Aspects of Immunisation of Calves against *Dictyocaulus viviparus*

by

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A thesis submitted for the degree of Doctor of Philosophy

in the Faculty of Veterinary Medicine

of the University of Glasgow

Department of Veterinary Parasitology

×Spring 1986

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ACKNOWLEDGEMENTS

I would like to thank Professor G.M. Urquhart who supervised this work, for his guidance and support and the academic staff of the Department of Veterinary Parasitology for their advice and discussion.

For their expert assistance I wish to thank Mr.K. Bairden and all the technical staff of the Parasitology Department, Mr.A. Finnie and Mr.A. May for their photographic help, Mr.J. Murphy and Mr.R. Irvine for post-mortem assistance and Mr.P. Tiebosch for looking after the animals.

In addition I would like to thank Dr.H. Birkbeck of the Department of Microbiology and Professor O. Jarrett of the Department of Veterinary Pathology without whose collaboration much of the work in this thesis would have been impossible.

The author was supported by the James Houston Cattle scholarship, Glasgow University funds and by Glaxo Animal Health Ltd. who also provided assistance with the cost of experiments.

My thanks are also due to the staff of Glaxo Animal Health at Harefield and Glaxo Operations at Ware for their technical information and encouragement.

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SUMMARY

The studies reported in this thesis were undertaken to explore ways in which an improved vaccine could be developed against the disease caused by the cattle lungworm Dictyocaulus viviparus.

The current vaccine is a live, non-sterile, oral preparation of x-irradiated larvae, although more recently a gamma-irradiated oral vaccine has been marketed in the Netherlands and the U.K. Each is administered orally in two doses 28 days apart.

It was thought that it would be advantageous from the viewpoint of future licensing requirements, if live vaccine could be rendered microbiologically sterile and that certain commercial benefits could be obtained from packaging the vaccine in plastic rather than glass bottles.

The first experimental chapter, **Chapter Three,** deals with the problems of producing a sterile suspension of larvae without harming their viability or infectivity. A suitable means of achieving this was found to be a one hour treatment with a 0.0024% w/v solution of the monosodium salt of 5,5'-Dichloro-2,2'-dihydroxydiphenylmethane (dichlorophen).

Chapter Four is concerned with the packaging of vaccine in plastic bottles. The main problem in this respect was that in earlier studies a large proportion of the larvae were found to adhere to the surface of the bottle and in consequence were not delivered to the calf at dosing. However, various newer plastics were examined in the present work and polyethylene tetraphthalate (P.E.T.) was found to give results comparable to glass.

As a stepping-stone towards the use of a killed antigen

extract as a vaccine, it was thought possible that, in a two dose vaccine, the initial dose could remain as the current oral preparation and be followed up with an injection of killed larval extract 28 days later. This regime was investigated in **Chapter Five** and, in the method attempted, gave poor protection against subsequent challenge.

Now that it was possible to render the irradiated larvae sterile, it was considered feasible to investigate the immunogenicity of parenterally administered irradiated larvae. The experiments on this aspect are presented in **Chapter Six.** It was found that subcutaneous administration of two doses of irradiated larvae 4 weeks apart gave 95% protection against experimental challenge.

In the concluding experimental chapter, **Chapter Seven,** the use of sterile, injectable vaccine was further studied and the process of sterilising the larval suspension simplified and refined. In addition, the required evidence of safety, efficacy and sterility was acquired in preparation for an application to be made for a Ministry of Agriculture certificate to permit field testing of this new formulation of bovine lungworm vaccine.

Chapter One.

General Introduction.

Taxonomy of the genus Dictyocaulus.

Species of the genus Dictyocaulus, Railliet & Henry, 1907, are parasitic phasmid nematodes which occur in the respiratory passages of certain ungulates.

The exact taxonomic position of the genus has been a matter of some debate. Soulsby (1968) and Dunn (1978) base their classification on the work of Skrjabin. In this system the genus is placed in the family Dictyocaulidae, Skrjabin, 1941 under the superfamily Trichostrongyloidea, Cram, 1927. Soulsby further assigns this superfamily to the suborder Strongylata, Railliet & Henry, 1913 under the Order Rhabditida, Chitwood, 1933. Dunn, however, raises the suborder to order status as the Strongylida. Smyth (1976) takes a different view and bases his classification on the work of Chitwood. Here, as in Dunn, the order is Strongylida but the genus Dictyocaulus is placed in the family Metastrongylidae, Leiper, 1908.

