VARIABLES IN THE RADIATION-ATTENUATION OF *NIPPOSTRONGYLUS BRASILIENSIS* LARVAE

DISSERTATION FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY
IN THE FACULTY OF VETERINARY MEDICINE

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PREFACE

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GENERAL INTRODUCTION

1. World Importance of Helminth Parasites of Man and Domestic Animals.

(a) Helminthic Infection of Man.

The world population is estimated at three thousand million and increasing at a rate of around fifty million per annum. It is somewhat ironic that this rapid growth in numbers is, in general, greatest in these regions where malnutrition and disease are most widespread. Infectious diseases pathogenic to man are caused by many different organisms, the principle offenders being bacteria, viruses, spirochaetes, fungi, protozoa and parasitic worms.

The relationship between incidence and disease in infections caused by bacteria, viruses, etc is profoundly different to infections caused by helminths. In, say, viral infections, a small inoculum can develop into a large population of infectious agents by multiplication inside the host without further additions from outside the host. In helminthic infections, accumulation within a host can not occur by multiplication inside but only by additional parasites entering from the outside. Thus, in general, small numbers of helminths mean little or no damage; large numbers mean a correspondingly greater damage.

The first comprehensive attempt to define the world incidence of helminthic infection in man was made by Stoll in 1947. From data gathered from the relevant journals over the previous 25 years, Stoll estimated that there were over 2257 million individual major flatworm or roundworm infections among a world population of 2170 million (1940 census).

The major contributors to this staggering total were the nematodes or roundworms. 75% of all human helminthiasis were due to five nematode groups: the ascarids, the hookworms Ancylostoma and Necator, the whipworm Trichuris, the threadworm Strongyloides and the pinworm Enterobius. All these nematodes live as mature adults in the human intestinal tract and their eggs, shed into the lumen, leave the host in the excreta.

A further 11% were due to the filaroid nematodes. This group require a period of development in a biting fly, or mosquito, prior to infecting man. The adult worms are found in the lymphatic vessels of the pelvis, extremities and genital organs and the microfilariae produced by the female worms appears in the peripheral blood.

Finally, the two other main contributors were the trematodes and cestodes. The trematodes or flatworms were estimated at about 7% of which the schistosomes or "blood flukes" were the most widespread. The cestodes or tapeworms accounted for about 4%.

As regards the geographical distribution of helminth infections, it is very evident that helminthiasis is most serious in the developing countries of the world, particularly in the tropics. In these parts, the effects of the worm diseases are often aggravated by malnutrition with the result that the lethargic and under-nourished native has to support a population of worms which are themselves a contributory cause to his lethargy. For example, hookworm disease or ancylostomiasis is prevalent in many parts of the world and especially so in tropical and sub-tropical countries. Hookworms enter the body usually through the feet, pass through the blood-stream to the lungs, then migrate up the bronchi and trachea, down the oesophagus to the stomach and finally end up in the mucosa of the small intestine. There, the worms feed by sucking the blood which can result in a serious anaemic condition of the host. As almost a quarter of the world's population harbour hookworms, one should not be too surprised by Stoll's (1962) estimate that the daily human blood loss to hookworms is equivalent to the total exsanguination of 1.75 million people.

Schistosomiasis is a helminthic disease of similar wide distribution and importance. Stoll (1947) estimated that 114 million were infected with one of these species:- Schistosoma haematobium, S. mansoni and S. japonicum, the first affecting the bladder, the other two, the intestines. Prior to infecting man, the schistosomes require an intermediate host, a certain species of water-snail. From this host, cercariae emerge and find their way into the human body either in drinking water or, more commonly, by burrowing through the skin of bathers. In Africa the disease caused by these blood-flukes is known as bilharzia. Due to the increase on the number of irrigation projects, this terrible disease is very much on the increase. For example, in lower Egypt, where perennial canal irrigation is carried out, as many as 95 per cent of the villagers suffer from this disease (Chandler, 1956).

A nematode species, not mentioned previously as it has a relatively low incidence rate, is the trichina worm, Trichinella spiralis. Unlike most other helminths, T. spiralis is almost entirely absent from the tropical areas. Trichinosis disease occurs chiefly in the United States, Europe and the Arctic regions. Man is infected from eating undercooked meat, especially pork.

Once in the intestine of the host, the trichina worms develop and the embryos produced travel into the blood and lymph streams, finally settling in the striated muscles. There the tissue around the trichina reacts by the formation of a connective tissue capsule, which ultimately becomes calcified. Early studies on the incidence of trichinosis in the United States revealed a figure of about 16% (Stoll, 1947). However, more recent studies have shown a significant reduction; the present incidence rate is probably around 4% or less (Most, 1965; Zimmerman, 1968).

In conclusion, it is clear from Stoll's 1947 figures and the above three examples that the health and economic standards of the world are influenced to a large extent by the prevalence of helminthic diseases. It is therefore discouraging to note that since 1947 there has been little or no evidence of a reduction in the incidence of any world species (Stoll, 1964). From this it would appear that man is now host to a total of over 3000 million helminthic infections.

(b) Helminthic infections in animals.

Helminths are responsible for three economically important diseases of cattle and sheep; parasitic gastro-enteritis, parasitic bronchitis and fascioliasis.

Parasitic gastro-enteritis is a disease complex caused by several species of nematodeswhich inhabit the abomasum and small intestine. The principal parasites involved are species of Haemonchus, Ostertagia, Trichostrongylus, Cooperia, Nematodirus and to a lesser degree, Bunostomum and Oesophagostomum. In Great Britain, the predominant species is Ostertagia ostertagi, occurring in some areas as a clinical disease in 15 per cent of farms with mortality ranging between 11 and 24 per cent (Anderson et al., 1965; Ross, 1965). Haemonchus contortus is a blood sucking worm, and in heavy infections especially in young animals, may cause severe anaemia resulting in sudden death even in apparently fit and healthy animals. Using radioactive chromium and iron to label red cells, Clark, Kiesel and Goby (1962) estimated the daily blood consumption in sheep per H. contortus worm as 0.05 ml. If one considers the average H. contortus worm burden in sheep as 3,000, this represents a daily loss of 150 ml.

Parasitic bronchitis in cattle is caused by the nematode, Dictyocaulus viviparus and in sheep, by the related D. filaria worm. In addition, species of Protostrongylus, Muellerius and Cystocaulus can cause serious outbreaks of disease in sheep in some parts of the world. These lungworms do their damage partly in the lungs where the adults live and partly in other sites of the body during the migration of the immature stages.

If present in sufficient numbers they can cause parasitic bronchitis or "husk". Pneumonia often follows and if not treated many animals may die.

The liver flukes, Fasciola hepatica and F. gigantica are prevalent in cattle, sheep and goats in nearly all parts of the world. In Great Britain, the areas of fluke infections are confined to the western counties as the land there is particularly suitable for the survival and multiplication of the fluke's intermediate host, the mud snail (Lymnaea truncatula). There are two basic types of liver fluke disease, acute and chronic. Acute disease, occurring usually only in sheep, is caused by a massive and sudden invasion of the liver by immature flukes. The damage to the liver by this invasion causes severe and often fatal haemorrhage. Chronic fluke disease, on the other hand, is caused by adult flukes which have developed in the bile duct over many weeks.

The total economic loss caused by worm infections in domestic animals is virtually impossible to assess, especially as it is now recognised that low to moderate levels of infestation, where there is continual interference with growth and development, are probably of greater economic importance to the livestock industry than the obvious outbreaks of helminthic diseases. The already mentioned nematodes of sheep have been shown, at a subclinical level, to

influence in a negative sense, the live weight gain and wool growth (Spedding and Brown, 1957; Spedding, 1965; Armour, Brown and Sloan, 1962).

Despite the inherent difficulties, several attempts have been made to assess the economic loss. For example, the U.S. Agricultural Research Service in 1954 estimated the total annual loss attributable to helminths of sheep, swine and cattle in the U.S.A. to be 227,672,000 dollars. However, this figure did not include the cost of parasitic control, i.e. research and treatment. Similarly, in a survey carried out by the Bureau of Agricultural Economics, Cummings (1964) estimated the cost of sheep parasites in Australia at almost thirteen million pounds of which 43% was calculated to be the control costs.

Finally, it should be pointed out that there is a distinct difference in the methods used for controlling helminthic diseases in man and animals. Whereas, in man, the risk of infection will obviously diminish as the standard of living conditions is improved, this is not so with animals. The demands of modern farm management require heavier stocking rates which, in turn, give rise to a greater contamination rate with helminth eggs resulting in a greater risk of reinfection. In the next section, the various methods used in the control of helminthic disease in animals are described.

2. Control of helminthic diseases in animals.

(a) Use of Anthelmintics

Drugs used for the treatment of parasitic worms are termed anthelmintics. Many of these drugs have been in use for centuries. For example, parts of plants or plant extracts such as the male fern used for the removal of tapeworm. Unfortunately, the majority were not highly effective and were frequently as toxic to the host as to the parasite. However, within the last few decades, considerable advances have been made in the pharmaceutical field and this has resulted in the appearance of new synthetic compounds of high efficiency and relatively low toxicity. Such drugs are now preferred to most of the older anthelmintics.

The ideal anthelmintic should effectively kill or expel the particular parasite for which it is used and, as many of the helminth diseases are caused by multiple infections, it should also possess a wide range of activity. This latter condition is, in many cases, not achieved and instead a combination of drugs must be used.

In the treatment of parasitic gastro-enteritis in cattle and

sheep, the older drugs such as copper sulphate, copper nicotine sulphate, and tetrachlorethylene have been largely replaced by thiabendazole (Brown et al., 1961; Campbell and Cuckler, 1962) and Tetramisole (Walley, 1966).

Tetramisole is also a very effective anthelmintic against lungworms in cattle and sheep.

Carbon tetrachloride has been used in the treatment of fascioliasis in sheep for over 40 years. An undesirable reaction of this treatment is the untoward reactions which sometimes follow its administration. It is also used in the treatment of fascioliasis in cattle in some parts of the world though in Britain hexachloroethane is generally preferred.

An extensive coverage of anthelmintic treatments of animals is given in a review by Gibson (1962).

(b) Immunilogical Control

Immunisation or vaccination has many advantages over treatment by chemotherapy in the control of infectious diseases. Firstly, with the latter, there is a definite delay between the onset of infection and diagnosis and treatment. During this delay period, the host's tissues may be extensively damaged and this may, at a

later time, produce clinical disease. Secondly, there is also the serious economic problem of sub-clinical infections which usually affect the whole flock or herd resulting, as mentioned previously, in reduced growth rates, etc. This loss would be overcome by the use of vaccine. Thirdly, it was suggested by Terry (1968) that with regard to food animals, anthelmintics may leave traces of potentially toxic drugs which could at a later stage build up to a dangerous level in man.

Studies on the immune reaction of hosts to helminth parasites are of fairly recent origin when compared to those on bacterial and viral infections. In fact, much of the current work stems from the observations of four workers, 30-40 years ago; Stoll, Searles, Taliaferro and Chandler.

Stoll (1929) infected worm-free lambs with H. contortus and turned them out to graze on a limited pasture area where they were subject to re-infection. After several weeks the faecal egg counts, having previously risen to a high level, suddenly dropped and subsequently these lambs were highly resistant to re-infection. Stoll attributed this dramatic change to the elimination of adult worms and termed the phenomenon "self cure".

Other effects of the immune reaction on the parasite will be mentioned later.

Since 1929, studies have shown in a wide range of helminth infections that animals can become immune to re-infection. Such studies have been the subject of reviews by several authors (Culbertson, 1941; Chandler, 1953; Soulsby, 1957; 1963; Stewart, 1959; Urquhart et al., 1962).

With nematode infections, immunity to re-infection has been demonstrated in domestic animals with H. contortus (Stoll, 1929), Trichostrongylus colubriformis (Stewart, 1950), T. axei (Gibson, 1952), Strongyloides papillosus (Turner, 1956), Ascaridia galli (Sadun, 1948), Ancylostoma caninum (Otto and Kerr, 1939) and in laboratory animals, with Nippostrongylus brasiliensis (Taliaferro and Sarles, 1939), Strongyloides ratti (Shelden, 1937) and Trichinella spiralis (Roth, 1939).

With trematode infections, experimental attempts to induce a strongly acquired immunity in the host have been largely unsuccessful. However, Boray (1967) showed that cattle, after a single infection with the liver fluke, Fasciola hepatica, were

markedly immune to re-infection; this work was confrimed by Doyle (1971). More recently, Corba et al (1971) demonstrated a strong resistance against the infection in rats using lymphoid cells. Lymphoid cells from rats infected with F. hepatica were able, when transferred to isogenic recipients, to confer a high degree of protection ranging between 66 - 100% against a primary challenge infection. Similar results were obtained using a pair of monozygous twin calves.

Associated with the immune response is an antibody response which can be measured by a variety of serological techniques. However, the precise role of these antibodies is complicated by the number and complexity of the potentially antigenic materials involved. The parasites themselves possess complex macromolecules which are genetically foreign to their hosts and are therefore one source of antigenic material. In addition to these "structural" antigens, there are those associated with the very metabolism of the parasite. Over the period of an infection, the host is therefore subjected to a whole range of antigens, varying both

quantitatively and qualitatively in the different stages of the parasite's life cycle and the problem is to determine which of the antigens give rise to protective antibodies and precisely how these antibodies operate.

In 1935, Chandler, on the evidence of his experiments with superinfections of N. brasiliensis in rats, put forward the theory that in the immune reaction, the effective antibodies were stimulated not by worm tissue, but by worm products; the fact that the worms were unable to grow normally or to reproduce was taken as evidence that the immune reaction was directed against the metabolic products of the parasites and not against the parasites' body substances. Following on from this, Sarles (1938) observed that when N. brasiliensis larvae were placed in immune serum, precipitates formed at the external openings and in the intestine, indicating again, an immune reaction to metabolic products. Studies by Jackson (1959) with T. spiralis using the fluorescent antibody technique enabled a more precise determination of the sites of antibody attachment on the parasite.

The effect of an immune response on the parasite is manifested by (1) slow development to the adult stage; (2) stunting of growth;

(3) inhibition of reproduction; and (4) premature expulsion of worms already harboured. Frequently, these effects occur just before or just after a moulting period. The significance of this period is well illustrated in the "self-cure" phenomenon in H. contortus infection in sheep. This reaction is induced when the challenge dose of larvae moults from the third to the fourth larval stage. At this point the abomasal mucosa of the host becomes markedly inflamed, thus rendering the environment unsatisfactory for the adult worms which are then eliminated.

The foregoing evidence clearly emphasises the importance of living worms, or alternatively, the antigenic material produced by them, in the stimulation of resistance. Attempts to vaccinate animals against parasitic disease have been made from two angles:-
(1) using non-living helminth material, and (2) using living helminths.

Vaccines made from dead whole worm extracts have, in the main, proved disappointing. The complete failure to stimulate immunity has been demonstrated with extracts of Ascaris lumbricoides (Oliver Gonzales, 1956; Soulsby, 1957), A suum

(Crandall and Arean, 1965) and H. contortus (Mayhew, 1944, 1949; Stewart, 1950). On the other hand, Jarrett et al., (1960a) were able to induce some protection against D. viviparus; the worm burdens in some groups of immunised animals were reduced to about half of those in the controls. The popular explanation for these failures is that the "functional antigens", those that stimulate resistance, are present in only minute amounts at any given time and in the dead worm type of vaccine, they are completely masked by the "non-functional antigens" which are present in far greater amounts (Terry, 1968).

Before describing attempts with "live" vaccines, it is worth mentioning the successful attempts to vaccinate using materials produced in "in vitro" cultures. In 1953, Thorson, showed that a solution of excretions and secretions was effective as an immunising agent against N. brasiliensis. Similar results were obtained with D. viviparus (Campbell, 1955; Chute, 1966). Although recent studies along these lines have been less fruitful, this method of vaccination, with the rapid progress being made in the "in vitro" cultivation of nematodes, holds some promise for the future.

Regarding "live" vaccines, it is obvious that the use of standardised doses of normal infective larvae is out of the

question because, in all probability, a patent infection will occur which may lead to clinical disease and infection of other animals.

The use of avirulent strains of helminths as a vaccine at first appeared promising as this method is widely used in other infectious diseases. Unfortunately, no satisfactory practical method exists at present. Allen and Samson (1961), in a preliminary communication, reported the isolation of a relatively non-pathogenic strain of Haemonchus from the pronghorn antelope, and were able to induce a significant resistance in sheep to challenge with H. contortus. However, this initially promising work does not seem to have been continued.

The most successful approach to a "live" vaccine has been the use of artificially attenuated infective larvae. Helminth larvae suitably treated with ionising radiation such as X-rays do not develop normally in the host animal. Thus, the ability of infective stages to reach patency is greatly reduced or eliminated. This effect was first demonstrated experimentally by Tyzzer and Honeij (1916) using T. spiralis. The use of irradiated larvae as immunising agents was first attempted by Levin and Evans (1941,

1942) with T. spiralis in rats. A significant practical advance in immunological control occurred in 1957 with the introduction of an X-irradiated larval vaccine for parasitic bronchitis in cattle by Jarrett et al., (1957, 1958a, b) at the Glasgow Veterinary School. The authors found that 40kr of X-irradiation would prevent D. viviparus larvae from developing to a stage where they would extensively damage the lungs of the host. Nevertheless, this level of irradiation did not destroy their property of conferring immunity on the host (Jarrett et al., 1960a). In field trials Jarrett et al., (1958a) showed that a single dose of 1,000 larvae irradiated at 40kr conferred a high degree of protection in calves from a subsequent lethal challenge. The disease, however, was not completely prevented if exposure to a very high challenge was encountered. Jarrett et al., (1959a) consequently experimented with double vaccination and demonstrated its advantages. Subsequent field trials in Great Britain (Poynter, 1964), the United States (Englebrecht, 1961, Edds, 1963), Sweden (Olson, 1962), Belgium (Vercruysse et al., 1963), Holland (Van Eck et al., 1960), France (Pierre, et al., 1961) and Germany (Enigk and Diuvel, 1963a, 1963b, Diuvel, 1963) have confirmed that the vaccine confers a high

degree of protection against challenge. The vaccine has been marketed successfully in Great Britain for about twelve years and has been used in many thousands of animals (Poynter et al., 1960; Jones and Nelson, 1960; Nelson, 1964). The undoubted success of this vaccine raised great hopes that the irradiation technique might be applied with equal success to other helminths. Initial studies with H. contortus were particularly promising (Jarrett et al., 1959b, 1961). Then, Manton et al., (1962) found that although a previous exposure to infection would confer a powerful protective immunity to reinfection with H. contortus in older sheep, this did not happen in young lambs. Later, Urquhart et al., (1963) showed that X-irradiated H. contortus larvae failed to protect young lambs against subsequent challenge although this procedure was effective in older animals. More intensive studies, using double vaccination, failed to successfully vaccinate young lambs (Urquhart et al., 1966a, 1966b).

Attempts have also been made to develop irradiated vaccines against T. colubriformis (Jarrett et al., 1960b; Mulligan et al., 1961) and D. filaria (Jovanovic, 1964; Jovanovic et al., 1965; Sokolic, 1964; Sokolic et al., 1965) in sheep; Cysticercus bovis

(Urquhart, 1961), Oesophagostomum radiatum (Riek and Keith, 1960), and H. placei (Ross et al., 1959) in cattle; and Uncinaria stenocephala (Dow et al., 1959, 1961) and A. caninum (Miller, 1964, 1965a, b, c, d, 1966) in the dog. On the basis of these studies, a vaccine against D. filaria is now produced in Yugoslavia and, in the near future, a dog hookworm vaccine against A. caninum will be marketed in the United States.

3. Radiation Biology of Helminth Larvae.

The deleterious effects of ionising radiation on helminths was first demonstrated by Tyzzer and Honeij (1916) who studied the effect of radium on the development of T. spiralis. The use of irradiated larvae as immunising agents greatly stimulated and widened the scope of this field of study with the result many host-parasite systems have now been investigated. These results are given below. In order to compare the results both within and between related species the data for each parasite has been categorised under the various headings - Class, Superfamily and Genus.

3. 1. Nematodes

(a) Trichuroidea

(i) Trichinella spiralis

Most of the early work on the effect of radiation on larvae was carried out with Trichinella infection of rodents. Following the initial study made by Tyzzer and Honeij (1916), Schwartz (1921), Semrad (1937) and Evans et al (1941) confirmed, using X-rays, the failure of irradiated larvae to develop normally in the host animal. Larvae exposed to 2,250r of X-rays developed to the adult stage but the females produced no eggs (Semrad, 1937). In more detail, Evans et al (1941) studied the effects of the radiation on embryonic development in the female. With 400r, the mean number of worm-shaped embryos that developed was small; with 2,000 to 2,500r, no embryos were found though many late cleavage forms were present; with 3,000 to 3,750r, only early cleavage forms were present; and with 5,000r, larval development was completely inhibited.

In 1942, Levin and Evans showed that irradiated Trichinella larvae were capable of stimulating immunity against challenge doses in the rat host and this has since been confirmed by

several workers (Hendricks, 1952; Gould et al, 1955; Zaiman et al, 1955a, 1955b, 1961; Kim, 1957; Denham, 1966). In detail, Levin and Evans induced immunity by feeding the rats larvae that had been irradiated with 3,250 to 3,750r of X-rays. This amount of radiation allowed the larvae to grow to maturity but most of the adult worms were sterile. In contrast, Magath and Thomson (1955) were unable to demonstrate any immunity in rats fed larvae irradiated at 30kr. As this radiation dose far surpassed the level by other groups in producing immunity it was suggested that the immune response might decrease as the radiation dose was increased. This, in fact, was shown by Zaiman et al (1961) over the range 8 to 20kr. Furthermore, they found that larvae exposed to either 8 or 12kr were more potent immunological agents than the non-irradiated controls.

The effect of ^{60}Co radiation on Trichinella has been studied by several workers, chiefly from the public health aspect where irradiation might serve as a means of sterilising infected meat (Alicata and Burr, 1949; Gomberg and Gould, 1953; Gould et al 1955, 1957; Taylor and Parfitt, 1959; Cabrera and Gould, 1964). Gomberg and Gould reported that a dose of 1,500r from ^{60}Co caused sterility in female worms and a dose of 18kr inhibited development

of the larvae.

(b) Trichostrongyloidea

(i) Dictyocaulus spp.

