinguish between dead and live worms which are the prerequisites for any effective method of assessing the viability of adult *O. volvulus* during drug evaluations, the enzyme method also has certain advantages. The technique can produce results in less than 24 hours after nodules are removed from study volunteers. It is therefore faster and easy to perform by

avoiding the lengthy protocols employed by *in-vitro* and histological techniques which sometimes takes days or even weeks before results are obtained. Enzyme histochemical stained slides are also available for review by other scientists and may still be available even after several years. This is not available in the *in-vitro* technique except in histology. Finally, there are strong indications that it can produce more dependable results than the existing techniques when used in assessing the viabilities of adult *O. volvulus* during drug evaluations. This project was designed as an exploratory project and therefore involved a few nodules. It is therefore recommend that further observations be made on a larger scale and if necessary in different environments to confirm the usefulness of the enzyme histochemical technique as a method of testing the viability of adult *Onchocerca volvulus* during macrofilaricide evaluations.

5.3 ACKNOWLEDGEMENTS

I wish to thank all the members of staff of the Department of Pathology of the University of Ghana Medical School (U.G.M.S.) especially all my colleagues in the Department for their cooperation throughout the project. My special thanks goes to Profs. E. K. Wiredu, A. A. Adjei, Drs. Y. Tettey and R. K. Gyasi, all of Pathology Department U.G.M.S., for their excellent guidance, encouragement, contributions and directions throughout the project without which I could not have completed this project.

I am grateful to Dr K. Awadzi, the Director of Onchocerciasis Chemotherapy Research Centre (OCRC) for his financial support in providing the chemicals and nodules for this project, Mr B. T. Quartey, the head of Laboratory services at OCRC for allowing me to use the OCRC laboratory facilities, Dr. N. O. Opoku for performing the nodulectomies to harvest nodules for this study and all members of staff of OCRC, Hohoe. I will also like to thank the management and staff of the Tetteh Quarshe Memorial Hospital, Mampong-Akwapim for the use of their Cryotome and other facilities in their laboratories.

I deeply appreciate the contributions of Dr. A. Dodoo, Research Fellow (Pharmacology) U.G.M.S. and Mr V. Korda, Executive Secretary, U.G.M.S., for their efforts in finding accommodation for me in Korle-Bu and Mampong-Akwapim while I was doing this project.

APPENDIX I

INFORMATION SHEET AND CONSENT FORM.

INTRODUCTION

Currently the only drug for the treatment of Onchocerciasis (river blindness) caused by a worm (*Onchocerca volvulus*), is ivermectin. Ivermectin affects only the larval stage of the worm so that to prevent the pathology of the disease, ivermectin has to be administered once a year for as long as the adult worm is alive which can take as long as 15 years or more.

There is the concern that after many years of ivermectin the worm may develop resistance to it. The ideal drug is the one that can kill the adult worm but, methodical difficulties involved in assessing the effects of promising compounds on *O. volvulus* has delayed the development of a safe drug. To speed up the development of such a drug, there is the need for a reliable method of assessing the effect of new compounds on *O. volvulus*. The first step of developing such a method is to obtain Onchocerca nodules from infected people to try a new technique that will allow us to differentiate between dead and live worms. You are being invited as one of 11 people to donate nodules for a project being organised by Mr. Seidu Mahmood Abdulai (M. Phil. Student) of the Department of Pathology, University of Ghana Medical School, in collaboration with Onchocerciasis Chemotherapy Research Centre (OCRC) at Hohoe Hospital to try the new technique.

PROCEDURE

If you agree to participate, you will be required to sign a consent form to attest that you have fully understood the contents of this information sheet and voluntarily decided to participate. You will be transported from your village to OCRC at the Hohoe Hospital where you will be

examined physically by a qualified Medical Officer for nodules. Your nodules will be removed under sterile conditions at an OCRC operating theatre and under local anaesthetic injection.