The principal difference between the Skrjabin and the Chitwood systems is whether the genus should be classified along with nematodes with a similar, non-parasitic phase outside the final host, or with those sharing a similar, pulmonary location in the final host.

In both classifications, the trichostrongyloid families typically have direct life-cycles employing no intermediate hosts. They are also non-migratory in their parasitic phase and lay unlarvated eggs. In contrast, typical metastrongylid genera, except Dictyocaulus, have indirect life-cycles utilising annelids or molluscs as intermediate hosts. In the final host they are all migratory, the majority of genera having their adult stage in

the lungs. They are also all ovo-viviparous with the solitary exception of Metastrongylus spp. which are oviparous.

The genus Dictyocaulus has a direct life-cycle as befitting a trichostrongyloid, yet these species are migratory, live in the lungs and are ovo-viviparous as are the metastrongylids. The genus can, therefore, be the only lung-dwelling, ovo-viviparous trichostrongyloid or it can be one of the few metastrongylids with a direct life-cycle.

Four species of Dictyocaulus are known: D.viviparus, Bloch 1782, is found in bovines, buffaloes and the Cervidae; D.filaria, Rudolphi 1809, in sheep and goats; D.arnfieldi, Cobbold 1884, in the Equidae and D.cameli, Boev 1952, in the camel.

Synonyms of D.viviparus used in the past include : Gordius viviparus, Bloch, 1782; Strongylus vitulorum, Rudolphi, 1809 and Strongylus micrurus, Mehlis, 1831. D.eckerti reported from the cervidae is now also thought to be synonymous with D.viviparus (Dunn, personal communication).

The Life-cycle of *Dictyocaulus viviparus* in Cattle.

Under natural conditions, infection with *Dictyocaulus viviparus* is initiated by the ingestion of ensheathed third-stage larvae (L_3) from contaminated pasture while grazing (Daubney 1920). Since the larvae exsheath readily under the stimulus of hypochlorite, it is assumed that they exsheath in the rumen and penetrate the wall of the small intestine (Smythe,1937; Jarrett, McIntyre, Jennings and Mulligan, 1957a; Poynter, Jones, Nelson, Peacock, Robinson, Silverman and Terry, 1960). These then, according to most authors, enter the lymphatic vessels and are carried to the mesenteric lymph nodes. Here the L_3 moults to become the L_4 (Smythe 1937). It is presumed that the L_4 is carried with the flow of lymph to the thoracic duct where it enters the blood-stream in the jugular vein. The blood-stream takes the larvae through the right heart and the pulmonary artery to the capillary beds of the lungs. Here they break out of the capillaries into the alveoli. The larvae then migrate up the respiratory tract. During this period they make their final moult and the adults are found in the larger bronchioles, the bronchi and the trachea (see Fig. 1.1). The adult males and females mate and larvated eggs are laid (Cobbold, 1886). The eggs are passed up the trachea by a combination of the ciliary/mucus movement and the animal's cough. They are then swallowed and are voided with the faeces. By the time they have passed through the alimentary tract the eggs have hatched and thus it is the L_1 which arrives on the pasture (see Fig. 1.2).

Unlike the free-living stages of the trichostrongyloid parasites, larvae of the genus *Dictyocaulus* apparently do not

feed (Daubney, 1920). However, their intestinal cells are filled with granules which are thought to be composed of lipid and provide an energy source.

The larvae of Dictyocaulus spp. are slow-moving in comparison to those of the trichostrongyloids. The L_2 is reached in one or two days but the L_1 cuticle is not shed. Under optimum conditions of 27°C and 95% humidity the infective L_3 is reached in about one week (Daubney 1920). This development is slower at lower temperatures (Rose, 1956). The L_3 retains both the L_1 and the L_2 sheaths although the L_1 cuticle is sometimes lost through mechanical damage.