The development of a highly effective X-irradiated larval vaccine for D. viviparus infection of cattle by Jarrett et al (1961) sparked off much activity in the field of immunological control of helminths. The studies made by the Glasgow group together with the subsequent field trials were mentioned previously. In detail, Jarrett et al (1960a) carried out immunological studies on D. viviparus irradiated at three different doses, 20, 40 and 60kr. At 20kr, the larvae were insufficiently attenuated to have been rendered non-pathogenic though they did not evoke resistance. At 40kr, the larvae were attenuated and did not produce disease but did produce immunity to subsequent challenge. At 60kr, the larvae were attenuated to such an extent that they produced no immune response in the host. In later studies, the fate of the irradiated larvae at variable intervals after infection were investigated both in calves (Poynter et al., 1960; Jarrett and Sharp, 1963) and guinea pigs (Poynter et al, 1960; Tomanek and Prochazka, 1965).

In both hosts, at 40kr, only a few stunted worms, mainly female, were found in the lungs after one week, and by the time that normal infections were patent no worms were found in the irradiated groups.

An irradiated vaccine for a related parasite of sheep, D. filaria, has been used widely in Yugoslavia for several years (Sokolic, 1964); the level of irradiation dose being 50kr X-rays. Concurrent studies showed that ^{60}Co could be applied successfully for the production of radiation vaccine, as almost identical results were found in animals treated with either 50kr X-rays or ^{60}Co (Jovanovic et al, 1965). It is noteworthy that Jovanovic et al (1965) X-irradiated two different stages of D. filaria and showed that 1st stage larvae were more sensitive to ionising radiation than 3rd stage larvae. A study by Casarosa (1966) on the fate of X-irradiated D. filaria larvae in guinea pigs showed identical results to those with D. vivíparus (Poynter et al, 1960).

(ii) Haemonchus spp.

H. contortus larvae subjected to 40 and 60kr of X-rays produced a good immunity to reinfection in 7-8 month-old sheep (Jarrett et al, 1959b; 1961a). Unfortunately, in two to three-

month old lambs similarly vaccinated no immunity was produced (Urquhart et al. 1963). Mulligan et al. (1961), in Australia, produced similar immunity in adult sheep though they found it necessary to use 60kr X-rays to produce the same degree of attenuation as that produced with 40kr by Jarrett et al (1959b). The authors suggested this was due to the possible strain difference of H. contortus in the two countries.

In similar studies, Ross et al (1959) reported the successful vaccination of calves against H. placei infection with larvae irradiated with 60kr X-rays.

(iii) Trichostrongylus spp.

The effects of different doses of X-rays on the infective larvae of the cattle nematode, T. axei in rabbits were reported by Ciordia and Bizzell in 1960. "In vitro" studies revealed that doses of up to 90kr failed to inhibit the motility of the infective larvae maintained in tap water for 28 days, whereas in the host, the number of worms recovered rapidly decreased as the dose of X-rays was increased above 10kr.

Extending their studies on immunisation against helminth

infections, Jarrett et al (1960) and Mulligan et al (1961) reported a high degree of immunity with irradiated larvae to experimental T. colubriformis in sheep. Whereas in the experiment reported by Jarrett et al, the X-ray dosage used (40kr) was insufficient to prevent numerous irradiated larvae developing into adults (largely sterile females), Mulligan et al, using a higher dose (80kr), prepared a vaccine which resulted in only small numbers of worms being recovered.

Immunisation against the same parasite in guinea pigs was reported by Gordon et al (1960). The results showed that larvae irradiated with either 40, 60 or 100kr of X-rays produced a strong immunity to challenge infection.

(iv) Nippostrongylus brasiliensis

The effect of X-rays under different irradiation conditions (variation of dose-rate, larval concentrations and faecal contamination of the irradiated suspension) of N. brasiliensis in the rat was reported by Jennings et al (1963). The results are described later in this Section.

Immunological studies on N. brasiliensis infections in rats

were reported by Prochazka and Mulligan (1965) using X-rays and Ashley (1964) and Shaikh (1965) using ^{60}Co. Over the dose range 50 to 180kr, Prochazka and Mulligan found larvae irradiated with either 50 to 100kr conferred as good an immunity as normal larvae but that larvae treated with 180kr had little or no immunogenic effect. Ashley reported that larvae irradiated at various dose levels from 10 to 115kr of γ - rays were as effective as normal larvae in stimulating resistance to reinfection. The worms that developed from larvae irradiated over the range 10 to 50kr showed no apparent alteration of their internal structure though there was marked decrease in their growth rate. Shaikh exposed larvae to γ-irradiation over a shorter range, 25 to 50kr and found that, after challenge, fewer worms were recovered from the 30 and 35kr irradiated groups than from the remainder. The author observed that the effect of γ - irradiation on the larvae over that range 10 to 50kr was fourfold; (1) delayed migration from the site of inoculation, (2) stunted growth; (3) wrinkling and thickening of the cuticle; and (4) considerable deposition of brownish granular material beneath the cuticle.

(c) Strongyloidea

(i) Ancylostoma spp.

Extensive studies by Miller (1964, 1965 a,b) have shown that dogs of various ages can be immunised against a challenge of normal Ancylostoma caninum by vaccination with 40kr X-irradiated larvae. The radiosensitivity of the larvae appeared to be similar to that of other nematode systems i.e., D. viviparus, etc. In experiments using ^{51}Cr - labelled erythrocytes, Miller (1966) showed that the worms which developed from irradiated larvae had a much reduced appetite for blood.

In a later study, Miller (1967a) demonstrated an interspecific immunity in dogs between A. caninum and A. brasiliensis, in that dogs could be protected against a challenge infection of the latter species by vaccination with X-irradiated A. caninum larvae.

(ii) Uncinaria sp.

Dow et al (1959, 1961) immunised dogs against Uncinaria stenocephala with larvae irradiated at 40kr. However a small number of adult worms developed to sexual maturity and these gave rise to some eggs in the faeces.

(iii) Syngamus sp.

The effect of X-rays on the eggs and larvae of S. trachea

has been reported by Varga (1964b). Up to 16kr, the number of larvae capable of developing normally was in proportion to the X-ray dosage, but beyond 16kr, no eggs developed beyond the morula stage. Larvae irradiated with 1 to 2kr developed into adults but produced few fertile eggs. At 3kr, no eggs were found and with 4 to 8kr, the larvae failed to reach the adult stage. In a further study, Varga (1965) demonstrated a strong immunity to S. trachea in chickens and turkeys by administration of larvae irradiated with 5kr and recently this has been extended to include satisfactory immunisation of pheasants (Varga, 1968).

(iv) Oesophagostomum sp.

The effects of 20kr X-rays on the development of O. radiatum in calves were similar to those found with D. viviparus (Riek and Keith, 1960).

(d) Rhabditoidea

(i) Strongyloides sp.

The effect of ^{60}Co irradiation of Strongyloides papillosus, a strongyle of sheep, was described by Katz (1960). Doses of 20kr and above was found to be lethal to all males and 40kr lethal for

females.

(e) Ascaridioidea

(i) Ascaris spp.

Villella et al (1958) examined the effects of ^{60}Co on development of unsegmented eggs of A. suum. Doses of 30 and 100k rep (roentgen-equivalent physical) retarded egg development. In addition, they compared the effects of ^{60}Co and X-rays on the ability of embryonated eggs to produce larval pulmonary infection in guinea pigs and found that a dose of 100 to 150k rep ^{60}Co or 100kr of X-rays prevented development of larvae in the lungs.

In a later study, Villella et al (1960a) induced immunity to Ascaris infection in mice by infection of 2nd stage Ascaris larvae irradiated with 180 or 200k rep of ^{60}Co.

Similarly, Casarosa et al (1964) reported an induced resistance in guinea pigs to embryonated Neoascaris vitulorum eggs irradiated with ^{60}Co - rays.

(ii) Ascaridia

The effect of X-rays on the development of the eggs of Ascaridia galli in chickens has been studied by Varga (1964a)

and Ruff et al (1966). Exposure to as little as 10kr prevented development of male worms and exposure to 40kr prevented development of the larval stages. In an immunisation experiment, Varga (1964b) reported that in chickens given a double vaccination dose of larvae irradiated with 10 to 20kr, the development of challenge worms was markedly retarded.

3. 2. Trematodes

(a) Schistosoma spp.

Standen and Fuller (1959) demonstrated that cercariae of S. mansoni after exposure to ultraviolet radiation, failed to mature in the mouse but were able to penetrate the skin and undergo the early stages of migration. Later, immunisation against challenge infection of human schistosomes (S. mansoni or S. japonicum) by administration of X or ^{60}Co irradiated cercariae of the human strain or cercariae of the closely related animal strain produced effective results in albino mice (Vilella, Gomberg and Gould, 1961, 1962; Sadun, 1964) albino rats (Erikson and Caldwell, 1962), and Rhesus monkeys (Hsu and Heu, 1961; Heu et al 1962; Sadun, 1964). The level of radiation in most cases was between 2 and 3kr. Doses

beyond this proved lethal to the cercariae (Sadun, 1964).

Comparing the results of Hsu et al (1962) with his own findings, Smithers (1962) suggested that cercariae of S.japonicum were slightly more radiosensitive than S. mansoni.

(b) Fasciola hepatica

The metacercariae of F. hepatica have been shown to have similar radiosensitivity properties as those of the schistosomes (Thorpe and Broome, 1962; Hughes, 1962; Lagrange, 1963; Sokolic, 1968). For example, Thorpe and Broome (1962) vaccinated rats with metacercariae irradiated with various doses of X-rays within the range 1 to 10kr and found evidence of attained immunity with metacercariae irradiated at 1kr, and "significant immunity" at 2.5kr.

3. 3. Cestodes

(a) Taenia spp.

The possible destruction of cysticeroi in meat by ^{60}Co has been the subject of some attention (Taylor and Parfitt, 1959; Pawel and Janicek, 1963). It appears that 400 to 500kr is required to render harmless C. bovis, C. cellulosae and C. pisiformis in meat.

Studies by Dow et al (1962) demonstrated almost complete immunity to infection with C. fasciolaris in mice vaccinated with oncospheres irradiated with either 40, 60, 80 or 100kr of X-rays.

The possible control of bovine cysticercosis with T. saginata irradiated with X-ray doses of 40kr has been discussed by Urquhart (1961).

(b) Hymenolepis spp.

Radiation studies with cestodes have been carried out by several workers; Hymenolepis diminuta (Villella, Gould and Gomberg, 1960b; Job, 1962) H. microstoma (Tan and Jones, 1966), and H. nana (Schiller, 1957, 1959). In general, doses of 15kr ^{60}Co or X-ray prevented cysticeroides from developing into adult tapeworms.

3. 4. Biological and Physical Factors

Miller (1967b) stated that the biological factors concerning the helminth parasite, such as its zoological classification (i.e. phylum, class, order, family) appear to have an important influence on radiosensitivity. Where the end point is death, it would appear

from the above results that the trematodes require the least radiation (> 5kr is lethal), next, the cestodes (> 15kr) and finally the nematodes (> 20kr). It is also apparent that within each order of classification, the results show a difference in radiosensitivity between the same stage of closely related parasites and, in the few studies that have been done, in the different stages of growth of a particular parasite.

Physical factors, such as different sources of radiation, type of radiation dish, depth of liquid in the radiation dish and the position of the radiation dish relative to the source, have important influences on the effect of radiation. Clearly, factors such as these should be kept constant to achieve a standard dose effect. The variation in the above results obtained by different workers using the same source, species of parasites and host may be due to one or more of the above physical factors. Unfortunately, there is no evidence that this is the case as the vast majority of the reports show insufficient data on the irradiation technique.

Jennings et al (1963) investigated three physical variables which were likely to be of practical importance in this field of study.

These variables were (1) the X-ray dose-rate; (2) larval concentration; and (3) faecal contamination of the larval suspension. The results showed clearly that (1) the attenuation of N. brasiliensis larvae by X-rays was independent of the dose-rate within the range 235-735r/minute; (2) the concentration of the larval suspension was unimportant within the range 9,000-50,000 larvae per ml. (the attenuating effect was enhanced at 1500 per ml); and (3) the faecal concentration was unimportant even when as high as 10% dry matter.

The aim of the present study was to investigate further factors which may be involved in the radiation-attenuation of helminth larvae.

Life Cycle of Nippostrongylus brasiliensis

Nippostrongylus brasiliensis (Travassos, 1914) is a trichostrongyle nematode occurring naturally as an intestinal parasite of the wild rat, rattus norvegicus. In the laboratory, experiments have shown that N. brasiliensis can attain sexual maturity in mice (Porter, 1934, 1935a, Wescott and Todd, 1942), hamsters (Lindquist, 1949, 1950; Haley, 1954), rabbits (Thorson, 1953) and guinea pigs (Newton et al, 1959). However, in some of these hosts, abnormal development occurs.

The life cycle and morphology of the nematode was first described by Yokogawa (1922). Later studies on the biology of the parasite include those by Schwartz and Alicata (1934), Lucker (1936), Twohy (1956) and Haley (1962). Basically, the life cycle consists of two moults in an external non-parasitic phase followed by two moults in a parasitic phase.

Preparasite stage

The eggs of N. brasiliensis are in the early stages of segmentation when they pass out of the rat host in the faeces (Haley, 1962). They are ellipsoidal in shape with an average

size range of 58 μ x 33 μ. Hatching of the rhabditiform larvae takes place at room temperature (18 - 22°C) within about 18 - 24 hours (Yokogawa, 1922). These first-stage larvae grow rapidly and moult to second-stage rhabditiform larvae within 48 hours (Lucker, 1936). Within the next 1 - 2 days the larvae, after further development, moult once again and become third-stage filariform (infective) larvae. In nature, the essential requirements in each stage of development are oxygen, moisture, and normal temperatures.

Parasitic stage

The natural mode of infection of the rat is by skin penetration (Yokogawa, 1922; Schwartz and Alicata, 1934). Parker and Haley (1960) reported that the filariform larvae were extremely sensitive to small temperature differentials and they suggested that such behaviour probably plays a vital part in the process of making contact with the rat host in nature.

The penetration of the larvae through the skin may be direct or via a hair follicle (Taliaferro and Sarles, 1939; Gharib, 1955)

and is accomplished within 5 minutes to 2 hours. According to Taliaferro and Sarles (1939), the larvae begin to feed soon after penetration of the skin and undergo rapid growth. Twohy (1956) however reported that the larvae first undergo a lag phase in growth as evidenced by a decrease in total length, increasing in size only towards the end of their time in the skin. After entry into the bloodstream, the larvae are carried via the heart to the lungs within about 11 - 20 hours post infection (Yokogawa, 1922; Twohy, 1956). It has been shown by Gharib (1961) that they may also migrate to the lungs via the lymphatic system. Once in the lungs, the larvae feed on whole blood and tissue cells (Taliaferro and Sarles, 1939; Twohy, 1956) and undergo rapid growth culminating in a third moult. It is at this stage that differentiation of the sexes occurs (Haley, 1962). After the moult, the now fourth-stage larvae migrate up the bronchi and trachea, down the oesophagus to the stomach and finally to the small intestine. The first larvae reach the small intestine in about 40 hours post-infection and by 60 hours nearly all the larvae have completed the migration. As in the lung phase, the larvae undergo another rapid increase in size finishing between 90 and 108 hours post-infection with the fourth and final moult, (Yokogawa, 1922).

Following this, the worms mature quickly and by the sixth day eggs appear in the host's faeces. In common with other nematodes, the reproductive capacity of the worms is large. According to Porter (1935a), an average number is 935 eggs/female worm/day. The adult males measure 3–4 mm and the females 4–6 mm in length.

M. brasiliensis was described by Travassos (1914) and Yokagawa (1920) as a parasite of the upper part of the small intestine of rats. There, according to Porter (1935b) and Taliaferro and Sarles (1939), the adults feed on tissue cells and blood. Mulligan et al (1965), using Cr^{51} labelled erythrocytes, found no evidence that blood was consumed. As intestinal flagellates have been found in the gut of the worm, it can be assumed that the intestinal contents also form part of the diet (Weinstein and Jones, 1956).

From the sixth to the tenth day post-infection the egg production rises rapidly, remaining at a high level for a further 3 to 5 days before falling to a low or zero level 15 to 20 days after infection (Africa, 1931; Schwartz and Alicata, 1934; Graham, 1934). After the twelfth day the number of worms found

in the duodenum and jejenum falls rapidly, the rate of this expulsion being directly proportional to the initial worm population (Haley and Parker, 1961). This so-called "self-cure" in N. brasiliensis infection has been reported by numerous authors (Africa, 1931; Chandler, 1932; Graham, 1934; Porter, 1935a; Haley, 1961; Mulligan et al, 1965; Neilson, 1965; Brambell, 1965).

In laboratory rats, the development of N. brasiliensis is influenced by such factors as the route of infection (Twohy, 1956; Haley, 1962), the age of the infective larvae (Haley and Clifford, 1958, 1960), the pretreatment of the larvae (Thorson, 1954; Haley, 1962), and the host's breed (Graham and Porter, 1934,) age (Chandler, 1932) and sex (Porter, 1935a).

MATERIALS AND METHODS

1. Experimental Animals

All rats used in the experiments were female, aged 6-8 weeks and of the Wistar Albino strain. The majority of these were obtained regularly from Animal Suppliers Ltd., London. In the experiments described in Section 2 the rats used were bred and reared in the Animal House of the Physiology Department, University of Glasgow.

The rats were housed ten to a cage in galvanised iron wire cages (24" x 11" x 9"). Urine and faeces were collected in metal trays placed under the cages. The Animal House temperature was maintained at approximately 23°C.

The animals were fed on a cubed diet (41B, supplied by W. Primrose & Son, Glasgow). This consisted mainly of wholemeal flour and ground oats. Water was supplied ad libitum. At the time of the experiments the rats weighed between 150 and 200g.

2. Parasite

a) Strain

The strain of N. brasiliensis used in all the experiments was obtained originally from Dr. A.C. Hopkins of the Zoology

Department, Glasgow University and had been maintained by regular passage through rats similar to those used for experimental purposes.

b) Culture of Infective larvae

Groups of 10 rats were infected each with 3000-5000 third-stage N. brasiliensis larvae by subcutaneous injection. Between days 6 and 10 of the infection, a sheet of paper was placed under the cage to collect the egg-bearing faeces. The faeces, once collected, was washed to remove food particles and other debris, then mixed with lukewarm water into a thick paste. With the aid of a spatula, small portions were spread on to the centres of 7.0 cm circles of Whatman's No. 1 filter paper. Each, in turn, were placed on to a pad of absorbent material (synthetic sponge) and saturated with water in a 9 cm. plastic Petri dish (Sterilin Ltd., Surrey). The lids were replaced and the cultures stored in a humid incubator at 26 ± 1°C for at least five days. The culture method described here is similar to that described by Bakaret (1951).

By the 5th day, the new third-stage larvae had migrated from the faeces to the edges of the filter paper where they

became fixed by their tails. Individual dishes that were found to be severely contaminated with fungal growth were discarded.

The larvae were harvested between 5 and 8 days after setting up the culture. This was done by filling Petri dishes with lukewarm water and waiting for several minutes while the larvae, thus stimulated, swam away from the filter paper. The filter paper and sponges were then removed and the warm water containing the larvae was filtered under suction through a coarse filter paper (Green's Hyduro 904, 18.5 cm. diameter) in a large Buchner funnel. This filter paper was then placed inverted on to a fine sieve (300 mesh to the inch) in a Baermann funnel filled with water at 35°C. The larvae swam down through the sieve and settled at the bottom of the funnel. After one hour the concentrated larval suspension was run off into a glass cylinder. A 1 ml. sample of the suspension was removed and diluted with water to a convenient volume, say, 10 or 100 ml. The larvae present in 0.025 ml. aliquots of this were counted under a dissecting microscope. Sufficient samples were taken until a total count of at least 400 was reached.

The number of larvae present in the original suspension was then calculated.

3. Infection Procedure

All irradiation treatments of larvae were carried out during the evening. The following day, each preparation of larvae (irradiated and normal) was diluted to a suitable volume with lukewarm water and counted as described above. The number of larvae present in each batch was then calculated and each was diluted until the desired number of larvae/ml. remained (usually 1,000/ml.).

The rats were lightly anaethsetised with "Trilene" (Trichloroethylene B.P., I.C.I. Wilmslow, Cheshire) and were injected subcutaneously in the groin region using a 1.0 ml. syringe fitted with a No. 15 needle (B.S.W.G.). Due to errors involved both in the dilution counting method and the inoculation procedure, it was not possible to determine the number of larvae given to each rat with great accuracy. However, on several occasions, the author, on carrying out the above procedure, emptied the syringe contents into a counting dish and on no

occasion found the numbers to be greater than ± 10% from the expected.

4. Post-Mortem Procedure

All rats were killed either by a blow to the head or by Trilene anaesthesia followed by a blow to the head. The skin and abdominal wall were slit along the mid-ventral line and the entire small intestine removed. The first two-thirds was slit longitudinally with blunt scissors, cut into 4 inch lengths and placed in a muslin bag suspended in a 250 ml. beaker half-filled with warm saline. This was then placed in an incubator at 37°C for one hour, by which time all the worms had swum out of the bag and collected at the bottom of the beaker. After decantation of the fluid, the worms were counted and sexed under a dissecting microscope. The procedure was essentially the same as that used by Neilson (1965).

5. Faecal Egg Counts

A random sample of infected faecal egg pellets was collected from paper placed under the cage for a period of one day. Three grams were weighed out and homogenised in 42 ml. water

to give a total volume of 45 ml. This was passed through a sieve (mesh 50) and 15 ml. of the filtrate centrifuged at 2000 rpm. for 2 minutes. The supernatant was first pipetted off and then the sediment containing the eggs was resuspended in 15 ml. of saturated sodium chloride solution. The suspension was first mixed thoroughly by shaking, care being taken to avoid air bubbles forming, then small samples were withdrawn by means of a broad mouthed Pasteur pipette and transferred into the two counting chambers of a McMaster slide. Both chambers, each representing a volume of 0.15 ml. were examined under the microscope for parasite eggs. The mean value from both chambers was obtained. This value, when multiplied by 100, gave the number of eggs present in 15 ml. of homogenate and thus 1 gram of faeces.

6. Irradiation Dish

The Perspex irradiation dish used in the studies described in the first two Sections is shown diagrammatically in Fig. 1. It was important that the diameter of the dish was such that, in the case of X-irradiation, the X-ray dose was the same at the centre as at the periphery of the dish.