This procedure produces mild to moderate discomfort. There may be minor bleeding at nodulectomy site during the removal and moderate pain after the nodulectomy which will be treated with oral analgesics. You will stay in hospital for two to three days after the nodulectomy, then transported back to your village where wound dressings will continue until wounds are healed. You will be advised to rest for a week for wounds to heal. During your stay in hospital, your sustenance (meals, clothing, laundry, entertainment etc.) will be the responsibility of the Centre. You may not smoke or drink alcohol while in the hospital.

WITHDRAWAL FROM STUDY

Your participation in the research is entirely voluntary. You can refuse to participate or withdraw from the study at any stage without a penalty. The investigators may terminate your participation for reasons that will be explained to you by the healthcare professionals.

CONFIDENTIALITY

All the information related to your participation in this study will be kept confidential and will not be revealed to anyone except where required by law and regulations. Any new information that may affect your willingness to participate will be made available to you.

BENEFITS

The benefits of participating in this project will include the removal of all your nodules which will reduce your symptoms of Onchocerciasis. The project may lead to the development of a

new method of assessing the effect of drugs on adult *O. volvulus* which can speed the development of a safe drug for the treatment of Onchocerciasis for the benefit of all infected people.

QUESTIONS

Dr. Awadzi (OCRC)/Dr Opoku (OCRC)/Prof. Wiredu (project supervisor) and Mr. Mahmood will be available for questions, clarifications or assistance in all matters relating to this project. You may also contact the OCRC coordinator in your village.

EXECUTION OF INFORMED CONSENT

I.....of.....

thereby certify that the contents of the above has been read by me/interpreted to me in

the.....language

by.....of.....I have

perfectly understood the same and thereby append my signature/mark (Right thumb print) to this consent form as an evidence of my agreement to participate in this project.

Signature/RTP.

Date.....

WITNESS

I.....of.....

thereby certify that I present when the contents of the above was read by/interpreted

to.....of.....in

the.....language

by.....of.....The

said.....seem to have perfectly understood

the contents of the above before appending his/her signature/making his/her mark (Right

Thumb Print) to this consent form in my presence as evidence of his/her agreement to

participate in this project.

Signature.....Date.....

Countersigned by OCRC team member

Name.....Signature..... Date.....

Title.....

APPENDIX II

FORM FOR HISTOLOGICAL ASSESSMENT OF ADULT ONCHOCERCA VOLVULUS

NODULE NUMBER.....

	F1	F2	F3	M1	M2
CUTICLE	Normal				
	Degenerated				
	Vacuolated				
HYPODERMIS	Normal				
	Degenerated				
	Vacuolated				
MUSCLE	Normal				
	Degenerated				
	Vacuolated				
INTESTINE	Normal				
	Degenerated				
	Pigmented				
G. T. WALL	Normal				
	Degenerated				
G. T. CONTENT	Empty				
	Embryo				
	Spermatozoa				

	F1	F2	F3	M1	M2
BODY CAVITY	Empty				
	Deposits				
CALCIFICATION	Organs				
	Worm				
GIANTT CELLS					
WORM NEARLY RESORTED					
MF. IN NODULE TISSUE					
WORM STATUS	'Alive'				
	'Dead'				
	'No judgement possible'				

SUMMARY

NO. OF FEMALE WORMS 'ALIVE'.....

NO. OF FEMALE WORMS 'DEAD'.....

NO. OF MALE WORMS 'ALIVE'.....

NO. OF MALE WORMS 'DEAD'.....

TOTAL NUMBER OF WORMS.....