Fig. 1.1.

Coiled thread-like masses of adult Dictyocaulus viviparus and frothy mucus in the bronchus of a diaphragmatic lobe.

Fig. 1.2.

Characteristic appearance of first stage Dictyocaulus viviparus larvae.

The Pathogenesis of Dictyocaulus viviparus Infection.

The pathology of lungworm disease has been the subject of a great deal of study and is described in detail in many publications. The description given here is based on the work of : Jarrett et al. (1957a); Jarrett, McIntyre and Urquhart (1957b); Simpson, Wade, Dennis and Swanson (1957); Jarrett, Jennings, McIntyre, Mulligan, Sharp and Urquhart (1960a); Poynter et al. (1960); and Pirie, Doyle, McIntyre and Armour (1971).

The severity of clinical signs which develop from an infection with D.viviparus depend on several parameters, the most important being the size of the infecting dose of larvae and whether or not the animal has been previously exposed to lungworm infection. Less important but significant are the animal's age, its size and possibly its nutritional state.

Jarrett et al. (1960a) divided the disease into four phases : penetration; prepatent; patent and postpatent.

The penetration phase is from the day of initial ingestion of infective larvae (day 0) to day 7. During this time the larvae penetrate the gut wall and migrate via the lymphatics and heart to the lungs. During this phase clinical signs are not apparent.

The prepatent phase lasts from day 8 to patency at around day 25. During this period the larvae appear in the alveoli and move up the bronchioles to the bronchi. Their presence causes inflammation typified by an influx of eosinophils, neutrophils, macrophages and multinucleated giant cells into the lumina of the alveoli and in the bronchioles these cells form plugs. This

blockage prevents inspired air reaching the lung tissue distal to the plug and these areas collapse. The bronchiolar walls, the connective tissues and the interlobular septa all become packed with inflammatory cells, mainly eosinophils. The bronchioles may become ruptured leading to interstitial emphysema. The emphysema makes expiration painful thus causing an inhibition of normal lung contraction. In heavy infections the dyspnoea coupled with the alveolar collapse leads to a state of anoxia which impairs normal heart function. This in turn leads to pulmonary oedema and then the formation of hyaline membranes, further reducing the respiratory surface and increasing the anoxia. In acute cases the anoxic dyspnoea and heart failure can cause death from Day 15 onwards and before adult worms are present in the lungs.

From around day 26 adult worms are present in the bronchi. These produce eggs detectable as L_1 in the faeces. The disease has thus reached patency. The adults cause bronchitis with an influx of inflammatory cells into the epithelium and lamina propria of the bronchi. This leads to a thickening and hyperplasia of the bronchial epithelium. Frothy, white mucus containing eggs, first-stage larvae, adult worms, epithelial cells and leucocytes is present on the bronchial mucosa. Extreme hyperplasia of the epithelium of the bronchioles plus an influx of neutrophils and eosinophils may block the passage of inspired air into the alveoli. As with the prepatent phase, interstitial emphysema at this stage can severely affect the breathing efficiency. In the alveoli there is a cellular infiltration of neutrophils, eosinophils, macrophages and giant cells. This is

in response to the aspiration of eggs and larvae (see Fig. 1.3) and, coupled with the collapse of the alveoli, leads to large areas of consolidation in the lungs (see Fig. 1.4). The majority of lungworm deaths occur in this phase and for the surviving animals the symptoms may start to decrease from day 35.

In untreated cases the post-patent phase of the disease is from day 60 onwards to about 13 weeks. Around day 60 the adult worms are expelled and the lesions regress. For 75% of the clinical cases the post-patent phase is simply a recovery period. There continues to be a cellular infiltration into the alveoli and bronchioles until all the worm debris has been removed. However, the epithelial hyperplasia remains and areas of fibrosis may be present. Complete recovery, at least clinically, eventually takes place although this may take several months.

In the remaining 25% of post-patent cases there is a sudden worsening of signs. This is due to a proliferative alveolitis characterised by a proliferation of type-2 pneumocytes on the alveolar epithelium. This is often, also, accompanied by interstitial emphysema, pulmonary oedema and hyaline membrane formation as described in pre-patent husk. Such cases usually die.