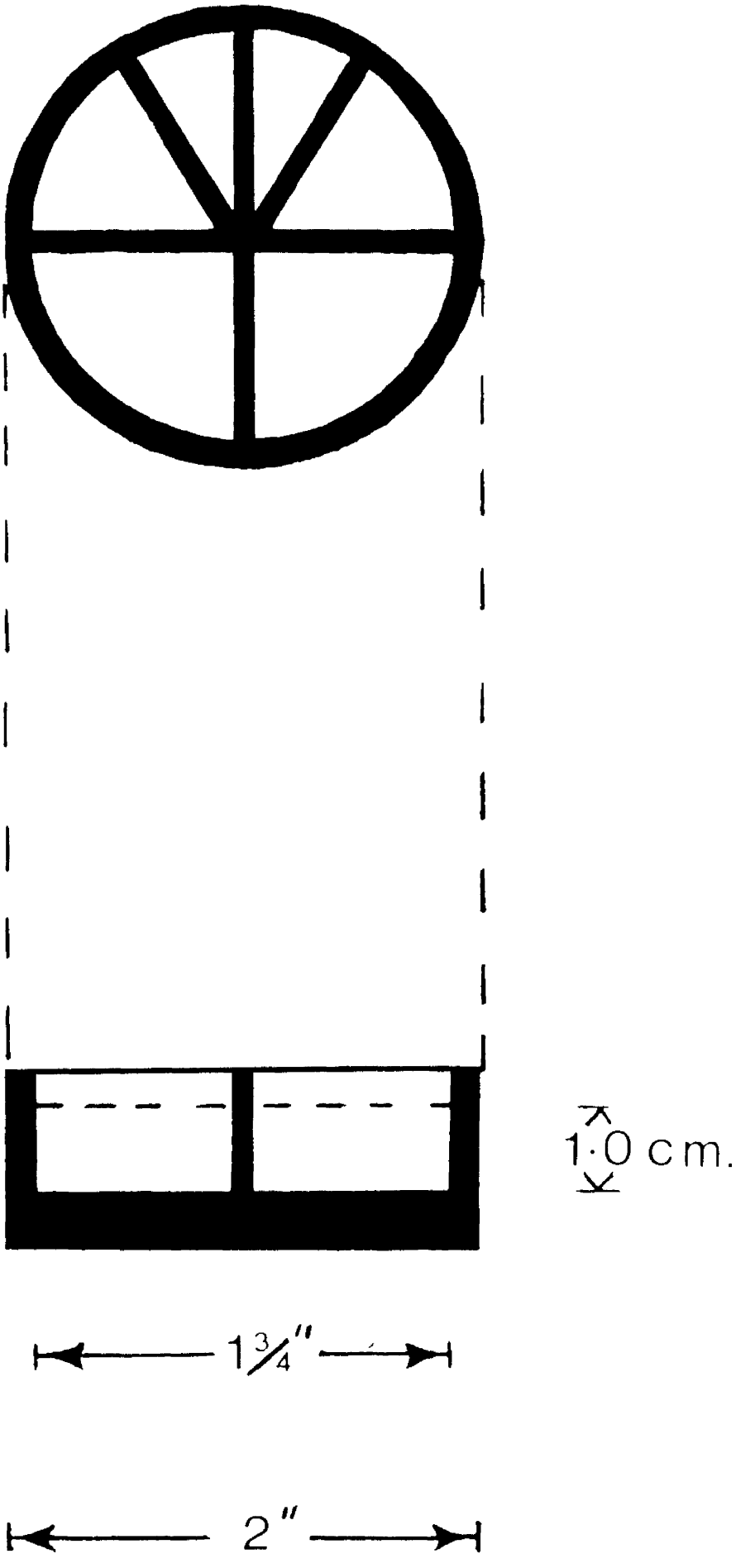


Figure 1 Segmented dish for larval irradiation.

As shown the overall diameter was 2". The total volume of the dish when filled to a height of 1 cm. was 13 ml. As, in some experiments, it was necessary to remove small volumes of the larval suspension during a long irradiation treatment, the dish was conveniently divided into several segments. Thus the total volume of 13 ml. was composed of 2 segments of 3.5 ml., 2 of 2 ml., and 2 of 1 ml.

7. Radiation Sources

All irradiation treatments were carried out in the Radiotherapy Department of the Western Infirmary, Glasgow. Three different types of radiation sources were used in the course of the work. These, together with each method of calibration, are described below.

(a) X-irradiation

The machine used was the Siemens Stabilipan (Siemens-Reiniger-Werke A.G., Erlangen, West Germany). This high output X-ray therapy Unit could be operated over a wide range of both current (2 to 20mA) and voltage (50 to 250kV). The H.V.L. was 8m.m. Al.

In the majority of the experiments to be described the machine was operated at 140kV, 20mA. However, in those experiments where the required dose-rate was less than the maximum (e.g. Section 2), the tube current was lowered accordingly.

During exposure to X-irradiation, the larval suspension will be subjected to both direct and indirect rays. The indirect rays will consist of radiation both scattered from the walls of the Perspex dish and backscattered from the material on which the dish is supported. Therefore, it is important that care is taken to standardise the scattered as well as the direct radiation. Such an arrangement is shown in Fig. 2. The dish was placed in a block of pressed wood which in turn was mounted into several thicker blocks of the same material. The depth of pressed wood was 30 cm. Fig. 3 shows a close-up of the dish seated in its pressed wood holder. The particulate material surrounding the dish was bolus (87% sucrose, 13% magnesium carbonate). Both the bolus and the pressed wood have the same general characteristics for absorption and scattering of radiation as the larval suspension.

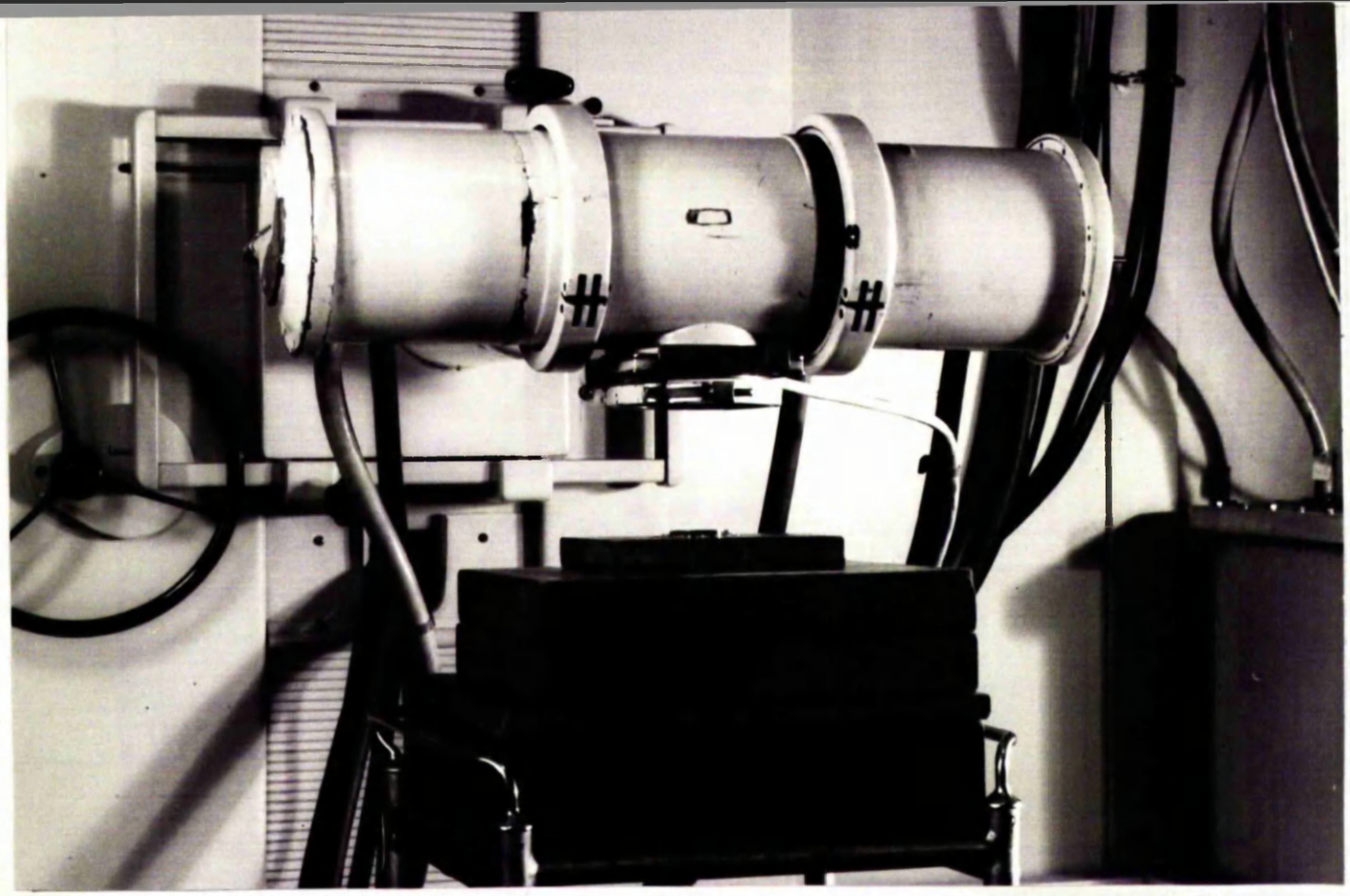


Figure 2 **X-irradiation of larvae; the X-ray tube is lowered until the window is 4mm above the surface of the pressed wood.**

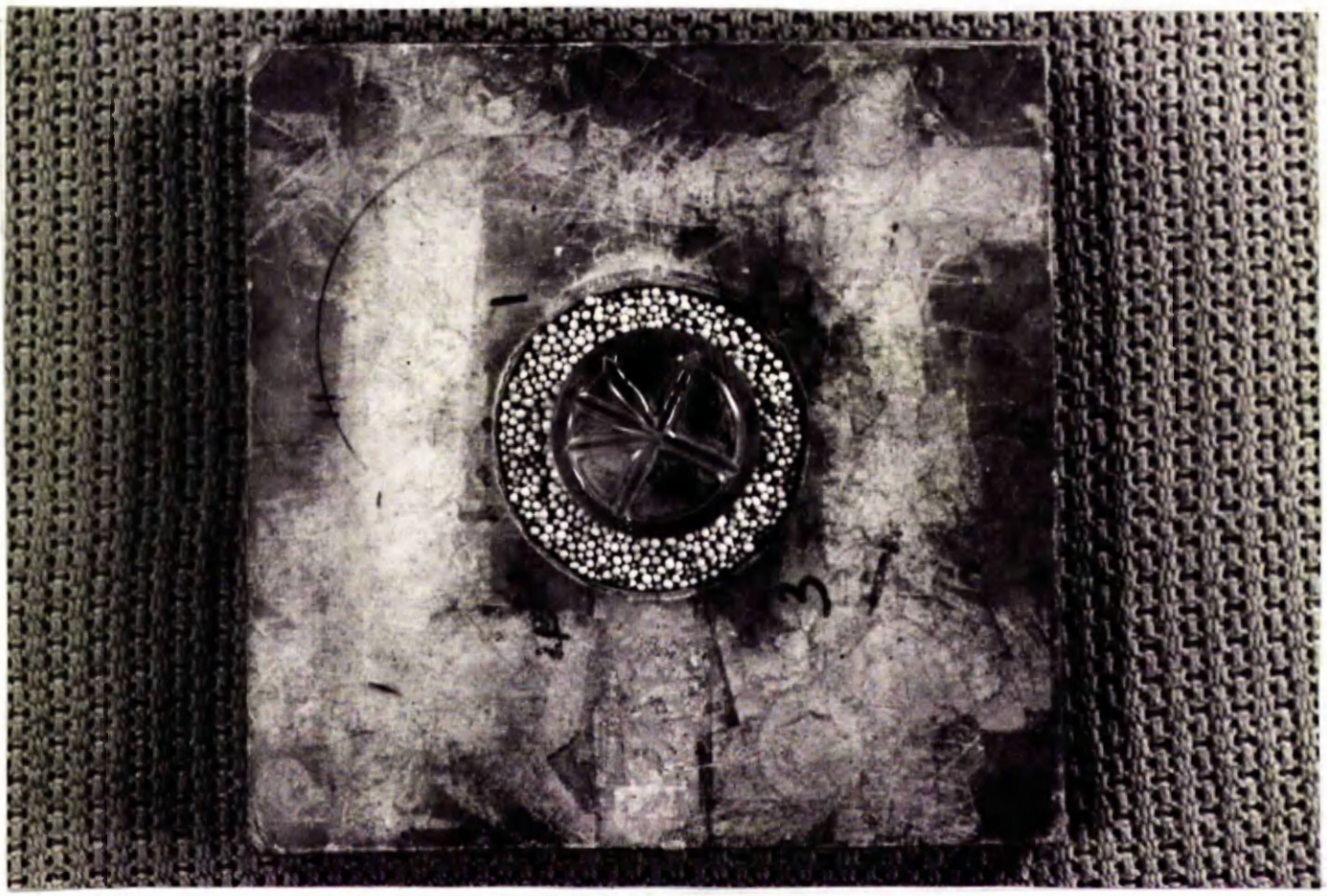


Figure 3 **Close-up of dish seated in the pressed wood holder.**

In this arrangement, care was taken to ensure that the dish was positioned below the centre of the window of the X-ray tube. Once in position, the tube was lowered until the window was at a height of 4 mm above the surface of the pressed wood. When, during an irradiation session, the machine was stopped and the dish removed e.g. for the withdrawal of some of the suspension, great care was taken to re-establish the correct distance of 4 mm as even a small variation would, because of the "inverse square" relationship, correspond to a significant difference in X-ray dose.

The dose-rate calibration was carried out as follows. A perspex dish identical to that shown in Fig. 1 was adapted as a calibration 'phantom' to contain the ionisation chamber probe of the dosemeter by having a hole drilled through its side and continuing as a groove of the same diameter halfway along the base. In this position the axis of the ionisation chamber corresponded to the level of the internal base of the irradiation dish proper. A horizontal hole in the pressed wood supporting the phantom dish allowed the dosemeter lead to be brought in. The ionisation chamber was covered with a layer of bolus to correspond to the volume of water and larvae present in an actual experiment.

The instrument used for the measurement of dose-rate was the Baldwin-Farmer Sub-Standard X-ray Dosemeter Mk2 (Baldwin Instrument Co. Ltd., Dartford, Kent). When the phantom dish was correctly positioned, the X-ray machine was switched on for a short period of time, say 5 seconds, and the dosemeter reading noted. This was repeated a further four or five times and the average reading calculated. The dose-rate in roentgens/minute was then calculated by multiplying the average reading by the time, temperature/pressure and instrument conversion factors.

(b) Gamma-irradiation

When ordinary metallic cobalt is exposed to a beam of high energy neutrons, the unstable isotope ^{60}Co is produced. This isotope emits a B-ray (energy 0.31MeV) plus two Gamma-rays (1.17 and 1.33 MeV). For most purposes, the gamma-irradiation may be considered as monoenergetic with an energy of 1.25 MeV. The isotopic half-life is 5.3 years.

The machine used was the Orbiton teletherapy Unit (Associated Electrical Industries Ltd.). The ^{60}Co source, initially of

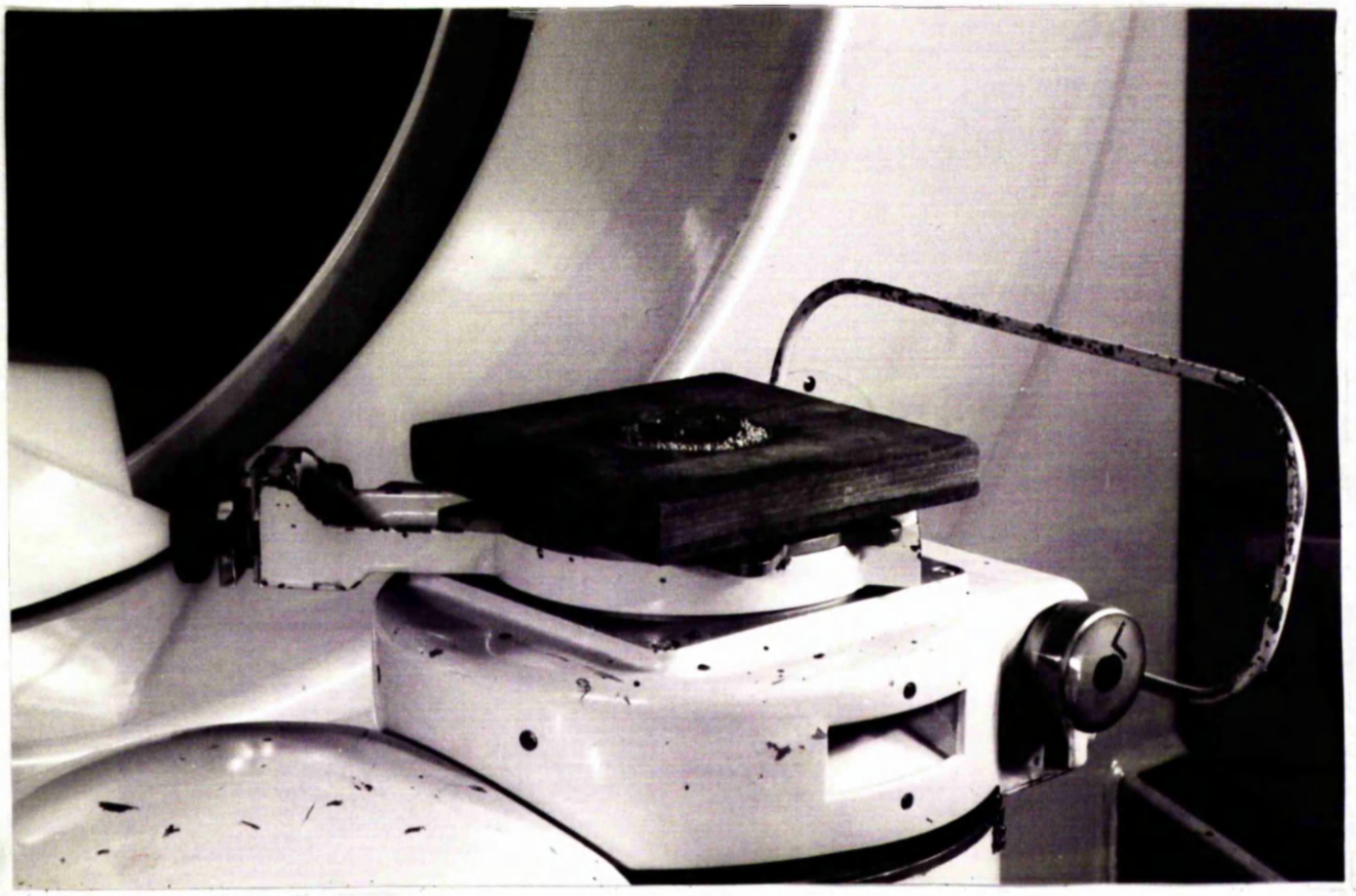


Figure 4 Gamma-irradiation of larvae. In this position the dose-rate was 170 r/min. For higher dose-rates, the dish was at calculated distances inside the radiation port.

2,000 curies strength but later replaced by a 4,000 curie source, was housed in two lead merged spheres. The source was secured in a recess on the edge of a protective disc which rotated in a plane at 90° to the beam axis. When not in use the source was at the centre of the larger sphere and for treatment, the disc was rotated through 180° bringing the source to the centre of the smaller sphere and opposite the radiation port. The distance from the source to the flat out surface on the sphere was 39.6 cms. In initial experiments the Gamma-ray treatment of the larvae was carried at this distance from the source. This is shown in Fig. 4. In later experiments, the irradiation dish was placed down into the radiation port, supported by a perspex sheet fixed 17.5 cms. above the source.

The procedure for the dose-rate calibration of the phantom dish when placed on the ledge, was identical to that for X-rays. The dose in rads was calculated from the formula given below.

$$D = RNC$$

where D = dose in rads

R = dosemeter reading corrected to Dry Air at 22°C, 760 mmHg.

N = National Physical Laboratory MeV factor to roentgens for Dry Air at 22°C, 760 mmHg and equal to 1.07

C = overall conversion factor which is a function of the radiation quality etc, and was equal to 0.944.

This formula was given in a report by the Hospital Physicist's Association (1964).

The dose-rate inside the radiation port was calculated by the "inverse-square" law from the known exposure dose at 150 cms from the source. This latter figure was routinely determined by the physicists at the Regional Physics Dept., Western Infirmary, Glasgow.

(c) High Energy X-irradiation.

In one experiment to be described in Section 2, the irradiation treatment of the larvae was carried out at a dose-rate of 3,000 rpm.

The machine used was the 4.3 MeV Orthotron Linear Accelerator (Associated Electrical Industries Ltd.). Calibration was carried out by the hospital physicists.

SECTION 1

The effect of differences in quality of radiation on the radiation - attenuation of *N. brasiliensis* larvae.

Introduction

The successful use of irradiated larvae in the control of helminthic disease depends first of all upon controlled treatment with ionising radiation. If the radiation treatment is such that there is too little attenuation, the vaccine will be too pathogenic and if the attenuation is too severe, the immunogenicity will be destroyed.

A preliminary step in immunological studies with irradiated larvae is usually that of measuring the effect of different doses of radiation on the infectivity and pathogenicity of the parasite. Three sources of ionising radiation have been employed in this type of experiment. The first source used was the radium needle, applied by Tyzzer and Honeij (1916) to encysted trichinae thereby rendering the larvae non-infective to mice. In 1921, Schwartz studied the effects of X-rays on the same parasite and since then the X-ray radiotherapy machine or its comparable industrial unit producing 50-250kV X-rays has been the most commonly used source in this type of study. Following on from Alicata and Burr's studies in 1949, an increasing number of studies have included investigations with larvae exposed to

gamma-radiation from the radioactive isotope ^{60}Co. Principle workers in this new field have been a group of Americans (Gomberg and Gould, 1953; Gould *et al.* 1955, 1957; Villella *et al.* 1958, 1960a, b).

Reports in the literature on the Relative Biological Effectiveness (RBE) of ^{60}Co gamma rays and X-rays (in the range 200-300 kvp) in a wide range of biological systems have shown a variation from about 0.35 to 1.35 with a peak of values around 0.75 and 0.95 (Kohn, 1958). For example, measurements of RBE in five biological criteria using 200 kvp X-rays as standard were determined by Sinclair (1962) as follows:-

Yeast, LD_{50} = 0.88; Mouse LD_{50} = 0.92; Rat LD_{50} = 0.91; ^{59}Fe uptake in rats = 0.90 and chicken embryo LD_{50} = 0.92.

Studies on the RBE of X-rays and ^{60}Co gamma-rays on helminth larvae have been few and variable in their results. Gould *et al* (1957) reported that the dose of ^{60}Co gamma-rays necessary to produce a given effect on *T. spiralis* larvae was approx. 3 times the dose of X-rays (250kv). Similarly, X-rays (245kv) were shown to be more effective in preventing

development of Ascaris eggs in the lungs of guinea pigs (Villella et al, 1958). In a later study, the same authors reported no difference in the effectiveness of the two qualities of radiation on the infectivity of cysticercoids of Hymenolepis diminuta (Villella et al, 1960b). A report by Sokolic (1964) on the effect of both types of radiation on D. filaria is the only case where ^{60}Co radiation has been shown to be more damaging than X-rays. This result is however, rather inconclusive as each irradiated group consisted of only two animals and, at autopsy, very few adult worms were recovered.