APPENDIX III

EXAMINATION FORM FOR ENZYME HISTOCHEMISTRY

	F1	F2	F3	F4	M1	M2	M3
Cuticle							
Hypodermis							
Muscles							
Intestines							
G. T. wall							
G. T. content							
Body cavity							
Worm status							

SUMMARY

No. of Female worms 'alive' _____

No. of Female worms 'dead' _____

No. of Male worms 'alive' _____

No. of Male worms 'dead' _____

Total no. of worms _____

ScoreInterpretation

0 - No purple colour (no reaction)

1 - Mild purple colour

2 - Moderate purple colour

3 - Deep purple colour

APPENDIX IV***MATERIALS AND CHEMICALS FOR ENZYME HISTOCHEMICAL METHOD***

1. Cryotome and accessories
2. Tissue tek gel
3. Water bath
4. Microscope slides and cover slips
5. Liquid nitrogen
6. Microscope
7. Nitro blue tetrazolium
8. Tris (hydroxymethyl aminomethane)
9. Magnesium chloride
10. Formalin
11. Sodium chloride
12. Methyl green
13. Sodium DL-lactate
14. Nicotinamide adenine dinucleotide
15. DPX mountant
16. Staining baths

PREPARATION OF REAGENTS AND SOLUTIONS FOR THE ENZYME HISTOCHEMISTRY METHOD

Solution 1

Stock Nitro Blue Tetrazolium (NBT)

Nitro Blue Tetrazolium.....	400 mg
Distilled water	100 ml

Solution 2

0.2M TRIS buffer (pH 7.4)

TRIS (hydroxymethyl) aminomethane.....	24.2 g
Distilled water.....	1000 ml.

Solution 3

0.05M Magnesium chloride

Magnesium chloride.....	1.01 g
Distilled water.....	100 ml

Solution 4

15% Formal saline

40% Formalin	150 ml
Sodium chloride	9 g
Distilled water.....	850 ml

Solution 52% Methyl green

Methyl green.....	20 g
Distilled water.....	1000 ml

Reagent ASTOCK 1M Sodium DL-lactate

Sodium DL-Lactate.....	12.5 ml.
Distilled water.....	100.0 ml

Reagent BTetrazolium (NBT) solution

Solution 1 (Stock NBT).....	25 ml
Solution 2 (TRIS buffer).....	25 ml
Solution 3 (Magnesium chloride).....	10 ml
Distilled water.....	30 ml

Reagent CIncubating Solution

Reagent A (Stock Sodium DL-Lactate).....	10 ml
Reagent B (Tetrazolium solution).....	90 ml
NAD.....	200 mg

STAINING PROCEDURE FOR ENZYME HISTOCHEMICAL METHOD

1. Unfixed Cryostat sections (5 - 7 μ m) mounted on light microscope slides
2. Incubate sections in incubating solution (reagent C) at 37°C for 60 minutes in a water bath.
3. Transfer sections to 15% formal saline (solution 4) for 15 minutes.
4. Wash in distilled water.
5. Stain in 2% methyl green (solution 5) for 5 minutes.
6. Wash in distilled water.
7. Dehydrate through ascending grades of alcohol (ie 30%, 50%, 70%, 80%, 95% and 100% in that order).
8. Clear in Xylene
9. Mount in DPX.

Results: Enzyme - purple formazan deposits

Nuclei - green

Background - not stained

Appendix V**REAGENTS AND MATERIALS FOR HISTOLOGICAL ASSESSMENTS**

1. 40% formaldehyde
2. Haematoxylin
3. Eosin Y
4. Paraffin wax (leica histowax mt. Pt. 57-58°C)
5. Ethanol,
6. Xylene
7. Methyl benzoate
8. Microtome
9. Microtome blades
10. Floating-out water bath
11. Hot air oven
12. Microscope, slides and cover slips
13. Tissue processing cassettes
14. Potassium alum
15. Citric acid
16. Chloral hydrate
17. Sodium iodate
18. Acetic acid
19. Sodium dihydrogen phosphate (monohydrate)
20. Disodium hydrogen phosphate (anhydrous)
21. Floating water bath
22. Staining baths
23. Forceps and brushes

PREPARATION OF REAGENTS FOR HISTOLOGICAL ASSESSMENTS**1. Reagent 1. 10% Phosphate buffered formalin**

40% Formaldehyde.....	100ml
Sodium dihydrogen phosphate monohydrate.....	4g
Disodium hydrogen phosphate anhydrous.....	6.5g
Distilled water.....	900ml

2. Reagent 2. Mayer's haematoxylin

Haematoxylin.....	1g
Potassium alum.....	50g
Citric acid.....	1g
Chloral hydrate.....	50g
Sodium iodate.....	0.2g
Distilled water.....	1000ml.