After repeated light infections calves develop a strong immunity to the disease. However, in response to a large challenge the immune response itself can cause clinical signs. This is known as the reinfection syndrome. The lesion starts as a necrotising bronchiolitis accompanied by a marked eosinophil infiltrate. This gradually regresses and eventually a lymphoid nodule forms around the dead larvae in the lungs (see Fig. 1.5).

This lesion causes temporary obstruction of the bronchioles resulting in coughing. It is rarely fatal as the lesion quickly resolves.

Occasionally, however, patent parasitic bronchitis is observed in adult cattle in circumstances where they have not been exposed to infections as calves and therefore have not developed immunity.

Fig. 1.3.

Aspirated eggs and first-stage larvae in the alveoli associated with cellular infiltration of neutrophils, eosinophils, macrophages and giant cells.

Fig. 1.4.

Typical areas of pulmonary consolidation in the diaphragmatic lobes associated with patent parasitic bronchitis. This is largely caused by the lesion shown in the previous figure.

Fig. 1.5.

Small lymphoid nodule on the pleural surface of the lung. This cellular reaction arises as a response to the death of a larva in the lungs of an immune animal.

The Epidemiology of Husk.

Clinical dictyocauliasis is largely a product of intensive grazing systems. In contrast, it is possible that if cattle lived as wild ruminants, the disease would rarely occur (Peacock & Poynter, 1980). The closest domestic example to wild existence is the spring-born beef-suckler calf. Such a calf tends to run with its dam on extensive grazing and gradually weans itself between four and eight months old. During this period the calf is increasing its intake of herbage and Dictyocaulus larvae and developing an acquired immunity. Under such circumstances the calf is unlikely to develop clinical husk before it is weaned in October by which time it is usually fully immune. This scenario is visualised by Taylor (1960) as " a group of red Hereford cows, with calves at foot, grazing the green infected grass, where they pick up infection and grow fat ".

In direct contrast to this picture is the dairy calf. In modern dairy systems the calf is removed from its mother as early as possible and pail fed until it is 5 to 7 weeks old. When such a calf is turned out at 3 - 4 months old, it is totally dependent on eating grass (see Fig. 1.6). If the grass is contaminated with lungworm larvae it will quickly pick up a large infection and since it has had no chance to acquire any immunity it will possibly contract severe parasitic bronchitis.

It must not be assumed, however, that lungworm outbreaks are confined solely to dairy herds. The autumn-born suckler calf is also at risk. Such a calf typically spends the important weaning period indoors where it will not experience a gradually increasing lungworm challenge. When turned out in the spring it

is, like the dairy calf, totally dependent on grass and immunologically, totally naive.

Infective larvae survive longest in cool damp conditions (Porter, 1942, Rose, 1956). In temperate climates larvae passed in faeces in the autumn can still be present on the pasture in the following spring (Jarrett, McIntyre & Urquhart,1954, Jarrett, McIntyre, Urquhart & Bell, 1955b, Allan & Baxter, 1957). While the actual bionomics preceding the onset of clinical signs is unknown, it has been suggested that, as is the case with Ostertagia infections, a light overwintered pasture infestation leads eventually to a build up of infection during the summer months. Others, in an effort to explain outbreaks, have suggested that larvae migrate into the soil in autumn to re-emerge in the spring and summer (Nelson, 1977). This migration is probably helped by the larvae being transported downwards by earthworms (Oakley, 1981) and dung beetles (Fincher, 1973) which have fed under the crust of the faecal pat.

It has also been shown that larvae which experience a prolonged period of chilling prior to ingestion may inhibit at the L_4 or L_5 stage (Taylor & Michel, 1952, Michel, 1955a, Oakley, 1979). Such inhibited forms possibly recommence their development throughout the winter and thus cause pasture contamination in the spring (Pfeiffer & Supperer, 1973).