Recent reports on the radiation-attenuation of helminth larvae and the application of this technique to the control of helminthic disease were reviewed and summarised in a report issued by the International Atomic Energy Agency in 1964. The panel responsible for this report stressed that further studies should be made of the variables that might be important in the radiation treatment of larvae. With the increasing availability of isotopic therapy units, it was natural that high on the priority list for such work was a comparison of the effects of radiation from the X-ray and ^{60}Co therapy machines.

The present section describes a comparison of the

attenuating effect of 140kV X-rays (h.v.l., 8mm Al) and gamma-rays from ^{60}Co on N. brasiliensis larvae. Experiments 1-3 described in this section were carried out in collaboration with Dr. T. Kassai.

Materials and Methods

Parasitological methods

Culture of N. brasiliensis larvae, infection of rats, and faecal egg counts were all as described previously. The post-mortem procedures were as described previously with the exception of the first two pilot experiments. In these experiments, both the small intestine and lungs were removed. The lungs were finely chopped with scissors, then suspended in a muslin bag in a beaker of warm saline. After one hour at 37°C the number of worms found at the bottom of the beaker were counted.

Radiation sources

The radiation sources used were as described previously. The X-ray machine was operated at 140 kV, (h.v.l., 8mm Al) 5 mA at a dose-rate of 170 r/min. and 140 kV, 20 mA at a dose-rate of 700 r/min. For a gamma-irradiation, the dish was placed at various distances above the source depending on the dose-rate required.

Irradiation of larvae

For each experiment a clean aqueous solution of N. brasiliensis larvae was prepared at a concentration of approximately 10,000/ ml. For radiation treatment the suspension was pipetted into the segmented Perspex dish to a depth of 1 cm. At appropriate times, samples of the larval suspension were removed corresponding to different total doses of radiation. This was done by completely emptying a segment of the dish and refilling to the same level with water. In this way, samples could be removed without altering the remaining suspension either in concentration or radiation geometry. All samples of larval suspension, including those of the non-irradiated control material, were kept at the same concentration until the end of the radiation treatment.

Results

Initially, three pilot experiments were carried out on rats infected with irradiated N. brasiliensis to determine the relationship between the radiation dose and the degree of attenuation of the parasite.

Experiment 1a

Batches of N. brasiliensis larvae were treated with an X-ray dose of 80 kr and others with the same dose of ^{60}Co gamma rays. The dose-rate, in both cases, was 170 r/min. After irradiation, each preparation of larvae was made up to a concentration of 2000/ ml. for injection. Two groups of 10 rats were infected with each of the irradiated larval preparations as well as a third group of 10 that were given normal larvae to act as controls. Ten days later, all the animals were killed and the worms present in both the small intestine and lungs were counted. In the control group, where the worm burden was high, dilution counting was used. The number of eggs present in the faeces on days 7 and 10 were calculated and expressed in the form - eggs/ g. The results are shown in Table 1.

It is clear from the number of worms found in the small intestine that a dose of 80 kr, whether from X-rays or gamma rays, produced a high degree of attenuation. In Table 1, this degree of attenuation was expressed in the form of a 'relative take', i.e. the percentage 'take' relative to that

TABLE 1

Number of Worms Recovered from Rats 10 Days after Infection with 2000 N. brasiliensis Larvae Irradiated with 80 kr of X-Rays or ^{60}Co Gamma-Rays

Group	No. worms from rats: Small Intestine	Lung	Total	Worms in the small intestine (mean)	Relative Take %	Eggs/g. 7th Day	Eggs/g. 10th Day
X-Ray	2	2	4	2.4 ± 2.3	0.4	0	0
	0	1	1				
	4	10	14				
	8	9	17				
	2	3	5				
	1	6	7				
	0	2	2				
	3	2	5				
	2	5	7				
	2	3	5				
^{60}Co γ-Rays	25	0	25	21.8 ± 7.9	3.5	0	0
	13	5	18				
	22	3	25				
	24	1	25				
	11	9	20				
	14	2	16				
	35	1	36				
	23	6	29				
	32	1	33				
	19	4	23				
Control	-	-	-	630 ± 280	100	54600	31800
	420	0	420				
	860	0	860				
	410	0	410				
	1160	0	1160				
	640	0	640				
	690	0	690				
	440	0	440				
	260	0	260				
	790	0	790				

of the control non-irradiated larvae as 100%. In the X - irradiated group, the relative take was 0.4% and in the gamma-irradiated group, 3.5%. No worms were recovered from the lungs of the control group and only a few from the irradiated groups. No eggs were found in the faeces of the irradiated groups.

Experiment 1b

Batches of N. brasiliensis larvae were treated with an X-ray dose of 40 kr and others with the same total dose of ^{60}Co gamma-rays. The dose-rate in each case was 170 r/min. After irradiation, each sample of larvae was made up to a concentration of 1000/ ml. for injection. The experimental groups were set up as before. All rats were killed 10 days after infection and the number of worms present in the lungs and small intestine is shown in Table 2.

The results of the intestinal worm burdens in the irradiated groups indicate that 40 kr ^{60}Co gamma-rays were less effective than the corresponding dose of X-rays. The relative takes of the two groups were 24.3% and 8.9%

TABLE 2

Number of Worms Recovered from Rats 10 Days after Infection with 1000 N. brasiliensis Larvae Irradiated with 40 kr of X-Rays or ^{60}Co Gamma-Rays

Group	No. worms from rats			Worms in the small intestine (mean)	Relative Take %	Eggs/g.	
	Small Intestine	Lung	Total			7th Day	10th Day
X-Ray	113	0	113	54 ± 42	8.9	0	0
	119	0	119				
	72	0	72				
	56	0	56				
	60	0	60				
	11	0	11				
	23	0	23				
	0	0	0				
	12	0	12				
	76	0	76				
^{60}Co γ-Rays	199	0	199	147 ± 61	24.3	0	0
	163	0	163				
	181	0	181				
	160	0	160				
	3	0	3				
	112	0	112				
	216	0	216				
	187	0	187				
	111	0	111				
	139	0	139				
Control	560	0	560	606 ± 188	100	33000	30100
	720	0	720				
	740	0	740				
	480	0	480				
	780	0	780				
	580	0	580				
	560	0	560				
	700	0	700				
	780	0	780				
	160	0	160				

respectively. No worms were recovered from the lungs of any groups.

No eggs were found in the faeces of the irradiated groups on Day 7 and Day 10.

Experiment 1c

Batches of N. brasiliensis larvae were treated with X-ray doses of 10 and 20 kr and others with the same total doses of ^{60}Co gamma rays in every case at a dose-rate of 170 r/min. Groups of 10 rats were infected with each of the irradiated preparations. The infective dose was 1000 larvae. The number of worms found in the small intestine of the rats 10 days after infection is shown in Table 3. In view of the results in the previous two experiments, the lungs were not examined.

Again the results indicate that the gamma rays from ^{60}Co were less effective than X-rays. In addition, both doses of gamma radiation appeared to have had a stimulating influence on the larval infectivity. Eggs were found in the faeces of all groups but only in the 20 kr X-ray group was there a

TABLE 3

Number of Worms Recovered from Rats 10 Days after Infection with 1000 N. brasiliensis Larvae Irradiated with 10 and 20 kr of X-Rays or ^{60}Co Gamma-Rays

Control	10 kr		20 kr	
	X	^{60}Co	X	^{60}Co
620	320	500	350	850
350	230	460	270	760
270	560	310	300	880
700	340	640	310	730
620	300	350	490	570
610	350	810	190	520
600	350	560	430	620
380	390	540	380	620
450	410	530	310	700
450	310		360	560
Total 5050	3560	4700	3390	6810
Mean 505 ± 144	356 ± 87	520 ± 149	339 ± 84	680 ± 124
Relative Take % 100	70.5	102.9	67.1	134.6
Eggs/g. Day 7 23500	18900	18000	550	13800
Day 10 27100	25300	22400	1100	29200

severe reduction compared to the control group.

The results of these three experiments showed, firstly, that the degree of attenuation was dependent on the radiation dose given to the larvae. Secondly, in each experiment, ^{60}Co gamma rays at the same dose were less effective than 140 kV X-rays. Finally, there was evidence from the results of Experiment 1c that small doses of radiation brought about an increase in infectivity.

Experiment 2

Comparison of the attenuating effects of X-rays and ^{60}Co gamma rays over the range 10-80 kr.

In this experiment, batches of N. brasiliensis larvae were treated with irradiation doses of 10, 20, 40, 60 and 80 kr of X-rays and others with the same total doses of gamma rays in every case at a dose-rate of 170 r/min. After irradiation, each batch of larvae was made up to a concentration of 1,000 larvae/ ml. for injection, with the exception of the 80 kr ^{60}Co group. Due to an insufficient number of larvae available, the rats in that group were given 700 larvae each. Ten days after infection all rats were killed and the results are shown in Table 4 and summarised in Fig. 5.

It is clear from Fig. 5 that the small doses of both types of radiation produced a marked stimulating effect on the larval infectivity. In the 10 kr X-ray group, the number of worms recovered was approximately three times the mean control worm burden and was 90% of the original infective dose. Likewise, the maximum 'take' in the ^{60}Co curve, though not so striking as in the X-ray curve, was found to be around 15 kr. Beyond

TABLE 4.

Number of Worms Recovered from Rats 10 Days after Infection with 1000 N. brasiliensis Larvae Irradiated with different doses of X-Rays and γ-Rays

Larvae X-Ray (kr)	Larvae γ-Ray (kr)	Mean No. of Worms ± S.D.	Relative Take %	Eggs/g.
-	-	329 ± 157	100	2200
10	-	901 ± 161	274	2050
-	10	625 ± 437	190	5550
20	-	334 ± 39	101	0
-	20	615 ± 143	187	0
40	-	9.8 ± 9.8	3	0
-	40	135 ± 79	41	0
60	-	4.3 ± 9.9	1.3	0
-	60	9.5 ± 8.5	2.9	0
80	-	0.4	0.1	0
-	80*	3.8 ± 3.8	1.6	0

* Injected Dose 700 larvae/rat.

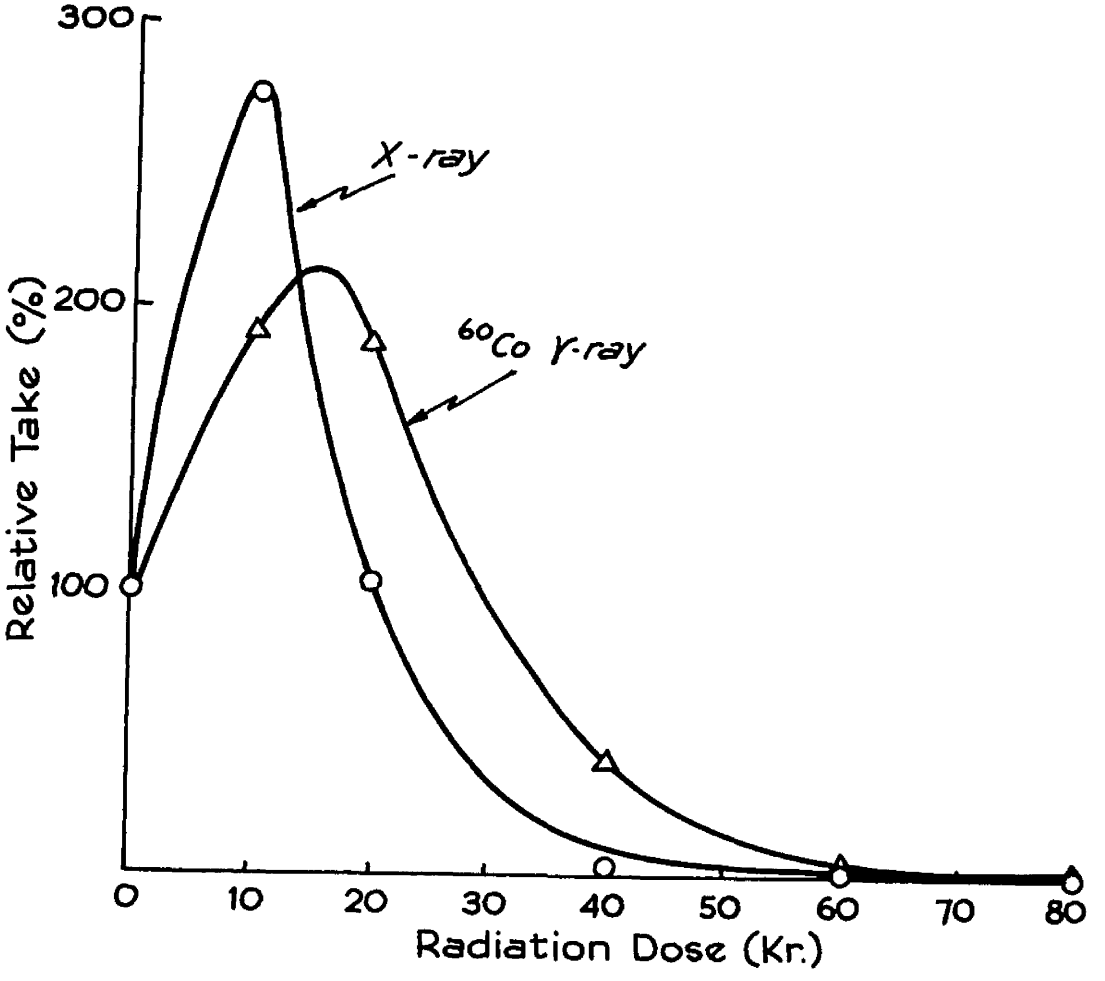


Figure 5 **The attenuating effects of X-rays and ^{60}Co gamma rays over the range 10-80 kr.**

these doses, the number of worms found decreased rapidly. No eggs were found in the faeces on day 10 in the irradiated groups 20 kr and above.

In radiation biology experiments, it is customary to refer to the Relative Biological Efficiency (RBE) of one type of radiation to another. Thus in this experiment, the RBE is defined by the ratio:-

$$\text{RBE} = \frac{\text{Dose of X-ray radiation to produce a given reduction in 'take'}}{\text{Dose of } {}^{60}\text{Co radiation to produce the same reduction in 'take'}}$$

The RBE of the two radiations for this experiment is shown in Table 5. The figures in columns 2 and 3 were obtained from the straight part of the curves in Fig. 5 at points where the reduction in 'take' was 75, 50, 25 and 12.5% of the non-irradiated control preparation. As shown, the RBE of the gamma rays was found to be about 0.7 compared to 140 kV X-rays taken as unity.

TABLE 5

Comparative Attenuating Effect of X-Rays and Gamma-Rays as Measured by the Curves in Figure 1

Take of irradiated larvae as % of control	Radiation dose to produce a standard reduction in take		RBE γ-rays/X-rays
	X-ray	γ-ray	
75	23	33	0.70
50	27	38	0.70
25	33	44	0.75
12.5	37	49	0.76

Experiment 3

Attenuating effect of X-rays over the range 5-60 kr.

Although the results of the previous experiment were convincing, it was felt that further experiments were desirable to cover the range of the previous one in greater detail. In this way, a more reliable attenuation curve would be obtained. It was also considered worthwhile to study the alteration in the sex-ratio of worms as a further measure of attenuation.

In this experiment, X-rays alone were used. Batches of larvae were treated with doses of 5, 10, 15, 20, 25, 40 and 60 kr of X-rays at a dose-rate of 700 r/min. Groups of rats, 10 in each, were infected with 1000 larvae each. Ten days later, all animals were killed and the number of worms found in their small intestines counted and sexed. The results are shown in Table 6 and summarised in Fig. 6.

From Fig. 6 it can be seen that there was again an increase in infectivity at the low doses of radiation, though not so spectacular as in the previous experiment. The maximum point in the curve was at 5 kr, 1.5 times the mean control

TABLE 6

Number of Worms Recovered from Rats 10 Days after Infection with 1000 N. brasiliensis Larvae Irradiated with Different Doses of X-Rays

Control	5kr	10kr	15kr	20kr	25kr	40kr	60kr
175	525	301	337	275	56	11	1
388	441	289	403	254	163	5	1
417	339	457	370	243	188	10	0
340	468	428	372	211	173	9	1
354	497	394	342	122	176	7	2
303	354	328	281	244	142	10	1
374	529	454	317	263	144	6	1
297	594	432	193	244	110	9	1
278	492	257	334	219	136	5	2
290		418	227	225	173	4	
Total 3216	4239	3758	3176	2300	1461	76	10
Mean 322 ± 66	471 ± 83	376 ± 75	318 ± 66	230 ± 42	146 ± 39	7.6 ± 2.5	1.1 ± 0.6
Relative Take % 100	146	117	99	71	45	2.5	0.3
Eggs/g. 20400	20300	7600	500	300	300	0	0
Sex Ratio M:F 52:48	44:56	32:68	21:79	8:92	3:97	0:100	0:100

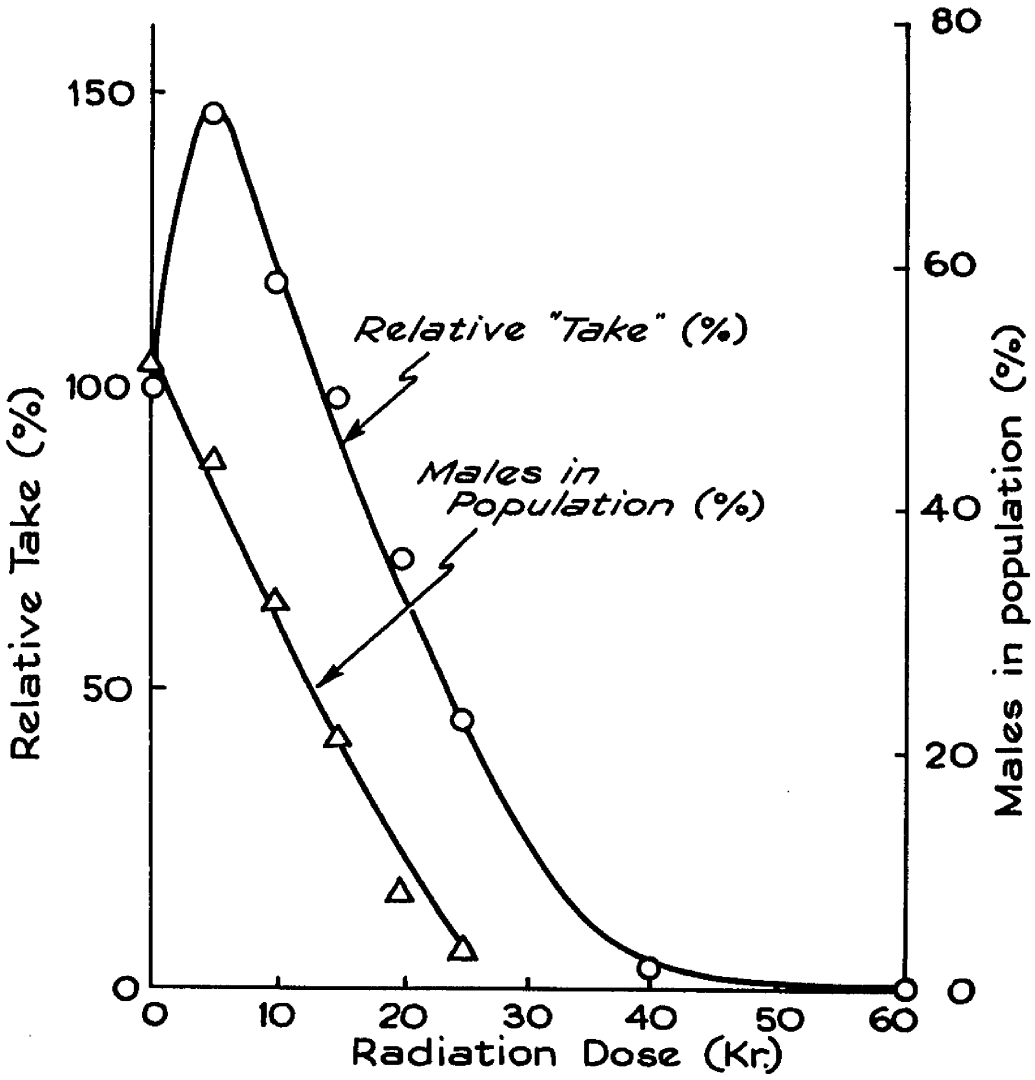


Figure 6 **The attenuating effects of X-rays over the range 5-60 kr.**

burden. Beyond 25 kr, no eggs were found in the faeces.

The sex-ratio of the worms, expressed in Fig. 6 as the percentage of males in the population, showed a consistent change from 0-25 kr. It appeared, therefore, to be a suitable means of assessing the radiation-attenuation of larvae. Beyond 25 kr, the sex-ratio was unreliable because of the small numbers of worms recovered in these groups.

Experiment 4

Attenuating effect of ^{60}Co gamma rays over the range 5-40 kr.

A similar experiment was carried out with larvae irradiated with ^{60}Co gamma rays. Batches of larvae were irradiated with 5, 10, 15, 20, 25 and 40 kr of ^{60}Co gamma rays at a dose-rate of 2000 r/min. Groups of rats were infected with 1000 larvae each, then killed 10 days later. The results are shown in Table 7. Although the results were slightly disappointing in that the mean control worm count was only 96, it is clear that the overall picture was the same as in the two previous experiments.

As expected, the sex-ratio, because of the less damaging gamma rays, changed less dramatically than in the previous experiment using X-rays. At 25 kr, 40% of the worms recovered were found to be males, compared with only 3% found at the corresponding X-ray dose.

TABLE 7

Number of Worms Recovered from Rats 10 Days after Infection with 1000 N. brasiliensis Larvae Irradiated with Different Doses of ^{60}Co Gamma-Rays

Control	5kr	10kr	15kr	20kr	25kr	40kr
61	470	160	208	204	112	57
117	355	181	313	285	61	155
190	328	343	246	14	0	2
49	107	368	116	169	124	17
59	339	159	292	179	159	8
57	188	244	213	235	180	53
89	146	253	387	192	123	29
134	425	89	252	226	178	3
86	236	340	309	262	155	94
122	194		235		76	115
Total 964	2788	2237	2571	1766	1108	533
Mean $96^{\pm}_{42}$	$279^{\pm}_{116}$	$249^{\pm}_{93}$	$257^{\pm}_{70}$	$196^{\pm}_{70}$	$111^{\pm}_{61}$	$53^{\pm}_{50}$
Relative Take%	291	259	268	204	116	55
Eggs/g. 1750	7150	6300	4100	4050	3250	-ve
Sex Ratio M:F 50:50	48:52	48:52	45:55	41:59	40:60	28:72

Experiment 5

Stimulating effect of small doses of radiation.