3. Reagent 3. Alcoholic eosin (stock)

Eosin Y.....	1g
Distilled water.....	100 ml

4. Reagent 4. Alcoholic eosin (working)

stock eosin solution.....	1 part
80% ethanol.....	3 parts
Conc. acetic acid.....	0.5 ml/100ml

HAEMATOXYLIN AND EOSIN STAINING PROCEDURE FOR HISTOLOGICAL ASSESSMENTS

1. Paraffin processed sections mounted on microscope slides.
2. Sections were put in two changes of xylene for 15 minute each
3. Section through three changes of absolute ethanol for 5 minutes each
4. Sections to 80% ethanol for 15 minutes
5. Sections to 50% ethanol for 15 minutes
6. Sections to tap water for 30 minutes
7. Sections to distilled water for 2 minutes
8. Sections to Mayer's haematoxylin stain for 20 minutes
9. Sections to tap water for 30 minutes
10. Sections to distilled water for 2 minutes
11. Sections to working eosin solution for 5 minutes
12. Sections to 96% ethanol for 5 minutes
13. Sections to two changes of absolute ethanol for 5 minutes
14. Sections to three changes for 5 minutes each
15. Sections mounted in DPX

RESULTS

Nuclei----blue black

Background and cytoplasm,---varying shades of pink

APPENDIX VI**EPI2000 STATISTICAL CALCULATION (2X2, 2X2n tables) COMPARING THE NUMBER OF WORMS DECLARED BY HISTOLOGY (H2) AND ENZYME HISTOCHEMISTRY (EH) ON THE SAME NODULES.**

PROGRAM: EPI2000\STATCALC.EXE (EpiInfo Version 6 Statcalc, 1993) Analysis of single and stratified tables.

The paraffin processed histology sections declared 34 female worms and 8 males while the enzyme histochemical examinations declared 34 female worms and 2 male worms. Using the two processes as exposures (H2 and EH) and gender of the worms as variable, the following calculations were obtained

EXPOSURE	FEMALE	MALE	TOTAL
EH	34	2	36
H2	34	8	42
TOTAL	68	10	78

Results of analysis of a single table (EpiInfo Version 6 Statcalc. 1993)

	<u>Chi-Squares</u>	<u>P - values</u>
Uncorrected	3.16	0.0755954
Mantel - Haenszel	3.12	0.0774952
Yates corrected	2.07	0.1506765

APPENDIX VII

EPI2000 STATISTICAL CALCULATION (2X2, 2X2n tables) COMPARING THE NUMBER OF WORMS DECLARED BY HISTOLOGY ON GROUP 1 NODULES (H1) AND HISTOLOGY ON GROUP 2 NODULES (H2).

PROGRAM: EPI2000\STATCALC.EXE (EpiInfo Version 6 Statcalc, 1993) Analysis of single and stratified tables.

The paraffin processed histology sections of group 1 nodules declared 32 female worms and 13 males from 27 nodules while the paraffin processed histology sections of group 2 nodules declared 34 female worms and 8 male worms from 30 nodules. Using the two processes as exposures (H1 and H2) and gender of the worms as variable, the following calculations were obtained

EXPOSURE	FEMALE	MALE	TOTAL
H1	32	13	45
H2	34	8	42
TOTAL	66	21	87

Results of analysis of a single table (EpiInfo Version 6 Statcalc, 1993)

	<u>Chi-Squares</u>	<u>P - values</u>
Uncorrected	1.15	0.2837585
Mantel - Haenszel	1.14	0.2865430
Yates corrected	0.67	0.4115183

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