In Britain, larvae which overwinter on the pasture are thought to be an important source of infection in the spring (Allen & Baxter, 1957, Burger, 1978, Oakley, 1978, Duncan, Armour, Bairden, Urquhart & Jorgensen, 1979). In colder regions,

such as the alpine areas of Europe, where survival on pasture is thought to be less common (Pfeiffer & Supperer, 1973), inhibited larvae are thought to be the more important source (Pfeiffer & Supperer, 1980). By one or a combination of these means larvae are present on the pasture in spring. These give rise to very light infections which cause few clinical signs and little immunity but which do, however, lead to an increase in pasture contamination. This reseeding may be aided by fresh contamination with larvae voided by older cattle acting as carriers (Cunningham, Jarrett, McIntyre & Urquhart, 1956) or by larvae reappearing from the deeper layers of the soil (Nelson, 1977). Thus the pasture larval burden is high enough by June or July to give rise to outbreaks of clinical disease in July to September.

Cattle are known to avoid the grass around faecal pats (Michel, 1955b) so that for efficient transmission the larvae must migrate as far from the pat as possible. Dictyocaulus larvae are sluggish in comparison to the infective larvae of the trichostrongyloids and probably do not leave the pat by their own movement (Michel & Rose, 1954). Instead they rely on mechanical translation (by means of rain splash, tractor wheels, hooves etc.) and their special relationship with the coprophagous fungus Pilobolus (Robinson, 1962). This fungus grows on the surface of the faecal pat from spores which have passed through the bovine alimentary tract. To facilitate the ingestion of spores, the sporangium explodes violently, discharging spores onto the surrounding herbage (see Fig. 1.7). Dictyocaulus larvae take advantage of Pilobolus by climbing into the sporangium

(Doncaster, 1981) (see Fig.1.8). On dehiscence the larvae are thrown along with the spores away from the faecal pat. By this means larvae can travel considerable distances especially if the airborne spores and larvae are caught by the wind.

For these two reasons no pasture in an endemic area can be thought of as "clean" - outbreaks of husk even having been reported from aftermath pasture (Armour, Al Saqur, Bairden, Duncan & Urquhart, 1980).

Taylor (1960) discussed some of the epidemiological questions raised by the use of the lungworm vaccine (Dictol, Glaxo Animal Health). He suggested that vaccinated calves should be kept away from heavily infected pastures, that vaccinates, after challenge, could act as immune carriers and that vaccination would never eradicate lungworm disease. While these suggestions were based on epidemiological theory rather than observation he was shown to be correct on all counts.

Cornwell (1959) and Cornwell & Berry (1960) reported an outbreak of husk in unvaccinated calves which had been initiated by vaccinated calves from another farm apparently, after field challenge, acting as immune carriers. Cornwell (1962a) subsequently studied 134 vaccinated calves. He noted that vaccinated calves could be killed by receiving too great a challenge and presumed that this was due to a severe lung reaction to dead or dying worms. However, he also pointed out that if all calves on a farm were vaccinated, such a high challenge would not arise. He suggested that it was desirable for all calves to receive some challenge to keep immunity at a

sufficient level and that this would almost certainly occur since all the vaccinates that he observed on pasture excreted larvae at some point following challenge. A low level of infection would cycle in the vaccinated herd without reaching levels where calves would die of a severe challenge and without pasture infestation being completely eliminated.

Fig. 1.6.

Typical paddock adjacent to farm buildings commonly used annually to graze young calves in early summer. Such paddocks are often a perennial source of lungworm infection, due mainly to the presence of overwintered larvae.

Fig. 1.7.

Pilobolus spp. fungus growing on the surface of the bovine faecal pat.

Fig. 1.8.

Close-up of a mature Pilobolus sporangium and proximal hypha, showing the presence of Dictyocaulus viviparus larvae on the latter.

Treatment and Control of Lungworm Infection.

The sucessful treatment of cases of clinical husk has long been a problem for the practitioner. Nicholls (1755) advocated fumigation with mercurials or tobacco. Smythe (1937), while admitting that " the value of all treatments ... has lain in their inefficiency" was of the opinion that intra-tracheal injections of oleum chaenopodii or pyrethrum in oil were the best available.

Gibson (1975) and Armour and Bogan (1982) have reviewed the recent history of anthelmintic medication. The drug of choice in the early 1960s was diethylcarbamazine citrate. This drug was more effective in prepatent cases. It was usually administered by intra-muscular injection daily on three consecutive days.