This experiment was carried out to cover the range 1-10 kr in smaller steps to obtain more information on this stimulating effect.

Batches of larvae were irradiated with 1, 3, 5, 10 and 40 kr of X-rays at a dose-rate of 700 r/min. After irradiation, each preparation of larvae was made up to a concentration of 1500/ml. for injection. Groups of 8 rats were each infected with the irradiated preparations and killed 10 days later. The results are shown in Table 8. It is clear that even at 1 kr there was slight increase in infectivity and it was not until a dose of 10 kr was given that the infectivity dropped below mean control worm burden.

TABLE 8

Number of Worms Recovered from Rats 10 Days after Infection with 1500 N. brasiliensis Larvae Irradiated with Different Doses of X-Rays

	Control	1kr	3kr	5kr	10kr	40kr
	414	707	792	704	560	33
	478	640	1104	727	398	10
	672	627	1074	664	475	37
	537	685	718	831	221	19
	744	551	1097	721	484	29
	22	548	869	627	547	32
	658	656	1026	704	529	21
	668	443	934	692	604	57
Total	4193	4857	7614	5670	3818	238
Mean	524 ± 232	607 ± 87	952 ± 147	709 ± 59	477 ± 121	30 ± 14
Relative Take %		116	182	135	91	5.7
Eggs/g.	11900	8100	27500	24800	11800	-ve
Sex Ratio M:F	48:52	42:58	42:58	40:60	32:68	2.98

Discussion

From the results of the three pilot experiments it is evident that the degree of attenuation of N. braziliensis larvae is directly related to the dose of irradiation and appears to be slightly higher for doses of 140 kV X-rays than for the same doses of ^{60}Co gamma rays.

The results of Experiment 1a show that out of 2000 non-irradiated larvae 31.5% developed to adults in the intestine of the host whereas only 1.1% and 0.1% of the larvae exposed to 80 kr of ^{60}Co and X-rays respectively so developed. In Experiment 1b, out of 1000 non-irradiated larvae 60.6% developed through to the adult stage, and 14.7% and 5.4% of the larvae exposed to 40 kr of ^{60}Co and X-rays respectively so developed. In both experiments, the number of worms present in the lungs were also counted but only in the 80 kr irradiated groups were any found. Both the gut worm burden and lung counts are in agreement with a similar experiment reported by Jennings et al (1963). They found that as the total dose of irradiation was increased from 40 kr to 160 kr the number of worms found in the intestine decreased and in

the lung increased. All the worms recovered from the intestine were sexually sterile and of stunted growth.

The results in Experiment 1c are interesting in that there appeared to be a slight increase in infectivity with the low doses of ^{60}Co. 52% and 68% of the larvae irradiated with 10 kr and 20 kr of ^{60}Co respectively developed in the gut compared with 50.5% in the control group. The faecal egg counts in the three groups were similar at day 10 which suggests that the radiation had no damaging effect on the larvae. This was not the case in the two X-irradiated groups. In these groups, approximately 35% of the irradiated larvae developed to adult worms.

In Experiment 2, a comparison was made of the attenuating effect of the two radiations over the range 10-30 kr. In this way, the RBE was determined. Reports in the literature on the RBE of X-rays and gamma rays on the infectivity of helminth larvae are few and conflicting. A comparison between the two radiations was first made by Gould, Gomberg, Villella and Hertz (1957) during their work on the radiation-attenuation of Trichinella spiralis. They compared the effectiveness of 250 kV X-rays and ^{60}Co gamma rays on the prevention of reproduction of

the worms, on the rate of elimination of the worms from the intestine of the host, and on the production of certain morphological changes. In each instance, they found that the dose of ^{60}Co necessary to produce a given effect was approximately three times the dose of X-rays. In 1958, Villella, Gould and Gomberg reported on the effects of 245 kV X-rays and ^{60}Co gamma rays on the ability of embryonated eggs of Ascaris lumbricoides suum to produce larval pulmonary infection in guinea pigs. From the results, it is not possible to calculate the effectiveness of the two radiations. However, they did find that within the range 3000-5000 r, fewer larvae were recovered from the lungs of guinea pigs fed with eggs exposed to X-rays than those fed with gamma irradiated eggs. In a later paper, Villella et al (1960) studied the effects of the same two radiations on the infectivity of the cysticercoids of Hymenolepsis diminuta in rats with similar results. It is interesting too that at the same time they reported on the effects of three different X-ray energies on the development of the cysts in rats. These results showed 120 kV and 245 kV to be slightly more damaging than 80 kV over the dose range where the tapeworms were

rendered sterile. So far, only one case has been reported where ^{60}Co gamma rays have been found to be more damaging than X-rays. Jovanovic, Sokolic, Movsesyan and Cuperlovic (1965) reported in the preparation of an irradiated vaccine against Dictyocaulus filaria, that a dose of 40 kr of ^{60}Co gamma rays appeared to produce a slightly higher degree of larval attenuation than the same dose of 200 kV X-rays. However, because of the smaller number of animals in each group and the very low worm counts in the irradiated groups, the result is somewhat in doubt.

In Experiment 2, where care was taken to ensure that the radiation treatment was carried out under identical conditions with both sources, ^{60}Co gamma rays were found to be slightly less effective than 140 kV X-rays. Where the comparison was made in the range where the attenuation was evident only as a reduction in total worm burden, the RBE, as shown in Table 5, was found to be about 0.7.

It is easy to see how in the present system difficulties of interpretation might arise where an attenuation experiment was carried out at one dose level only. From Fig. 5, it is

clear that an experiment carried out at 30kr would show considerable attenuation in the case of the X-ray dose with a 'take' reduced to less than one-third of that given by the normal larvae. On the other hand, the same dose of radiation from ^{60}Co would seemingly give no attenuation if judged by the worm burden alone. The reasons for apparently these anomalous results are twofold. First, the enhanced larval infectivity caused by the low doses of radiation and secondly, the two curves slightly out of phase due to the greater effectiveness of the X-rays.

This mis-interpretation would not arise if the alteration in sex-ratio, rather than the reduction in worm burden, were used as the index of attenuation. In Fig. 6, the sex-ratio of the worms, expressed as the percentage of males in the population, shows a linear change from 0-25 kr. This general trend is evident in all the later experiments.

In view of these results, the alteration in sex-ratio does seem to be an accurate test for judging radiation effects on parasitic worms, especially in the region where the dose is too small to bring about a significant reduction in the total

worm burden.

The alteration in sex-ratio with increased radiation dose was first observed by Kiek and Keith (1960) in their work with Oesophagostomum radiatum in cattle, and it has been found to occur with all species of helminths so far studied.

The stimulating effect of small doses of radiation on larval infectivity found in Experiment 2 and onwards has been found in other nematode systems. For example, Villella et al (1958) recovered an average of 10,128 Ascaris larvae from the lungs of guinea pigs after exposure of the infective eggs to a dose of 3000 rep (roentgen equivalent physical), whereas only 4348 larvae were recovered from those hosts infected with non-irradiated eggs. Other nematode systems in which this phenomenon has been demonstrated include Haemonchus contortus (Jarrett et al, 1959b), Trichostrongylus axei (Giordia and Bissell, 1960) and Ascaridia galli (Ruff, Hansen and Ostlind, 1965). Exactly how small doses of radiation enhance the larval infectivity is not known.

Summary

1. Experiments are described on the effects of ^{60}Co gamma rays and 140 kV X-rays on the infectivity and development of N. brasiliensis larvae.

2. The use of the alteration to the sex-ratio as a criterion for assessing radiation effects is discussed.

3. A stimulating effect of small doses of radiation on larval infectivity was observed.

4. In the dose range where the attenuation was manifested as a decrease in total worm burden the gamma rays had an RBE of 0.7 relative to the X-rays as unity.

Section 2

The effect of dose-rate on the radiation-attenuation of *N. brasiliensis* larvae.

Introduction

Studies on the effect of different dose-rates on the radiation-attenuation of larvae are important in connection with the preparation of vaccines. Clearly, a high output machine/low irradiation time would be advantageous. Until recently, most of the studies on larval irradiation had been carried out with X-ray machines. However, with high-energy sources such as the linear accelerator and γ-emitting sources now readily available, it is obvious that further studies on the radiation-attenuation of larvae should be made.

Mulligan (1964) suggested that the rate of delivery could be important as a factor involving the 'oxygen effect'. Almost all biological systems are more radiosensitive in the presence of oxygen. Therefore, on the assumption that during the time of irradiation the larvae use up oxygen in the suspension, then at a low dose-rate a large part of the radiation will be delivered when the suspension is low in oxygen. Thus, for a standard dose of radiation, a high dose-rate would be expected to be more effective than a low dose-rate.

Jennings *et al* (1963) have shown that the X-ray attenuation of *N. brasiliensis* larvae is independant of the dose-rate within

the range 235-735 r/min. This section describes further experiments on the effect of different dose-rates on the radiation-attenuation of N. brasiliensis.

Materials and Methods

Experimental animals

The rats used were bred and reared in the Physiology Department Animal House, University of Glasgow. They were female and of the Wistar albino strain. At the time of each experiment, they were 6-8 weeks of age and weighed 150-200 g.

Parasitological methods

Culture of N. brasiliensis larvae, infection of rats, faecal egg counts and post mortem procedures were all as described previously.

Radiation sources

The radiation sources used were as described previously. The X-ray machine was operated at 140 kV, 5 mA at a dose-rate of 170r/min. and 140 kV, 20 mA at a dose-rate of 700r/min. For γ-irradiation,

the dish was placed at various distances above the source depending on the dose-rate required.

In Experiment 2, one larval suspension was irradiated at a high dose-rate of 3000 r/m. The machine used was a linear accelerator (4.3 Mev Orthotron). The larval suspension, contained in a plastic bottle, was placed at a calculated distance from the window of the tube.

Experiment 1

The effect of 40kr X-rays delivered at different dose-rates on the radiation-attenuation of N. brasiliensis larvae.

As stated above, Jennings et al (1963) showed that with X-rays, the radiation-attenuation of N. brasiliensis larvae is independent of the dose-rate within the range 235-735r/min. In their experiment, the larvae were treated with X-ray doses of 40, 80, 120 and 180kr at dose-rates of 235, 495 and 735 r/min. The X-ray machine used was the same 'Siemens Stabilipan'.

Experiment 1 covered the same dose-rate range as above. Batches of larvae of 10,000/ml. were irradiated with 40kr X-rays at dose-rates of 170 and 700r/min., non-irradiated larvae were

used as controls. After irradiation, each sample was diluted to a suitable concentration and stored overnight at room temperature. The following day the larvae were counted, diluted to 1000/ml. and inoculated subcutaneously in rats. The number of rats in each group was 10. Ten days later, the rats were killed and the number of worms present in the small intestine counted. At the same time, the intestinal contents were examined for tapeworms, (Hymenolepsis nana) because it was then the author's view that the variability in worm burdens within each group was possibly linked with the presence or absence of tapeworms. The sex-ratio of the recovered worms and group egg counts were determined. The results of the experiment are shown in Table 9.

Results

It is evident from the control group, that the overall infectivity of the larvae was low. Only, 135 larvae succeeded in becoming established. Of these, an unusually high figure of 62% were males.

In the irradiated groups, there was no significant difference in 'take' between the groups but, as in the control group, the variation from the mean was very high. A higher proportion of males was found in the low dose-rate group.

TABLE 9

Number of Worms Recovered from Rats 10 Days after Infection with 1000 Larvae Irradiated with 40 kr X-Rays at Different Dose-rates

	Non Irradiated	170r/min	700r/min
	245 (1)	48	14
	277	36	4 (5)
	410	36	4 (4)
	192 (30)	10 (5)	13
	145	0 (19)	1 (4)
	33	7	3
	4	0	46 (2)
	35	3	11
	11 (2)	4	66
	2 (14)	0	58
Total	1354	144	220
Mean	135 ± 147	14 ± 18	22 ± 25
Relative Take %	100	10.4	16.3
Eggs/g.	680	-	-
Sex Ratio M:F	62:38	46:57	20:70

( ) No. of tapeworms.

The number of tapeworms recovered from small intestine of each rat is shown in brackets. A total of one-third of the rats were found to harbour H. nani but there was no evidence from these results that the tapeworms interfered with N. brasiliensis worm burdens.

Experiment 2.

The effect of 40kr ^{60}Co γ-rays delivered at different dose-rates and 'hard' X-rays on the radiation-attenuation of N.brasiliensis larvae.

The maximum dose output from the X-ray machine was 700 r/min. In order to carry out a wider range of dose-rates than in Experiment 1, two high energy sources (^{60}Co γ-rays and the linear accelerator machine) were used.

Batches of larvae at a concentration of 10,000/ml. were irradiated with 40kr ^{60}Coγ-rays at dose-rates of 600, 1200, 1500 and 2000r/min. In addition, a further batch was irradiated with 40kr X-rays from a 4.3 Mev linear accelerator at a dose-rate of 3000r/min. The post-irradiation treatment was the same as in Experiment 1. Six groups of 10 rats were infected with 1000 larvae each and the results are shown in Table 10.

Results

It is clear in this experiment that the γ-ray attenuation of N. brasiliensis was independent of the dose-rate within the range 600-2000r/min. The relative take in the 4 groups ranged from 8 to 11.8. In the X-irradiated group (3000r/min.) there was a slight

TABLE 10

Number of Worms Recovered from Rats 10 Days after Infection with 1000 Larvae Irradiated with 40 kr ^{60}Co γ-Rays at Different Dose-rates

	Non Irradiated	600 r/min	1200 r/min	1500 r/min	2000 r/min	3000* r/min
	613	22	141	4	38	6
	269	13	10	45	16	36
	13	42	57	7	2	102
	308	18	20	19	64	16
	203	17	2	26	47	1
	419	5	7	36	17	65
	633	64	15	3	32	33
	65	2	2	54	7	76
	249	6	88	35	19	55
	344	62	31	-	81	145
Total	3116	271	371	229	313	535
Mean	312±207	27±27	37±46	26±18	31±26	53±45
Relative Take %	100	8.6	11.8	8.0	10.0	17.0
Eggs/g.	17200	-ve	-ve	-ve	-ve	200
Sex Ratio M:F	46:54	71:29	72:28	77:23	83:17	68:32

* 4.3 Mev X-irradiation (Linear accelerator)

decrease in the degree of attenuation and some eggs were found in the faeces.

Discussion

The object of these two experiments was to determine the effect of different dose-rates on the radiation-attenuation of N. brasiliensis larvae. Because of the wide range of dose-rates covered, three different sources were employed.

In Experiment 1, the degree of attenuation with 140kV X-rays was found to be independent of the dose-rate within the range 170-700r/min. This was in agreement with the findings of Jennings et al (1963). In parallel work in this laboratory, Urquhart et al (1966) investigated the degree of protection in sheep vaccinated with Haemonchus contortus prepared at two different rates of irradiation. Batches of larvae were irradiated with 40kr X-rays from either the Newton-Victor G.X.10 machine (dose-rate 200r/min.) or the 'Siemens Stabilipan' (dose-rate 750r/min.). The results showed no apparent difference in the immunogenicity of the larvae. Prechazka and Tomanek (1968) reported the irradiation of Dictyocaulus viviparus larvae with 40kr X-rays at dose-rates of 250 and 500 r/min. No significance

difference was found in the results as shown by larval counts recovered from guinea-pig lungs. In a further experiment, they exposed larvae to the same dose of radiation using different filtrations, 25 mm Al and 0.5 mm Cu i.e. a difference in the quality of the radiation. The dose-rates then were 795 and 280r/min. respectively. Again, no difference was found in the degree of attenuation. Thus, from the results of Experiment 1, as well as from the data presented above, it would appear that within the range 170 to 800r/min., the dose-rate is not an important factor in the X-irradiation of parasites. In Experiment 2, batches of larvae were treated with 40kr ^{60}Co -rays over the higher range 600 to 2,000 r/min. Again, it appears from the results that the radiation-attenuation of the larvae was unaffected over that dose-rate range. It is noteworthy that the degree of radiation-attenuation was very high, much higher than one would expect using 40kr ^{60}Co -rays in the light of the studies reported in Section 1. Clearly a feature other than total dose is involved and one possible explanation is explored in the following Section.

In the final group in Experiment 2, the larvae were subjected to 'hard' X-rays from a 4.3 Mev linear accelerator. The maximum output of this machine was in the region of 10,000r/min. and this,

of course, would be a considerable advantage in vaccine production. The results, (Table 10) show the degree of attenuation to be similar to that of the γ - irradiated groups. However, the fact that some eggs were found in the faeces indicate that the effectiveness of the 'hard' X-rays is probably slightly less than ^{60}Co γ-rays.

Finally, from the results of Experiment 1, the possibility that tapeworms already present in the rat might affect the resulting worm burden was not evident; H. nana was found in rats that had high as well as low worm burdens.

Summary

1) Experiments are described on the effect of different dose-rates on the radiation-attenuation of H. brasiliensis larvae.

2) The degree of attenuation was found to be independent of the dose-rate within the range 170-700r/min. using 140kv X-rays and 600-2000r/min. using ^{60}Co γ-rays.

3) The effectiveness of 'hard' X-rays from a 4.3 Mev linear accelerator was found to be slightly less than ^{60}Co γ-rays.

Section 3

The Effect of Temperature on the radiation-attenuation of *N.brasiliensis* Larvae.

Introduction

The influence of temperature during irradiation on the radiation response of biological systems has been extensively studied by numerous workers. Most of this work has been involved with the study of the changes in radiosensitivity of unicellular organisms such as bacteria, yeast and mammalian cells over a wide range of temperature, i.e. - 250°C to +50°C. Lea (1946) has covered the early studies in this field while the more up-to-date work is included in a monograph by Bacq and Alexander (1963). To date little work has been done on helminths.

In the course of the experiments described in the previous two sections, the room temperature was routinely recorded prior to the start of all X-irradiation experiments. This was necessary because the radiation output from the X-ray machine is influenced by changes both in temperature and pressure. In the majority of experiments, the temperature recorded was in the narrow range 20°C to 22°C. However, occasionally, due to the on/off of the central heating or a window left open or shut, the room temperature dropped to as low as 18°C or reached as high as 25°C. In the main, this fluctuation was found only during the winter months, though in one experiment to be described in Section 4, due to an

exceptional warm summer's day the recorded room temperature was 27°C.

Thus, it was considered worthwhile to carry out a series of experiments varying the temperature over a range ± 10°C around normal room temperature and studying the effect on the radiation-attenuation of *N. brasiliensis* larvae.

Materials and Methods

Experimental animals

All experimental animals used were as described previously.

Parasitological methods

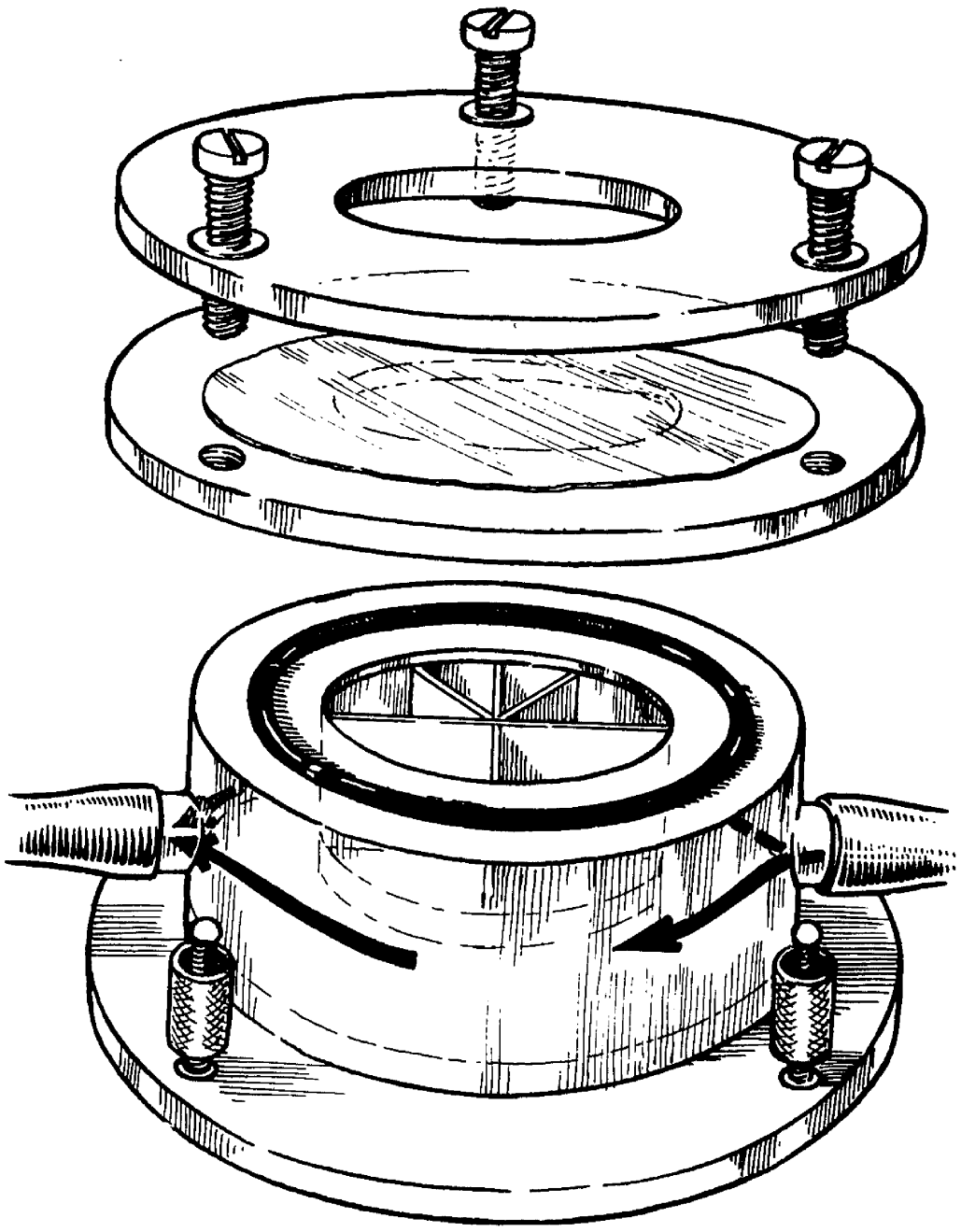
Culture of *N. brasiliensis* larvae, infection of rats, faecal egg counts and post mortem procedures were all as described previously.

Radiation sources

The X-ray machine and γ-ray machine were operated as described previously.

Irradiation vessel and calibration

The irradiation vessel used for the control of temperature is shown in Fig. 7. Basically, it consisted of a closed-in irradiation dish surrounded by a water jacket. The segmented dish was the same as that used in Sections 1 and 2. As before, each batch of larvae at a concentration of 10,000/ml. was pipetted into a segment to a height of 1 cm. The total volume of the dish was 13 ml. (2x 3.5 ml.; 2 x 2 ml.; 2 x 1 ml.). Vacant segments were filled with tap water. To maintain the dish suspension at a constant temperature water flowed continuously around the dish as indicated in the Figure 7. The water circulation was carried out by means of a pump situated in a constant temperature water bath (Shandon 'Circotherm') placed about one metre from the vessel. The water travelled to and fro along two glass rods fixed at either end by rubber tubing. To minimise the temperature difference between the bath and the irradiation dish, the glass rods were encased in a thermal insulating material. Irradiation treatment commenced 15 minutes after the larval suspension in the dish reached the required temperature. During irradiation at room temperature and above, the temperature variation never exceeded one degree. At



gure 7 **Irradiation dish with temperature controlled water jacket.**

temperatures well below the room temperature ice cubes were added periodically to the water bath and this caused a variation of around $2^{o}C$.

To maintain the temperature in the larval suspension, the dish was sealed off from the atmosphere during irradiation. The lid was built up from a cellophane sheet 0.003 inches thick sandwiched between two 0.25 inch thick pieces of Perspex, the centres of which were drilled out to a size equivalent to the diameter of the dish itself, i.e. 2 inches. To ensure a good seal, a narrow circular length of rubber tubing was placed between the lid and the dish face and the lid was clamped to the base of the vessel by three ORA screws coupled to 4BA screws with union nuts.

Calibration was carried out with the vessel placed on blocks of pressed wood in such a way that the top of the lid was level with the window of the X-ray tube. In this position, the dose-rate at the bottom of the irradiation dish was 470r/min.

Experiment 1 Preliminary experiment using X-rays to investigate the effect of temperature on the radiation-attenuation of N. brasiliensis larvae.

Results

Batches of N. brasiliensis larvae were exposed to 40kr of X-rays at different temperatures, non-irradiated larvae being used as controls at each stage. The dose-rate was 470r/min., thus the total time for each irradiation was just over 85 minutes. Temperature readings were taken every 15 minutes to ensure that each batch remained at constant temperature to within $\pm$ 0.5°C. During irradiation at 11°C, ice cubes were added to the water bath as required. Each control batch of larvae was suspended in a universal bottle in the water bath during the appropriate irradiation.

After irradiation, each sample was diluted to 3-4000/ml. and stored overnight at room temperature. The following day the suspensions were counted, diluted to 1000/ml. and inoculated subcutaneously into rats. There were 8 rats in each control group and 10 rats in the irradiated groups. Unfortunately, during this procedure, the high 32°C control larval suspension

was lost. The results are shown in Table 11.

In the control groups the mean worm burden in the 11°C group was less than the 20°C group but because of the scatter within each group, the difference was not statistically significant.

The results in the three irradiated groups indicate that the temperature during the irradiation treatment influenced the radiation-attenuation of the larvae to some extent. Although the worm burden in each group was low, an increase in temperature was matched by an increase in 'take' (The relative take for the 32°C irradiated group was based on the control group at 20°C).

Discussion

This preliminary experiment was carried out for two reasons. First, to see whether there was any indication that the temperature at the time of irradiation influenced the radiation-attenuation of the larvae and, secondly, to test the apparatus. The results above showed evidence of a slight enhancement of 'take' with increased temperature. However, it is obvious that for a more detailed study on this effect the total dose must be reduced as 40kr produces far too great an attenuation for comparisons to be

TABLE 11

Number of Worms Recovered from Rats 10 Days after Infection with 1000 Larvae Irradiated with 40kr X-Rays at Different Temperatures

	Control 11°C	X-ray 11°C	Control 20°C	X-ray 20°C	X-ray 32°C
	121	1	422	9	21
	341	2	289	7	34
	299	0	333	32	52
	233	0	256	1	3
	396	0	364	3	1
	84	0	454	3	19
	380	1	343	17	54
	457	1	545	3	47
	-	1	-	7	22
	-	0	-	8	13
Total	2311	6	3006	90	266
Mean	289±133	0.6	376±94	9±9.3	27±19
Relative Take % (Room Temp.)		0.21	100	2.4	7.2

mede. As regards the two control groups, there was no indication that the temperature influenced the infectivity of the larvae.

Experiment 2a, b The effect of temperature at the time of irradiation on the radiation-attenuation of N. brasiliensis larvae by X-rays.

These two experiments were carried out using a reduced total dose of X-irradiation. The range in temperature in both cases lay between 15°C and 30°C.

Results

Experiment 2a

Four batches of larvae were irradiated with a total dose of 30kr at different temperatures. Non-irradiated larvae were used as controls at each stage of the experiment. The time for each irradiation was just over 64 minutes.

Eight groups of 10 rats were infected with 1000 irradiated or non-irradiated larvae and killed 10 days later. The sex-ratio of the recovered adult worms in each group was assessed. In cases where the number of worms/group was large, a minimum of 200 were sexed. The results are shown in Table 12.

In this experiment it is apparent that the range of temperatures in no way influenced the infectivity of the larvae in the control groups. The worm burden was uniform

TABLE 12

Number of Worms Recovered from Rats 10 Days after Infection with 1000 N. brasiliensis Larvae Irradiated with 30 kr X-Rays at Different Temperatures

	Temp. (°C)	Mean Worm Burden	Relative Take %	Egg/g.	Sex Ratio (M: F)
Control Groups	15	330 ± 181		5350	54 : 46
	21	289 ± 172		11150	55 : 45
	25	287 ± 139		12200	54 : 46
	30	288 ± 116		3550	53 : 47
Irradiated Groups	15	88 ± 62	26.6	-ve	7 : 93
	21	61 ± 32	21.1	-ve	5 : 95
	25	254 ± 73	88.5	-ve	3 : 97
	30	320 ± 121	111.1	-ve	14 : 86

throughout and in each case the sex-ratio was similar, slightly more males than females recovered.

In the irradiated groups, the results were dramatic in that an increase in temperature of a few degrees above normal room temperature had a profound effect on the radiation-attenuation of the larvae. In the group in which irradiation had taken place at 21°C, the average worm burden was 61. In the group irradiated at 25°C, the average worm burden was increased to 254. In other words, increasing the temperature by 4°C brought about a fourfold increase in 'take'. It is interesting to note that despite this fourfold increase in 'take', the sex-ratio remained at what one would expect from 30kr X-irradiation, i.e. less than 5% males recovered (c.f. Section 1).

In the group irradiated at 30°C, there was a further increase in 'take'. The very interesting point here being that the recovered worm burden was greater than the corresponding control. In fact, the Relative Take was 111. The percentage of males recovered rose to 14%, a figure equivalent to 20kr in a total dose verus infectivity experiment carried out at room temperature.

In the only group irradiated below room temperature, the 'take' was similar to that of the 21°C group. However, the percentage of males recovered was slightly higher.

No eggs were recovered in the faeces of any of the irradiated groups.

Experiment 2b

The above experiment was repeated one month later. This time the four irradiation temperatures were the room temperature (21°C), 16°C, 24°C, and 29.5°C. The results are shown in Table 13.

It is immediately apparent from the control groups that the overall infectivity of the larvae was very much lower than in the previous experiment. This was a phenomenon that occurred every so often in this work. No explanation can be offered because in all experiments the culture and pre-irradiation treatment of the larvae remained unaltered.

Nevertheless, it is again clear that the temperature did not affect the infectivity of the larvae in the control groups. The worm burdens, egg counts and sex-ratios were all similar.

TABLE 13

Number of Worms Recovered from Rats 10 Days after Infection with 1000 N. brasiliensis Larvae Irradiated with 30 kr X-Rays at Different Temperatures

	Temp. (°C)	Mean Worm Burden	Relative Take %	Eggs/g.	Sex-Ratio (M : F)
Control Groups	16	127 ± 95		4400	45 : 55
	21	113 ± 92		3050	40 : 60
	24	157 ± 51		2450	46 : 54
	29.5	129 ± 70		1750	46 : 54
Irradiated Groups	16	0.2	0.16	-ve	2♂
	21	20 ± 13	17.7	-ve	11 : 89
	24	18 ± 11	11.5	-ve	13 : 87
	29.5	54 ± 43	41.9	-ve	21 : 79

In the irradiated groups, the worms burdens were also very low and the difference in take in the 4 groups over the range of temperature 13.5°C was significant only at the extreme temperatures, 16 and 29.5°C.

Only two worms were recovered from the ten rats in the 16°C group and both were surprisingly males. In the high temperature group the relative take was more than twice the room temperature group.

No eggs were recovered from the faeces of any of the irradiated groups.

Experiment 3 The effect of temperature at the time of irradiation on the radiation-attenuation of N. brasiliensis larvae with ^{60}Co γ -rays.

Radiation Procedure

The arrangement for controlling the temperature of the vessel was the same as described before. The vessel itself was placed above the radiation source, seated on a sheet of hardboard over the radiation outlet. The total distance from the source to the bottom of the dish was 39.6 cm.

Calibration was carried out with a phantom dish using the Baldwin-Farmer substandard dosemeter. The dose-rate was found to be 506r/min.

Results

The results are shown in Table 14. Batches of larvae were irradiated at three different temperatures, 16°C, 24°C and 29°C. No irradiation was carried out at the room temperature of 20°C. The inoculum was increased to 2000 larvae.

TABLE 14

Number of Worms Recovered from Rats 10 Days after Infection with 2000 N. brasiliensis Larvae Irradiated with 30 kr ^{60}Co γ-Rays at Different Temperatures

	Temp. (°C)	Mean Worm Burden	Relative Take %	Eggs/g.	Sex Ratio (M : F)
Control Groups	16	908 ± 337		15350	49: 51
	24	835 ± 446		15350	50 :50
	29	751 ± 437		13850	49: 51
Irradiated Groups	16	70 ± 63	7.7	150	25: 75
	24	253 ± 198	30.3	250	25: 75
	29	250 ± 131	33.3	50	24: 76

Because individual counts in the control groups were large, all numbers greater than 600 were approximated by a dilution counting method. Again, there was no significant difference between the worm burdens in the control groups.

In the irradiated groups, raising the temperature from four degrees below the room temperature ($20^{\circ}C$) to four degrees above increased the 'take' fourfold. Despite this large increase the egg count and sex-ratio in each group remained the same. In the final group, raising the temperature a further $5^{\circ}C$ did not alter the 'take'.

Experiment 4a, b The effect of temperature before and during the time of irradiation on the radiation-attenuation of N. brasiliensis larvae using X-rays.

It is clear from all the experiments above that a variation in temperature of a few degrees around the normal room temperature has a profound effect on the radiation-attenuation of N. brasiliensis larvae. On the other hand, it is possible that this variation in 'take' may be due to an oxygen effect. The larvae do use up oxygen and it has been shown by Wilson (1965) that 3rd stage N. brasiliensis larvae undergo a high rate of oxygen consumption immediately after they are subjected to a temperature rise. As the irradiation vessel was sealed off from the atmosphere, this increased respiratory rate will have caused an oxygen depletion of the medium. Furthermore, it is well-known that most biological systems are less radiosensitive at low oxygen concentrations and if this was the case here an increase in 'take' would be expected.

In the final two experiments in this Section, the larvae were subjected to different temperatures before and during the period of irradiation. It was assumed that the oxygen tension

in the suspension decreased uniformally over a period greater than the irradiation time when the larvae were subjected to a temperature rise.

Results

Experiment 4a

Three batches of larvae were irradiated with 30kr X-rays at different temperatures, non-irradiated larvae serving as controls at each stage of the experiment. Six groups of rats were each infected with 1500 larvae. The results are shown in Table 15.

The groups were divided as follows:-

Group A - stored at 19°C for 1 hour prior to irradiation, irradiation temperature 19°C

Group B - stored at 19°C for 1 hour prior to irradiation, irradiation temperature 27°C

Group C - stored at 27°C for 1 hour prior to irradiation, irradiation temperature 27°C.

TABLE 15

Number of Worms Recovered from Rats 10 Days after Infection with 1,500 N. brasiliensis Larvae Irradiated with 30 kr X-rays under different Temperature Conditions

	CONTROL A	CONTROL B	CONTROL C	X-RAY A	X-RAY B	X-RAY C
	217	236	632	210	214	392
	426	387	349	167	181	404
	426	468	202	185	319	380
	620	656	651	54	185	259
	764	598	269	121	231	332
	696	408	488	19	208	391
	328	500	407	103	256	341
	395	517	591	134	199	240
	693	245	489	132	171	377
	636	456	581	142	87	
TOTAL	5201	4471	4659	1267	2051	3116
MEAN	520 ± 187	447 ± 135	466 ± 155	127 ± 56	205 ± 60	346 ± 60
RELATIVE TAKE %				24.4	45.9	74.2
EGGS/g.	23,400	3,450	3,150	-ve	-ve	-ve
SEX RATIO M : F	48:52	50:50	50:50	17:83	14:86	11:89

A - Stored at 19°C, 1 hour prior to irradiation, irradiated at 19°C

B - Stored at 19°C, 1 hour prior to irradiation, irradiated at 27°C

C - Stored at 27°C, 1 hour prior to irradiation, irradiated at 27°C

As expected from the results of the previous experiments, no difference was found between the worm burdens in the controls.

In the irradiated groups, the experimental procedure in Groups A and B was essentially similar to that performed in Experiments 2a, b; one batch was irradiated at room temperature, in this case 19°C, and the other irradiated at a higher temperature. The results again were much the same. An increase of eight degrees in the irradiation temperature brought about a twofold increase in the Relative Take. In Group C, where the larvae were stored at 27°C for 1 hour prior to irradiation at 27°C, the Relative Take was increased further. Statistically, the significance between the means of the irradiated groups was as follows:- Group A/Group B, $P<0.001$; Group B/Group C, $P< 0.001$.

No eggs were found in the irradiated groups. In the control groups, for no apparent reason, the egg count in Group A was abnormally high.

Experiment 4b

Three batches of larvae were irradiated with 30kr X-rays at different temperatures, non-irradiated larvae serving as

TABLE 16

Number of Worms Recovered from Rats 10 Days after Infection with 1000 N. brasiliensis Larvae Irradiated with 30 kr X-rays under Different Temperature Conditions

	CONTROL A	CONTROL B	CONTROL C	X-RAY A	X-RAY B	X-RAY C
	158	118	156	55	39	183
	215	213	281	59	34	92
	322	294	172	111	38	111
	267	307	207	24	45	182
	194	295	235	50	20	139
	169	216	233	18	17	103
	142	163	278	25	132	189
	263	197	157	35	111	137
		205	154		20	
TOTAL	1730	2008	1873	377	456	1136
MEAN	216 ± 63	223 ± 64	208 ± 51	47 ± 30	51 ± 42	142 ± 39
RELATIVE TAKE %				21.8	22.9	68.3
EGGS/g.	6,800	7,500	5,400	-ve	-ve	50
SEX RATIO M : F	56:54	47:53	44:56	17:83	13:87	11:89

A - Stored at 19.8°C, 1 hour prior to irradiation, irradiated at 19.8°C

B - Stored at 19.8°C, 1 hour prior to irradiation, irradiated at 25°C

C - Stored at 25°C, 1 hour prior to irradiation, irradiated at 25°C

controls at each stage of the experiment. As before, the experiment was divided into 3 groups:-

Group A - stored at 19.8°C for 1 hour prior to irradiation, irradiation temperature 19.8°C

Group B - stored at 19.8°C for 1 hour prior to irradiation, irradiation temperature 25°C

Group C - stored at 25°C for 1 hour prior to irradiation, irradiation temperature 25°C.

Each rat was infected with 1000 larvae and the results are shown in Table 16. The Relative Take in the irradiated Group A was 21.8 which was similar to the corresponding group in Experiment 4a. In Group B, where the irradiation was carried out at 25°C, it was surprising to find no increase in 'take'. In view of the previous experiments, an increase in 'take' was to be expected. In Group C, where the larvae were subjected to 25°C for an hour before irradiation, a significant increase in 'take' was found. In addition, a few eggs were found in the faeces.

Discussion

As mentioned earlier, it is well-known from the literature that temperature during irradiation may influence the radiation

response of biological systems. Unfortunately, indications as to the precise effect of temperature are conflicting as it depends largely on the particular biological system under investigation and conditions of irradiation.

As far as nematodes are concerned, Alicata (1951) compared the radiation response of encysted Trichinella spiralis larvae in pork at room temperature (24°) and at freezing point. Using a radiation dose of 9000r, found previously to be the minimum dose required to produce 100% sterility in female worms, he observed no difference in the two temperatures.

In the experiments described in this Section, 3rd stage N. brasiliensis larvae were irradiated with either X-rays or ^{60}Co γ-rays at different temperatures ranging ± 10°C around the normal room temperature. In cases where either the radiation dose was too severe or the overall infectivity of the larvae as noted in the control groups was below average, the effect of temperature was not conclusive. But where experimental conditions were normal, it is clear from the results that a variation in temperature of a few degrees around the normal room temperature has a profound effect on the radiation - attenuation of N. brasiliensis larvae.

For example, in Experiment 2a, batches of larvae were irradiated with 30kr X-rays at the following temperatures, 15°C, 21°C, 25°C and 30°C. The Relative Take in these groups were 26.7, 21.1, 88.5 and 111.1 respectively. Thus, within the range of room temperatures normally recorded, i.e. 18°C to 25°C, there was a four-fold increase in 'take'. Despite this huge increase to a level just below that of the corresponding control group, it was surprising to find that the sex-ratio of the worms was virtually unaltered. In Experiment 3b, using γ-rays, an increase in temperature from four degrees below the room temperature (20°C) to four degrees above produced a fourfold increase in 'take'. However, it should be noted that a further increase in temperature made no difference to the resulting 'take'.

The use of irradiated helminth larvae for immunological purposes depends on a reproducible relationship between the radiation dose and the degree of attenuation (I.A.E.A. Report, 1964). In view of the results shown here with N. brasiliensis it would appear vital that fairly strict temperature control should be maintained during the irradiation treatment of larvae. In addition, in order to compare results obtained by different

workers, the temperature should be quoted along with the conditions of radiation exposure.

In Experiments 4a and 4b, the possibility of an oxygen effect was studied. The pre-treatment of the larvae was such that during the irradiation treatment, the oxygen tension in Group A Group B Group C. The results did indicate that something more than a temperature effect was involved. Clearly, before any real conclusions can be reached concerning an oxygen effect, further experiments will be necessary in which the larvae are irradiated in either an oxygen-saturated or an oxygen-depleted suspension. Either method would eliminate the difficulty of the suspension being aerobic at the beginning of irradiation with the oxygen content being depleted during the irradiation time. Such studies are covered in Section 4.

Finally, Miller (1968), working here at the Glasgow University Veterinary Hospital, has recently demonstrated a similar temperature effect on the radiation-attenuation of Ancylostoma caninum, a hookworm disease of dogs.

Summary

The radiation-attenuation of *M. brasiliensis* larvae has been investigated at various temperatures. It has been shown that there is a significant decrease in the radiosensitivity of the larvae with increasing temperature in the range 20-30°C. In view of this result, it would appear vital that fairly strict temperature control should be maintained during the irradiation treatment of larvae and that the temperature should be recorded in reported experimental work.

This decrease in radiosensitivity may well be due to an oxygen effect.

Section 4

The effect of oxygen on the radiation-attenuation of *N. brasiliensis* larvae.

Introduction

The results of the experiments described in the early part of Section 3 showed very clearly that the temperature of the larval solution during the irradiation treatment was a significant factor in the radiation-attenuation of *S. braziliensis* larvae. The final two experiments in Section 3 were designed to indicate either that the increase in take in groups irradiated at temperatures above normal was purely a temperature effect or, as has been shown in other biological systems, an oxygen effect. Obviously, at high temperatures, less oxygen would be present in the larval suspension due to both an increase in the larval metabolic rate and a decrease in the oxygen solubility in the water. Thus, further studies are described in this Section in which the oxygen content of the larval suspensions were measured during irradiation treatment at different temperatures.

The role of oxygen as potentiating agent for radiation effects has been the focus of considerable attention for many years and is distinguished by three features :-

1) The deleterious effects of X or γ- rays are reduced in the absence of oxygen or at reduced oxygen pressure.

2) The oxygen must be present during the irradiation treatment, exposure to oxygen before or after irradiation would not influence the radiosensitivity.

3) The oxygen effect is always linked with the type of radiation employed. The effect is greatest with the sparsely ionising radiations such as X, γ or B-rays, and least with α-radiations.

The oxygen effect was first demonstrated by Holthusen (1921) who showed that anoxic Ascaris eggs were more resistant to X-rays. Since then, the effect has been found to occur in virtually all biological systems. The influence of oxygen on the response of cells and tissues to ionising radiation has, of course, proved to be of great practical importance in the field of clinical radiotherapy (Gray, 1957; Goldfeder, 1958). In the majority of systems, the radiosensitivity is increased two or threefold by changing from anaerobic conditions to one containing a few percent of oxygen. In one case, Trowell (1953) reported the extreme difference in radiosensitivity of rat lymphocytes to be a factor of 11. However, the relationship really depends on the techniques used to measure it.

The nature of the influence of oxygen on radiosensitivity is still a subject of great debate. The simplest interpretation, according to Bacq and Alexander (1963) is that the radiation - modifying actions occur at the level of the primary lesion. That is, the presence of oxygen will increase the chances that a given amount of radiation energy will produce a chemical change that proves damaging to the cell. If the initial chemical lesion is caused by indirect action, they suggest the oxygen present will alter the nature of the attacking radicals to ones which may be more damaging. For example, the oxygen may combine with the radical (R) formed after exposure of organic substances to radiation with the result that production of this formed peroxide radical may lead to damage when the formation of R alone does not. A similar situation can be envisaged with direct action except that the radical R may be different.

In the irradiation of aqueous solutions, oxygen has two principal roles (Bacq and Alexander p. 138, 1963):

1) To react with H atoms to give an oxidising radical

$$H + O_2 \rightarrow HO_2$$

2) To add on to organic radicals to give a peroxy radical

$$R + O_2 \rightarrow RO_2$$

Here again, both these reactions may be responsible for the oxygen effect. Irradiation of water containing dissolved oxygen by X - or γ-rays leads to the formation of relatively large amounts of hydrogen peroxide. Initially, it was thought that the enhancing effect of oxygen could be accounted for by the HO_2 peroxide radicals affecting the primary damage but now this view is no longer held.

The amount of dissolved oxygen in larval suspensions is determined both by the temperature of the suspension and the uptake by the larvae. Using data from published tables (Handbook of Chemistry and Physics, 33rd Edition, p 1480-1481, 1951-52), the amount of dissolved oxygen from air in water at the relevant temperatures was calculated as follows:

Temperature (°C)	O_2 Conc. (ml./l)
19	6.48
20	6.36
28	5.46

Total pressure 760 mm.