Diethylcarbamazine has since been superseded by levamisole and the benzimidazoles. Levamisole has proved to be an efficient drug against developing larvae, adults and even inhibited larvae. Of the benzimidazoles, fenbendazole, albendazole and oxfendazole have proved effective although only fenbendazole, to date, has been shown to have any action against arrested forms of Dictyocaulus viviparus.

In any outbreak of parasitic bronchitis, while only one or two animals may be clinically ill, the rest of the calves may have the disease in the early prepatent phase. In such an instance it may be desirable to treat the whole herd to pre-empt the appearance of clinical signs. In the treament of adult dairy cattle, the choice of anthelmintic will be limited to those which are licensed for use in the lactating animal. In this respect only levamisole and fenbendazole are of practical use.

Even in use of these, milk cannot be sold for human consumption during a period of one day for levamisole, or three days for fenbendazole, following treatment.

McEwan, Oakley & Robinson (1979) compared levamisole and fenbendazole in the treatment of experimental husk. They reported that fenbendazole-treated animals showed more serious lesions than the levamisole treated group. These lesions were not associated with poor anthelmintic activity but it was postulated that these were reactions to adult worms dying in-situ. Fenbendazole kills the worms by preventing their absorption of nutrients, thus starving the worms in the lungs. In contrast, levamisole causes neuro-muscular paralysis in the worms and allows their clearance by muco-ciliary movement while still intact. McEwan, Oakley & Robinson (1979) therefore thought that only fenbendazole-killed worms released worm material into the lungs causing the severe reactions that they noted. However, Jarrett, Urquhart & Bairden (1980) found that both these drugs caused adverse reactions when used in the treatment of patent husk and related the degree of lung damage to the length of time between infection and treatment.

The prophylactic use of anthelmintics to prevent outbreaks of husk has never proved very successful. The fact that the bionomics of the parasite on pasture are poorly understood makes the calculation of the optimal time for prohylactic treatment impossible. The use of anthelmintics at three and six weeks after turn out has been shown to prevent the build-up on pasture of high levels of the infective larvae of the gastro-intestinal

pasrasites and therefore prevent outbreaks of parasitic gastro-enteritis in mid-summer. Such a treatment regime has little value in the prevention of husk, possibly because infective larvae reappear on the pasture from the soil in mid-summer and also because low numbers of larvae on the herbage can cause clinical husk.

Immunity to Lungworm Infection.

The fact that cattle which survive an initial infection of Dictyocaulus viviparus are subsequently resistant to reinfection has been known for some years (Taylor 1951, Jarrett, McIntyre and Urquhart 1954) and has been reproduced experimentally (Porter & Cauthen, 1942; Rubin & Lucker, 1956; Jarrett, Jennings, McIntyre, Mulligan, Thomas & Urquhart, 1959b). The speed with which this immunity is acquired and its efficacy is quite unusual in helminth infections.

The reasons for this relative absence of effective immunity to nematodes generally, are closely linked to the successful exploitation of parasitism by this group. While these complex metazoan creatures could be expected to present a greater amount of potentially antigenic material to the host compared with microbial pathogens, it is apparent that their size renders such potent immunological mechanisms as agglutination, precipitation and opsonisation relatively ineffective. In addition, the fact that these parasites have several stages of their life-cycles within the host may enable them to change their external antigens before the host has had a chance to respond (Philipp, Parkhouse & Ogilvie, 1980). Furthermore, the nematode cuticle appears to be less immunogenic than the tegument of cestode parasites. This is not surprising in view of the complex metabolic functions of the tegument of the tapeworms in comparison with which the nematode cuticle is relatively inert. One point in the host's favour, however, is the fact that helminths (with a very few exceptions) do not multiply within the host and therefore cannot rely on a

massive reproductive potential to outstrip an individual host's immune response.

In recent years the immunology of parasitic diseases in laboratory models has possibly been the most researched area of parasitology. However, despite the massive literature on the subject, little has been learned which adds to our knowledge of the mechanisms of immunity to D.viviparus infection. It is, perhaps, somewhat paradoxical that the only helminth infection for which a vaccine is commercially available is also one of the least understood from the point of view of the immune responses involved.