The respiratory metabolism of N. brasiliensis has been studied by several workers (Rogers, 1948; Schwabe, 1957; Wilson, 1965). As nematodes possess no circulatory system, the oxygen must pass through the body wall to the internal organs by diffusion through the pseudocoelemic fluid. This process is helped considerably by locomotory movements of the parasite which bring about movements of the fluid (Lee, 1965). Wilson (1965) showed that the uptake of oxygen was profoundly influenced by change of temperature, a period of two to three hours being necessary before the response to a rise in temperature subsided. In his studies, the larvae were initially stored at 25°C. Measurement of oxygen uptake (Q_{O_2} - µl. of oxygen taken up per mg. of carbohydrate-free dry weight) was made at 25°, 30°C and above by Warburg's direct method. Over a period of one hour, which is equivalent to the irradiation time in the experiments to be described the uptake in the 30°C batch was about twice the rate of the 25°C batch (Q_{O_2} at 30°C = 4.04; at 25°C = 2.36). From these above results it would appear that the oxygen concentration of a larval suspension over a range of temperatures depended more on the oxygen uptake by the larvae than the simple temperature effect on the oxygen solubility.

The section, therefore, describes a study made of the effect of oxygen on the radiation-attenuation of N. brasiliensis larvae.

Materials and Methods

Experimental animals

All experimental animals used were as described previously.

Parasitological methods

Culture of N. brasiliensis larvae, infection of rats, faecal egg counts and post mortem procedures were all as described previously.

Radiation source and vessel

The X-ray machine, 'Siemens Stabilipan' was operated under the conditions described previously.

The irradiation vessel was the same as described in Section 3. The terms 'open' or 'sealed in' when applied to the vessel refers simply to whether or not the lid was used.

Gas mixtures

The gases, oxygen and oxygen-free nitrogen (specially prepared) were obtained from the British Oxygen Company Ltd.

Measurement of dissolved oxygen concentration

Fox and Wingfield (1938) described a method based on the well-known Winkler iodimetric technique,(1888) for the determination of oxygen dissolved in a small volume of water. The principle is as follows. An aqueous solution of manganous chloride is added to the water sample, followed by a solution of sodium hydroxide containing potassium iodide. The following reaction first takes place:

$$2MnCl_2 + 4NaOH \longrightarrow 4NaCl + 2Mn(OH)_2$$

Because of the oxygen dissolved in the water sample, part of this manganous hydroxide is converted into manganic hydroxide as shown:

$$2Mn(OH)_2 + H_2O + O \longrightarrow 2Mn(OH)_3$$

Finally the solution is acidified by adding a few drops of o-phosphoric acid:

$$2Mn(OH)_3 + 2KI + 6HCl \longrightarrow 2MnCl_2 + 2KCl + 6H_2O + I_2$$

It is clear from these equations that each atom of oxygen in the water sample finally liberates one molecule of iodine. The iodine is titrated against a standardised sodium thiosulphate solution using a dilute starch solution as an indicator and the amount of oxygen in the water sample then determined.

The apparatus used for the procedure is fully described in the above paper. It consisted of a glass syringe pipette and an ordinary micrometer syringe burette. The pipette was so designed that contamination of the water sample with atmospheric air was avoided. According to Fox and Wingfield, the accuracy of the method is within 2%, even at low oxygen concentrations. Analysis made in this laboratory of the oxygen content of ordinary tap water at different temperatures were found to be within this order of accuracy when compared with the data given in the Handbook of Chemistry and Physics.

In the studies to be described in this Section, the sample volume taken for analysis was about 1.5 ml. In all cases, this volume was removed from either of the two large segments (3.5 ml.) of the irradiation dish. The utmost care was taken to avoid disturbing the larvae settled in the bottom of the dish as this would undoubtedly affect the accuracy of the results. After

removal of the sample, the segment was refilled with water to the same level as previous and at the end of the irradiation run, that batch of larvae was discarded.

Experiment 1a, b

Determination of the change of oxygen concentration in larval suspensions irradiated at different temperatures

In Section 3, the experiments were carried out using a sealed-in irradiation vessel especially designed for temperature control, whereas in the two previous sections and, in fact, in most reported radiation-attenuation studies on larvae, an open dish has been employed. With either type of vessel, it is highly unlikely that the atmospheric oxygen would affect the oxygen concentration of the larval suspensions during the period of irradiation unless, of course, the surface of the suspension was in some way broken. To show whether, in fact, this really was the case, the irradiation of the larvae was carried out with the irradiation dish either open or closed to the atmosphere and the amount of dissolved oxygen in the suspensions was determined both prior to and at the end of each treatment. The experimental procedure in both experiments was as follows:

Group A - irradiated at room temperature (19°C in Expt. 1a, 20°C in Expt. 1b) in dish sealed from the atmosphere.

Group B - irradiated at 28°C in dish open to the atmosphere.

Group C - irradiated at 28°C in dish sealed from the atmosphere. The total irradiation dose was 30kr at a dose-rate of 470r/min. (sealed dish) and 680 r/min. (open dish). Once the larval suspension reached the appropriate temperature, 15 minutes elapsed before the irradiation was begun. During the irradiation, temperature readings were taken every 15 minutes and in all three groups, the recorded temperature was constant to within 1°C. Non-irradiated larvae were used as controls at each temperature. After irradiation, each batch was diluted to a suitable concentration and stored overnight at room temperature. The following day, groups of rats were infected with 1000 larvae.

Results

Experiment 1a

The results of the experiment, including the oxygen measurements, are shown in Table 17. It is clear from the worm burdens of the irradiated groups that the radiation-attenuation of the larvae was significantly influenced by the temperature during irradiation; the takes in both Groups B and C were four times that of Group A. Furthermore, the close similarity between the results of Groups B and C suggest that the attenuation

TABLE 17

Number of Worms Recovered from Rats 10 Days after Infection with 1000 N. brasiliensis Larvae Irradiated with 30 kr X-Rays under different Temperature Conditions

6	Control A	Control B	X-ray A	X-ray B	X-ray C
	649	315	19	144	223
	419	532	43	140	147
	283	386	18	201	137
	691	406	36	86	273
	298	612	50	221	179
	339	607	53	120	180
	488	507	25	196	252
	489	691	33	255	185
	422	564	26	217	266
	480	566	117	224	146
Total	4558	5186	420	1812	1988
Mean	456 ± 135	519 ± 119	42 ± 29	181 ± 55	199 ± 50
Relative Take %			9.2	34.9	38.3
Eggs/g.	14,000	16,650	-ve	1100	-ve
Sex Ratio M : F	45:55	47:53	10:90	12:88	8:92

A - irradiated at 19°C sealed dish
O_2 dissolved at beginning = 4.46ml/l.
O_2 dissolved at end = 4.28ml/l.

B - Irradiated at 28°C open dish
O_2 dissolved at beginning = 2.97ml/l.
O_2 dissolved at end = 2.78ml/l.

C - irradiated at 28°C sealed dish
O_2 dissolved at beginning = 2.85ml/l.
O_2 dissolved at end = 2.46ml/l.

of larvae is not altered when a closed irradiation dish is used instead of the commonly used 'open' dish.

The amount of oxygen dissolved in the larval suspension held at room temperature over the period of irradiation was found to fall only slightly, in that time the difference being 0.18 ml./l. In Group B, larvae irradiated at 28°C in the open-type dish, the difference was exactly the same, but in Group C, where the dish was closed, the difference was almost double. This difference between Groups B and C presumably was partly due to the difference in the time of exposure at 28°C because of the different dose-rates. The irradiation times for Groups B and C were 43 and 64 minutes respectively.

Experiment 1b

The above experiment was repeated and the results are shown in Table 18. Because of an exceptional hot summer's day, in which the room temperature was 27°C, this experiment was carried out in reverse. Ice cubes were added periodically to the water bath to maintain the temperature of the suspension at the normal room temperature of 20°C.

Although the overall radiation-attenuation of the larvae (compare Group A in both experiments) is low by normal standards,

TABLE 18

Number of Worms Recovered from Rats 10 Days after Infection with 1000 N. brasiliensis Larvae Irradiated with 30 kr X-Rays under different Temperature Conditions

	Control A	Control B	X-ray A	X-ray B	X-ray C
	515	591	202	326	456
	581	200	239	316	312
	541	533	221	412	504
	267	634	157	424	289
	340	418	169	422	191
	251	397	142	451	128
	531	476	209	427	386
	680	265	152	410	334
	451	545	156	395	377
	426	713		244	376
Total	4853	4772	1647	3827	3353
Mean	458 ± 138	477 ± 155	183 ± 35	383 ± 64	335 ± 114
Relative Take %			40	80.3	75
Eggs/g.	37,600	17,600	600	6,700	6,600
Sex Ratio M : F	45:55	47:53	17:83	21:79	20:80

A – irradiated at 20°C sealed dish — O_2 dissolved at beginning = 4.58 ml./l.; O_2 dissolved at end = 4.48 ml./l.

B – irradiated at 28°C open dish — O_2 dissolved at beginning = 3.41 ml./l.; O_2 dissolved at end = 3.17 ml./l.

C – irradiated at 28°C sealed dish — O_2 dissolved at beginning = 3.28 ml./l.; O_2 dissolved at end = 2.87 ml./l.

the effect of temperature was again evident; the takes in Groups B and C were twice that of Group A. As to why the radiosensitivity of the larvae in the two experiments was so different when, at the same time, the controls were equal, it is not known. As far as the author is aware, the physical conditions in each experiment, i.e. the distance from source to larvae, were identical.

The initial oxygen concentration measurements were higher, particularly in Groups B and C, than in the corresponding groups in Experiment 1a, yet the oxygen depletions over the irradiation period were practically the same.

Experiment 2

A study of the effect of oxygen on the radiation-attenuation of N. brasiliensis larvae at different temperatures

The data from Experiments 1 (a, b) showed a marked decrease in radiosensitivity in the two groups, B and C, irradiated at above normal temperature. The measured oxygen concentration in these groups was considerably lower than that of the corresponding controls. If the increase in 'take' is due solely to the lower oxygen content of these suspensions then it should be possible to nullify this enhancement by increasing the O_2 content of the high temperature suspension to the region found in normal temperature suspensions.

The experimental procedure was as follows:

Group A - irradiated at room temperature (20°C)

Group B - irradiated at 28°C

Group C - irradiated at 28°C under aerobic conditions

The total dose was 30kr (dose-rate 470 r/min.) and each suspension was irradiated with the dish sealed from the atmosphere: To achieve an aerobic state, oxygen gas was bubbled through the

suspension for a few minutes prior to the irradiation treatment. During the irradiation itself, oxygen was passed through the dish but over the suspension by means of two hypodermic syringes fixed on the underside of the lid. Measurements of the oxygen concentration of the suspension was made only at the end of each run. Two non-irradiated batches of larvae were used as temperature controls. Groups of rats, mainly 9/group, were infected with 1000 larvae and the results are shown in Table 19.

Results

It is clear from the worm burdens that addition of oxygen in Group C reduced the effect of temperature considerably. Comparing Groups A and B, irradiation at 8°C above room temperature resulted in a threefold increase in take. Yet, in Group C the take was reduced to that of Group A. Thus, from these results, it would seem that the important factor in the radiation-attenuation of the larvae was not the temperature at the time of irradiation but the oxygen concentration of the suspension.

The oxygen concentration measurements of the larval suspensions in Groups A and B were similar to those in the previous experiments, whereas the addition of oxygen in Group C increased

TABLE 19

Number of Worms Recovered from Rats 10 Days after Infection with 1000 N. brasiliensis Larvae Irradiated with 30 kr X-Rays under different temperature and gas conditions.

	Controls		Irradiated Groups		
	20°C	28°C	20°C	28°C	28°C (Oxy)
	110	516	79	192	102
	513	520	69	290	13
	333	445	47	302	112
	327	388	137	213	119
	525	498	99	298	169
	564	542	45	192	64
	471	462	124	354	127
	327	435	121	270	157
	367	498	96	376	138
	456				
Total	3975	4304	817	2487	1060
Mean	397± 135	477± 49	91± 34	276± 67	106± 48
Relative Take %			23	58	22
Eggs/g.	3500	9000	-ve	-ve	-ve
Sex Ratio M:F	47:53	47:53	9:91	12:88	2:98

Oxygen Concentrations at end of irradiation treatment :

20°C - 4.371 ml./l.
28°C - 2.604 ml./l.
28°C (Oxy) - 5.208 ml./l.

the dissolved oxygen concentration to a level somewhat higher than the room temperature readings.

Experiment 3

A further study on the effect of oxygen at the time of irradiation on the radiation-attenuation of N. brasiliensis larvae.

In time sequence, this experiment is not a follow-on of the previous one as it was carried out prior to the studies made on the effect of temperature (Section 3) and before the experiments on the oxygen effect were seriously considered. Nevertheless, it is included because of its obvious connection with the previous experiment, i.e. the addition of oxygen during the irradiation.

The experimental procedure was as follows. Two batches of larvae were irradiated with 40kr X-rays at a dose-rate of 700 r/min. in the small segmented irradiation dish as used in Section 1. During the irradiation, oxygen gas was bubbled through the suspensions every 10 minutes for 30 seconds. The gas flow-rate was sufficiently low to avoid frothing of the suspension. As the total irradiation time was just over 58 minutes, five such bursts were given. The oxygen concentrations of the suspensions were not measured. A single batch of non-irradiated larvae was used as controls.

TABLE 20.

Number of Worms Recovered from 10 Rats 10 Days after Infection with 1000 N. brasiliensis Larvae Irradiated with 40 kr X-Rays under different gas conditions.

	Non Irradiated	700 R/min.	700 r/min. + O_2*
	3	42	7
	104	66	9
	169	58	0
	75	21	6
	75	40	15
	302	2	12
	94	5	3
	346	15	3
	296	98	16
	272	24	13
Total	1736	371	84
Mean	174 ± 121	37 ± 30	8.4 ± 5.5
Relative Take %	100	21.2	4.8

* O_2 passed every 10 minutes for 30 seconds.

Results

The results are shown in Table 20. The sex-ratio of the recovered worms and egg counts were not measured. Despite the low infectivity, it can be seen that the addition of oxygen during the irradiation resulted in a fivefold decrease in take.

Experiment 4

A study of the radiation-attenuation of N. brasiliensis larvae under normal and anaerobic conditions

In Experiment 2 it was shown that the addition of oxygen during irradiation at several degrees above normal temperature prevented any change of radiosensitivity with respect to temperature. By the same token, if an oxygen effect is involved, a reduction in the oxygen concentration during irradiation at room temperature should induce a decrease in radiosensitivity.

The experimental procedure was as follows:

Group A - irradiated at room temperature (21°C) under normal conditions.

Group B - irradiated at room temperature (21°C) under anaerobic conditions.

The total dose was 30 kr (dose-rate 470 r/min.). For anaerobic conditions, oxygen-free nitrogen gas was passed continuously throughout the irradiation treatment in the same manner as described in Experiment 2. Oxygen concentrations of the suspensions were determined only at the end of the irradiation treatment. The results are shown in Table 21.

TABLE 21.

Number of Worms Recovered from Rats 10 Days after Infection with 1000 N. brasiliensis Larvae Irradiated with 30 kr X-Rays under different gas conditions.

	Control	X-ray A	X-ray B
	229	67	168
	215	82	90
	160	14	108
	138	59	103
	233	52	60
	194	30	95
	351	13	179
	198	67	144
	206	69	166
		52	109
Total	1924	505	1222
Mean	214 ± 60	50.5 ± 26	122 ± 46
Relative Take %		23.6	57
Eggs/g.	4500	-ve	-ve
Sex Ratio M:F	47:53	10:90	14:86

Room Temperature = 21°C.

A - Oxygen Conc. = 3.92 ml./l.

B - Oxygen Conc. = 0.47 ml./l.

Results

Together with the information from Experiment 2, it is clear that the oxygen concentration of the larval suspension during irradiation was a vital factor in the radiation-attenuation process. In this experiment, the addition of nitrogen gas reduced the final oxygen concentrations from 3.92 to 0.47 ml./l. and, as a result, the take was increased from 23.6 to 57.

Discussion

Studies, in Section 3, on the irradiation of N. brasiliensis larvae at different temperatures showed a marked increase of radiosensitivity with increasing temperature. In Experiments 4 (a, b) of Section 3 a further decrease in radiosensitivity was observed in these groups in which larval suspensions were maintained at these high temperatures both before and during the irradiation treatment. From these observations it was considered such effects were probably due to a depletion in the oxygen content of the suspensions during irradiation. In this Section, further experiments were designed to examine the role of oxygen during irradiation at different temperatures.

The amount of dissolved oxygen in ordinary tap water at room temperature (19°C) and 28°C is 6.48 and 5.46 ml./l. respectively. In Experiments 1 (a, b) the amount of dissolved oxygen in larval suspensions held at similar temperatures was found to be in the ranges 4.58/4.28 and 3.41/2.87 ml./l. respectively. The difference in these two sets of figures is almost certainly due entirely to the metabolic activity of the larvae. In both experiments a dramatic decrease of radiosensitivity with increased temperature was observed. As the later experiments showed that conclusively only the oxygen concentration was involved, it is possible from the above figures to indicate approximately the threshold below which the $^{O}2$ effect is likely to occur. Therefore, as a rough guide, the oxygen content of the larval suspension must drop below 4 ml./l for the oxygen effect to occur.

It was shown by Jennings et al (1963) that the radiation-attenuation of N. brasiliensis larvae was influenced to some extent by the larval concentration during irradiation; the radiation-attenuation of the larvae irradiated at a concentration of 1500/ml. was greater than in the higher concentrations, i.e. 50,000/ml. They suggested that this was due to an oxygen effect as high concentrations would produce anaerobiosis more quickly.

It was therefore thought worthwhile to measure the oxygen concentration in different larval concentrations and note if they are in agreement with the data in Experiments 1 (a, b). Two suspensions of larvae were prepared at different concentrations, 10,000 and 50,000/ml and left for one hour in an irradiation dish at room temperature (20°C). The oxygen concentration measurements at the end of this period were 4.37 and 3.35 ml./l. respectively.

The argument as to whether the decrease in radiosensitivity at high temperatures was due either to an oxygen effect or temperatures or both was resolved in the results of Experiments 2 and 4. In Experiment 2, irradiation at 28°C under normal conditions resulted in an increase in take from 23% at room temperature to 58% but under aerobic conditions the take was reduced to the former level. Conversely, in Experiment 4, irradiation at room temperature under anaerobic conditions resulted in an increase in take from 23.6% under normal conditions to 57%. Clearly, the radiosensitivity is related only to the oxygen concentration of the suspension and not the temperature during irradiation.

In Experiments 1a, b, both an open and closed irradiation vessel was used and the oxygen concentration measurements indicated a slightly greater drop in the latter. Taking into

account the different irradiation times, it would appear unlikely that the atmospheric oxygen influenced the oxygen concentration of the larval suspensions.

Summary

1. Experiments are described on the effect of oxygen on the radiation-attenuation of N. brasiliensis larvae.

2. The degree of attenuation was found to be dependent on the oxygen concentration of the larval suspension during irradiation.

3. The radiation-attenuation of larvae irradiated in either an open or closed irradiation was compared.

Section 5

Effect of long-term storage on the infectivity and radiation-attenuation of N. brasiliensis larvae

Introduction

Several reports in the literature show that 3rd stage infective nematode larvae can be stored for a reasonable length of time with no reduction in motility or infectivity. With N. brasiliensis, Haley and Clifford (1958, 1960) showed no loss in infectivity up to a larval age of 4 weeks.

The maintenance of stocks of N. brasiliensis require repeated cultures through successive numbers of rats. This method is very time consuming. As the majority of cultures result in the harvest of larvae far greater than is required at that time, it would be a decided advantage if larvae, not immediately required, were stored away for later use. This, of course, would be true only if the infectivity was unimpaired and also, if the system was part of a radiation vaccine production the radiosensitivity was unaltered. As far as the author is aware no work has been done on the effect of larval age on radiosensitivity.

Experiments are described in this Section on the effect of long-term storage on both larval infectivity and radiosensitivity. One of the difficulties in this type of study was the problem of overcoming the presense of microbial contamination. The preparation

of a clean larval suspension was therefore a laborius procedure, involving many washes by centrifugation.

Haley (1962) and Wilson and Dick (1964) reported that water was unsuitable as a storage medium and that a salt solution of some kind was necessary to maintain larvae in good condition. Two salt solutions were selected for this study, one, Tris buffer solution, used frequently in cell culture work and, two, a modified quarter strength mammal Ringer-Locke solution (¼ RLA +) used successfully by Wilson and Dick (1964) as a storage medium. Bacterial and fungicidal antibiotics were added to control contamination.

This section, therefore, describes a study made of the effect of long-storage on the infectivity and radiation-attenuation of N. brasiliensis larvae.

Materials and methods

Experimental animals

All experimental animals used were female and of the Wistar albino strain. They were 6-10 weeks of age and weighed 150-200 gms.

Parasitological methods

Cultures of N. brasiliensis larvae, infection of rats, faecal egg counts and post-mortem procedures were all as described previously.

The utmost care was taken in the preparation of a clean larval suspension for storage in one or other of the salt solutions described below. The suspension was first washed several times in water and then further cleaned by successive centrifugations. During centrifugation, the larvae were transferred to the storage medium. 20ml. aliquots of the larval suspension at a concentration of 1,000/ml. were stored in glass bottles (8 oz medical flat) tightly sealed with screw caps, the linings of which were of non-toxic rubber. The bottles were stored at room temperature.

Storage Medium

(a) Tris Buffer solution

Tris (tris (hydroxy-methyl) amino methane) organic buffer solution is commonly used as a diluent for virus preparations and for washing cell cultures. The solution, made up in the Institute of Virology, University of Glasgow, contained the following per litre:

sodium chloride, 8.0g. potassium chloride, 0.38g. di-sodium hydrogen phosphate, 0.1g. glucose, 1.0g tris (hydroxyl-methyl) amino methane, 3.0g. phenol red (1%), 1.5ml. penicillin, 50,000 units, streptomycin, 0.1g. distilled water, 950 ml. To this was added 0.02 gm. "Actidione", a fungicidal antibiotic. The pH of the solution was adjusted by adding N/1 hydrochloric acid.

(b) $\frac{1}{2}$ RLA +

This solution, described by Wilson and Dick (1964) as being suitable for prolonged storage of N. brasiliensis larvae, contained the following per litre: sodium chloride, 2.3g. potassium chloride, 0.01g., calcium chloride, 0.044g. sodium bicarbonate, 0.04g. streptomycin sulphate, 0.025g. benzl penicillin (Na salt), 0.025g. and "Actidione", 0.01g.

Radiation source and vessel

The X-ray machine 'Siemens Stabilipan' was operated at 140kV, 20mA. The dose-rate was approximately 700 r/min.

The irradiation vessel used was the same as in Sections 1 and 2, that is, the small open dish of volume, 13 ml.

Experiment 1

Effect of long-term storage in Tris buffer solution on the infectivity and radiosensitivity of N. brasiliensis larvae

A pool of 900,000 N. brasiliensis larvae was harvested in 50 ml. of water and cleaned by successive centrifugation. Approximately 200,000 were withdrawn and prepared for use in Experiment 2 (Section 3), the results of which were taken as zero time of storage in this experiment. During centrifugation, the remainder were transferred to the Tris buffer solution. The pH of the resulting suspension was measured as 8.3. Finally, the suspension was diluted to 1,000/ml. and stored in 20 ml. aliquots in sterilised glass bottles at room temperature. By dividing the pool of larvae into small volumes, in this way, it was hoped that contamination, if present, would not be sufficiently widespread to cause a premature end to the experiment.

One week later, two bottles were removed and examined for larval viability and contamination. The larvae were then transferred back into water, washed three times and concentrated to 10,000/ml. for irradiation treatment. The X-ray irradiation dose was 30 kr. delivered at 700r/min. The following day, 2 groups of 10 rats

were infected each with 1,000 larvae. This procedure was repeated each week until the pool of stored larvae was exhausted. The results are shown in Table 22.

The experiment was terminated after eight weeks. Contamination was found in 2 bottles after 1 week's storage, a further 4 after 2 weeks and progressively more in most of the remaining weeks. Most of the contamination present was of a bacterial nature and only a very few bottles contained fungal growth. There was no apparent change in the viability of the larvae until after the fourth week. From then on the percentage of dead larvae/bottle rose from 2% to over 50% by the eighth week. The $_{p}H$ value of the medium after 4 weeks had dropped to 7.4. In an attempt to restore the value to above 8, the medium in all remaining bottles was replaced with fresh Tris buffer solution. This had little effect, as by the 6th week, the $_{p}H$ value had dropped to below 7.

In the control groups, the initial mean larval infectivity was 397 representing 40% of the infected dose. This figure is consistent with the results of the majority of the experiment reported so far and can thus be termed a normal infection. The mean infectivity of the larvae stored for 1 week was 160, 16% of the injected dose. Thereafter the figure fell to around 8 - 12%

for storage over Weeks' 2 to 5 and finally to 3 - 5% for Weeks' 6 to 8. The sex ratio remained more or less the same throughout this period but no eggs were recovered beyond Week 4.

The mean larval infectivity in the irradiated groups dropped dramatically from 91 initially to 4 after 1 week's storage. Thereafter, apart from the Week 4 group, the worm burden was zero.

TABLE 22.

Number of Worms Recovered from Rats 10 Days after Infection with 1000 N. brasiliensis Larvae Stored in Tris Buffer Solution then Irradiated with 30 kr X-Rays.

	Week	Mean Worm Burden.	Eggs/g.	Sex-ratio (M:F)
Control Groups.	0	397 ± 135	3500	47:53
	1	60 ± 75	2050	47:53
	2	82 ± 68	600	46:52
	3	126 ± 79	750	47:53
	4	78 ± 68	300	44:56
	5	71 ± 46	-ve	46:52
	6	30 ± 16	-ve	47:53
	7	55 ± 38	-ve	45:55
	8	41 ± 21	-ve	46:54
Irradiated Groups.	0	91 ± 34	-ve	9:91
	1	4 ± 2.5	-ve	9:91
	2-3	0	-ve	-
	4	2.4 ± 3.3	-ve	0:100
	5-8	0	-ve	-

Experiment 2

Effect of long-term storage in ¼RLA+ solution on the infectivity and radiosensitivity of N. brasiliensis larvae.

The procedure for storage in the ¼RLA+ solution was exactly as described in Experiment 1 except that each bottle contained 25 ml. at 1,000 larvae/ml. The experimental plan was slightly different in that the first irradiation treatment was carried out 2 days after storage and, thereafter, at 7 or 14 day intervals. Also, the injected dose was increased to 2000 larvae/rat. The results are shown in Table 23.

The mean larval infectivity in the initial control group (stored for 2 days) was 1157, 58% of the injected dose compared to 40% in Experiment 1. Again, as before, there was a sharp fall in infectivity in the group stored for 9 days, in this case, to 15%. The general pattern in the remaining groups was similar to those in Experiment 1 though there appeared to be a slight recovery in the infectivity in the final group which had been stored for 44 days.

The effect of storage on the results of irradiated groups was less than had been demonstrated in Experiment 1 even allowing

TABLE 23.

Number of Worms Recovered from Rats 10 Days After Infection with 2000 N. brasiliensis Larvae Stored in ½ RLA + Solution then Irradiated with 30 kr X-Rays.

	Day	Mean Worm Burden	Relative Take %	Eggs/g.	Sex-Ratio (M:F).
Control Groups.	2	1157 ± 294		7900	44:56
	9	295 ± 96		1300	47:53
	16	243 ± 87		2,250	44:56
	30	75 ± 56		550	47:53
	44	176 ± 113		1550	47:53
Irradiated Groups.	2	80 ± 62	7	-ve	2:98
	9	64 ± 41	21.7	-ve	0:100
	16	9 ± 6	3.7	-ve	0:100
	30	6.5 ± 6.0	8.6	-ve	0:100
	44	11 ± 11	6.3	-ve	0:100

for the difference in infective dose. This was particularly evident in the first two groups (Day's 2 and 9). No eggs were recovered in any groups and, apart from the first group, all worms recovered were female.

The condition of the larvae throughout the 44 days in the ¼RLA+ solution showed little or no signs of deterioration. Only 3 out of 14 bottles were lost by contamination, 2 after 9 days and 1 after 30 days. The percentage of dead larvae/bottle at the end of the experiment was less than 5%.

In conclusion, the infectivity and radiosensitivity of the larvae was, again, severely affected by storage beyond a few days.

Experiment 3.

Effect of storage - ¼RLA+ solution on the radiation-attenuation of N. brasiliensis larvae.

In Section 1, investigations were carried out on the effects of different doses of radiation on the infectivity of N.brasiliensis larvae. The studies showed that at low doses of radiation the infectivity was increased compared to the irradiated control but beyond 20 kr X-rays the infectivity decreased markedly. In order to examine the effect of storage in the radiosensitivity in more detail, radiation-dose titrations were carried out at various times during larval storage. The experimental procedure is described below.

From the information obtained - Experiments 1 and 2-it appeared that the ¼RLA+ solution was more reliable for maintaining the larvae in reasonable condition. A pool of 650,000 larvae were stored away in 40 ml. aliquots at 1,000 larvae/ml. A further 200,000 larvae that had been subjected to the same pre-storage treatment were re-suspended in water at a concentration of 10,000/ml in preparation for radiation treatment. The radiation doses were 5,10,15 and 30kr delivered at a dose-rate of 687r/min. The following day, the different batches of larvae were diluted to 3,000/ml and injected

into groups of rats. The results were taken as for zero time (Week 0). This procedure was repeated twice more, at three and 5 weeks after storage. Unfortunately, the bulk of the larval suspension irradiated at 30 kr on Week 3 was accidentally lost. Hence only 3 rats were injected in that group. The results for the three different periods are shown in Tables 24, 25, 26.

In the control groups, the average number of worms recovered was 30% of the infected dose at Week 0, 9.5% at Week 3 and 13% at Week 5. At similar periods of storage in Experiment 2, the percentages were 58%, 4% and 9% respectively. No reasons can be given as to why there was an appreciable rise in infectivity beyond 3 weeks in both experiments.

The radiation-titration pattern for Week 0 was in agreement with the results of similar experiments in Section 1. At 5 kr the infectivity was 113% compared to the control group and at 10 kr and beyond, the infectivity decreased with increasing dose.

The results of the Week 3 groups were of a similar pattern to Week 0 at, of course, a reduced infectivity. The only apparent change was found in the sex-ratio of worms recovered at the two high irradiated dose-groups. In the 15 kr groups the percentage

TABLE 24.

WEEK 0.

Number of Worms Recovered from Rats 10 Days after Infection with 3,000 N. brasiliensis Larvae Irradiated with Different Doses of X-Rays.

	Control	5kr	10kr	15kr	30kr
	957	607	505	607	249
	845	942	1132	509	246
	1041	1143	491	722	428
	956	1189	411	153	163
	877	1086	351	696	571
	912	1230	788	666	374
	956	1214	781	676	283
	731	828	787	788	199
	987	912		580	559
		1240		563	198
Total	8262	10391	5246	5960	3270
Mean	918 ± 86	1039 ± 200	656 ± 245	596 ± 167	327 ± 140
Relative Take %		113	71.5	65	35.5
Eggs/g.	15,050	17,250	11,150	6,100	150
Sex Ratio M:F	43:57	30:70	25:75	22:78	9:91

TABLE 25

WEEK 3.

Number of Worms Recovered from Rats 10 Days after Infection with 3,000 N. brasiliensis Larvae Stored for 3 Weeks in ¼ RLA+ Solution then Irradiated with Different Doses of X-Rays.

	Control	5kr	10kr	15kr	30kr
	208	316	142	116	4
	254	317	246	32	8
	335	182	283	106	1
	215	239	237	115	
	344	174	89	193	
	186	394	82	72	
	244	269	166	293	
	497	667	272	55	
	297	416		44	
Total	2577	2974	1517	1026	21
Mean	286± 90	330± 145	190± 65	114± 78	7± 3.7
Relative Take %		115	66.5	40	2.4
Eggs/g.	1150	6150	50	100	-
Sex Ratio M:F	44:56	31:69	25:75	7:93	0:100

TABLE 26.

WEEK 5.

Number of Worms Recovered from Rats 10 Days after Infection with 3,000 N. brasiliensis Larvae Stored for 5 Weeks in ± RLAt Solution then Irradiated with Different Doses of X-Rays.

	Control	5kr	10kr	15kr	30kr
	516	527	76	146	0
	202	743	486	205	6
	236	612	378	79	0
	303	502	252	178	6
	248	718	408	139	9
	638	620	158	191	0
	635	676	228	180	5
		380	368	133	30
Total	2778	4778	2354	1251	56
Mean	397± 179	597± 114	294± 130	156± 38	7± 8
Relative Take %		150	74	40	1.7
Eggs/g.	7250	9200	800	-ve	-ve
Sex Ratio M:F	45:55	25:75	29:71	7:93	0:100

fell from 9% to 0.

The results of the Week 5 groups were again of the same pattern. It should be noted that the increase in infectivity was significantly more than the above two, an increase in Relative Take to 150 as opposed to 115, 113 respectively. Unfortunately, the experiment could not be continued beyond the five weeks as, by that time, largo number of bottles were contaminated.

Discussion

The results of the experiments described in this Section show decisively that storage of larvae for even a short period of one week in either of the two salt solutions caused a reduction of infectivity by at least a factor of two. Such an effect obviously precludes the use of these larvae for experimental purposes.

It is probably that other storage methods, if tried, would have retained the initial high larval infectivity for a longer period. Haley and Clifford (1958, 1960), using N. brasiliensis larvae that varied in age from 3 to 111 days, reported that infectivity was high and showed little change for larvae that ranged from 3 days to about 4 weeks of age, but beyond that time infectivity decreased as larval age increased. It appears from their report that the larvae were retained in the charcoal-faeces cultures until required for injection into rats. This method of storage was tried by the author in the laboratory but was unsuccessful because of contamination growth appearing in all the cultures within one month. Beach (1964) found that infective larvae of Cooperia punctata retained their infectivity for 15 months when stored on moistened sphagnum moss at 6 to 8°C and,

similarly, Herlich (1966) reported that larvae of Trichostrongylus colubriformis, stored on moistened filter paper at 4°C, retained their infectivity for as long as 12 months. In the author's search for a suitable storage medium, larvae were stored in either tap water or 0.9% saline solution at refrigerator temperature (4°C). After storage for one week, the infectivity had dropped to under 10% of the control and so was considered no further.

The radiation groups in experiments 1 and 2 had very low worm burdens and no real conclusions can be made. The results of the 3rd experiment did indicate that although the infectivity of the larvae fell with storage the radiosensitivity remained more or less unaltered. Both the final control and irradiated groups in experiments 2 and 3 showed increased worm burdens, contrary to the general pattern. Unfortunately with each experiment contamination of the storage bottles terminated the experiments prematurely.

Summary

1. Experiments are described on the effect of storage in different salt solutions on the infectivity and radiosensitivity of N. brasiliensis larvae.

2. The storage mediums were generally unsatisfactory. Larval infectivity fell considerably after 1 week's storage. In the main experiment (3) the fall in infectivity was not accompanied by a similar fall in radiosensitivity.

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APPENDIX 1a - TABLE 4.

Number of Worms Recovered from Rats 10 Days after Infection with 1000 N. brasiliensis Larvae Irradiated with Different Doses of X-Rays or Co^{60} Gamma-Rays.

	Control	10 kr		20 kr	
		X	Co^{60}	X	Co^{60}
	290	1200	480	210	520
	550	930	300	260	850
	250	830	700	490	490
	500	950	1130	350	750
	410	980	10	280	620
	80	670	470	320	780
	420	690	430	390	720
	470	920	1150	370	530
	100	750	1150		400
	220	1090	430		490
Total	3290	9010	6250	2670	6150
Mean	$329^{\pm}$ 165	$901^{\pm}$ 173	$625^{\pm}$ 462	$334^{\pm}$ 35	$615^{\pm}$ 151
Relative Take %	100	274		101.5	
			190		187
Eggs/g.	2200	2050	5550	0	0

APPENDIX 1b - TABLE 4.

Number of Worms Recovered from Rats 10 Days after Infection with 1000 N. brasiliensis Larvae Irradiated with Different Doses of X-Rays or Co^{60} Gamma-Rays.

	40 kr		60 kr		80 kr	
	X	Co^{60}	X	Co^{60}	X	Co^{60}*
	1	50	1	12	2	8
	13	130	3	13	0	11
	14	190	34	6	0	1
	9	100	2	5	0	6
	32	180	0	0	0	1
	19	40	0	27	0	4
	0	270	1	18	0	2
	1	250	1	0	1	0
	9	80	0	14	1	1
	0	60	1	0	0	
Total	98	1350	43	95	4	34
Mean	9.8± 10.3	135± 83	4.3± 10.5	9.5± 8.9	0.4	3.8± 3.8
Relative Take %	3		1.3		0.1	
		41		2.9		1.1
Eggs/g.	0	0	0	0	0	0

* Injected Dose 700 larvae/rat.

APPENDIX 2a - TABLE 12.

Number of Worms Recovered from Rats 10 Days after Infection with 1000 N. brasiliensis Larvae Irradiated with 30kr X-Rays (470 roentgens/min.) at different temperatures.

CONTROL GROUPS	15°C	21°C	25°C	30°C
	265	79	185	254
	155	234	280	362
	617	175	328	127
	389	96	471	525
	289	640	71	183
	537	382	380	290
	104	306	211	310
	458	332	393	208
	157	361	108	330
			444	
Total	2971	2605	2871	2589
Mean	330 ± 181	289 ± 172	287 ± 139	288 ± 116
Relative Take %				
Eggs/♀.	5350	11150	12200	3550
Sex Ratio M:F	54:46	55:45	54:46	53:47

APPENDIX 2b - TABLE 12.

Number of Worms Recovered from Rats 10 days after Infection with 1000 N. brasiliensis Larvae Irradiated with 30kr X-Rays (470 roentgens/min.) at different temperatures.

IRRADIATED GROUPS	15°C	21°C	25°C	30°C
	107	61	258	340
	58	25	328	194
	235	48	203	267
	67	40	253	499
	81	102	116	135
	51	8	212	291
	31	56	250	297
	154	90	220	365
	45	81	318	489
	54	102	381	
Total	883	613	2539	2877
Mean	88± 62	61± 32	254± 73	320± 121
Relative Take %	26.6	21.1	88.5	111.1
Eggs/g.	- ve	- ve	-ve	-ve
Sex Ratio M:F.	7:93	5:95	3:97	14:86

APPENDIX 3a - TABLE 13.

Number of Worms Recovered from Rats 10 Days after Infection with 1000 ± 100 Nippostrongylus brasiliensis Larvae Irradiated with 30kr X-rays (470 roentgens/min) at different temperatures.

CONTROL GROUPS	16°C	21°C	24°C	29.5°C
	1	192	216	224
	77	79	205	107
	62	0	87	107
	3	166	105	109
	120	92	112	65
	116	5	116	133
	246	271	193	1
	283	8	214	156
	196	186	140	152
	166	130	182	239
Total	1270	1129	1570	1293
Mean	127 ± 95	113 ± 92	157 ± 51	129 ± 70
Relative Take %				
Eggs/g.	4400	3050	2450	1750
Sex Ratio M:F	44:55	40:60	46:54	46:54

APPENDIX 3b - TABLE 13.

Number of Worms Recovered from Rats 10 Days after Infection with 1000 ± 100 Nippostrongylus brasiliensis Larvae Irradiated with 30kr X-rays (470 roentgens/min) at different temperatures.

IRRADIATED GROUPS	16°C	21°C	24°C	29.5°C
	0	8	11	2
	0	15	31	53
	1	42	35	2
	1	28	21	104
	0	24	12	40
	0	3	8	48
	0	41	0	6
	0	10	21	81
	0	17	29	85
	0	14	16	123
Total	2	202	184	544
Mean	0.2	20 ± 13	18 ± 11	54 ± 43
Relative Take %	0.16	17.7	11.5	41.9
Eggs/g.	-ve	-ve	-ve	-ve
Sex Ratio M:F	2 ♂	11:89	13:98	21:79

APPENDIX 4a - TABLE 14.

Number of Worms Recovered from Rats 10 Days after Infection with 2000 N. brasiliensis Larvae Irradiated with 30kr Co^{60} Gamma-Rays (506 Roentgens/Min) at Different Temperatures.

CONTROL GROUPS	16°C	24°C	29°C
	1300	1700	820
	940	1100	1520
	1020	1200	940
	960	820	840
	880	880	128
	1120	840	198
	840	584	800
	900	19	760
	1160	621	
	57	589	
Total	9077	8353	6006
Mean	908 ± 337	835 ± 446	751 ± 437
Relative Take %			
Eggs/♀.	15350	15350	13850
Sex Ratio M:F	49:51	50:50	49:51

APPENDIX 4b - TABLE 14.

Number of Worms Recovered from Rats 10 Days after Infection with 2000 N. brasiliensis Larvae Irradiated with 30kr Co^{60} Gamma-Rays (506 Roentgens/Min) at Different Temperatures.

CONTROL GROUPS	16°C	24°C	29°C
	75	73	19
	43	263	316
	13	562	176
	86	172	179
	145	57	185
	22	88	329
	3	465	451
	176	15	186
		417	238
		414	425
Total	563	2526	2504
Mean	70 ± 63	253 ± 198	250 ± 131
Relative Take %	7.6	30.3	33.3
Eggs/g.	150	250	50
Sex Ratio M:F	25:75	25:75	24:76

APPENDIX 5a - TABLE 22.

Number of Worms Recovered from Rats 10 Days after Infection with 1000 N. brasiliensis Larvae Stored in Tris Buffer Solution then Irradiated with 30kr X-Rays.

	CONTROL GROUPS								
WEEK	0	1	2	3	4	5	6	7	8
	110	137	47	273	32	153	8	72	8
	513	98	33	142	204	68	18	32	54
	333	89	42	195	30	94	10	52	22
	327	59	2	218	19	84	48	74	53
	525	246	103	98	152	48	43	14	47
	564	110	207	1	91	131	24	17	46
	471	244	191	117	2	75	44	64	22
	327	196	69	22	117	9	45	147	76
	367	145	41	111	134	45		17	
	456	272		88	0	2		62	
TOTAL	3975	1596	735	1259	781	709	240	551	328
Mean	397± 135	60± 75	82± 68	126± 79	78± 68	71± 46	30± 16	55± 38	41± 21
Eggs/g.	3,500	2,050	600	750	300	-ve	-ve	-ve	-ve
Sex Ratio M:F	47:53	47:53	46:52	47:53	44:56	46:52	47:53	45:55	46

APPENDIX 5b - TABLE 22.

Number of Worms Recovered from Rats 10 Days after Infection with 1000 N. brasiliensis Larvae Stored in Tris Buffer Solution then Irradiated with 30kr X-Rays.

	IRRADIATED GROUPS				
WEEK	0	1	2/3	4	5-8
	79	8	0	0	0
	69	2	0	0	0
	47	2	0	9	0
	137	4	0	0	0
	99	2	0	0	0
	45	0	0	1	0
	124	6	0	0	0
	121	6	0	10	0
	96	6	0	0	0
TOTAL	817	36	0	24	0
Mean	91 ± 34	4 ± 2.5	0	2.4 ± 3.3	0
Eggs/g.	-ve	-ve	-ve	-ve	-ve
Sex Ratio M:F	9:91	9:91	-	0:100	-

APPENDIX 6a - TABLE 23.

Number of Worms Recovered from Rats 10 Days after Infection with 2000 N. brasiliensis Larvae Stored in ½ RLA+ Solution then Irradiated with 30kr X-Rays.

Control Groups	Day 2	Day 9	Day 16	Day 30	Day 44
	770	304	165	32	76
	1120	333	262	29	142
	1390	338	189	40	19
	960	327	204	46	288
	1200	206	296	19	392
	880	154	288	68	116
	910	185	416	143	219
	1260	360	120	126	190
	1730	454	245	170	144
	1350				
Total	11570	2661	2185	673	1586
Mean	1157 ± 294	295 ± 96	243 ± 87	75 ± 56	176 ± 113
Relative Take %					
Eggs/g.	7900	1300	2250	550	1550
Sex Ratio M:F	44:56	47:53	44:56	47:53	47:53

APPENDIX 6b - TABLE 23.

Number of Worms Recovered from Rats 10 Days after Infection with 2000 N. brasiliensis Larvae Stored in ½ RLA+ Solution then Irradiated with 30kr X-Rays.

Irradiated Groups	Day 2	Day 9	Day 16	Day 30	Day 44
	153	31	5	3	33
	58	35	7	8	0
	104	107	25	1	4
	19	31	7	1	0
	11	37	10	0	20
	176	132	11	5	6
	46	123	10	16	17
	157	50	4	4	8
	27	60	8	16	10
	50	30	2	11	
Total	801	636	89	65	98
Mean	80± 62	64± 41	9± 6	6.5± 6.0	11± 11
Relative Take %	7	21.7	3.7	8.6	6.3
Eggs/g.	-ve	-ve	-ve	-ve	-ve
Sex Ratio M:F	2:98	0:100	0:100	0:100	0:100