The bulk of the work on immunity to nematodes has been concerned with gastro-intestinal worms. In particular a great deal has been written about Nippostongylus brasiliensis and Trichinella spiralis in the rat (reviewed by Wakelin, 1984). Immunity to a primary infection of these parasites is typified by what is known as "immune expulsion" some days after the establishment of the worms. During immune expulsion adult worms are expelled from the intestine, often still alive. In response to a secondary infection fewer worms establish and immune expulsion occurs earlier (Jarrett, Jarrett & Urquhart 1968). The mechanisms involved in this phenomenon are still a matter of debate but appear to be associated with an IgE-mediated anaphylaxis (Ogilvie 1964). Mast cells in the intestinal mucosa increase in numbers and bind specific anti-worm IgE via the F_c receptor. When antigen binds to the IgE the mast cell degranulates releasing various factors including vaso-active amines. These cause an increase in mucosal permeability allowing

plasma to leak into the gut lumen. In addition to involvement with mast cells, IgE may be instrumental in causing goblet cell hyperplasia with a resultant increase in mucin secretion (Miller & Nawa, 1979). The increased amount of mucus is thought to prevent the worms from taking up their normal position between the villi (Miller, Huntley & Wallace, 1981). This is known as immune exclusion. Perhaps also, specific anti-worm antibody which has leaked into the intestine acts directly on the worms (Barth, Jarrett & Urquhart, 1966).

Turning to parasites which undergo tissue migration, one of the common features is eosinophilia (Wakelin, 1984). Recently in this respect an important advance was the finding of Butterworth, Sturrock, Houba & Rees (1974) that peripheral blood leucocytes could kill schistosomula of Schistosoma mansoni in vitro. The same group (Butterworth, Sturrock, Houba, Mahmoud, Sher, & Rees, 1975) found that the best mediator of schistosomula damage was the eosinophil. Since then, much has been written on the subject including several reviews (Kay 1979, Butterworth, Taylor, Veith, Vadas, Dessein, Sturrock & Wells 1982).

Anwar & Kay (1977a) discovered that eosinophils had receptors for the F_c portion of IgG and for the C_3b fraction of complement. Butterworth, Remold, Houba, David, Franks, David & Sturrock (1977) showed that eosinophils would mediate schistosomula killing in the presence of antibody alone. Anwar, Smithers & Kay (1979) finally showed that while eosinophil mediated killing could be achieved with antibody alone or complement alone it was greatly enhanced when antibody and

complement were both present.

Anwar and Kay (1977b) had previously shown that the mast-cell derived products Eosinophil Chemotactic Factor of Anaphylaxis (ECF-A) and histamine increased the number of complement receptors on the eosinophil. Gathering all this together Kay (1979) was able to propose the following theory.

He suggests that when the cercaria of a schistosome enters the skin of a previously exposed animal the schistosomulum becomes coated with IgG and also activates the alternative pathway of complement. Mast cells in the skin adhere to the schistosomulum by means of specific IgE and complement receptors. These mast cells degranulate as in a type I hypersensitivity reaction releasing ECF-A and histamine. Eosinophils are attracted to the site and display an increased number of complement receptors due to the enhancement by the mast cell products. These eosinophils then adhere to the schistosomulum by their complement and IgG receptors. They are now in a position to damage the schistosomulum by degranulating on to its tegument.

Eosinophil granules have been shown to contain peroxidase (McLaren, MacKenzie and Ramalho-Pinto 1977), a major basic protein (MBP) (Gleich, Loegering & Maldonado, 1973) and eosinophil cationic protein (ECP) (Olsson and Venge 1974). Studies using electron microscopy have shown that schistosomulum damage is indeed associated with eosinophil degranulation (McLaren 1980). These ultrastructural studies show that holes appear in the surface tissues of the parasites at the site of degranulation. The damage is further increased by the active movement of the eosinophil into the hole forcing apart the

already damaged tissues.

While many papers concerning immunity to Dictyocaulus have been written (especially in the early '60s) few authors have been willing to speculate on the mechanisms involved in this immune response. As this parasite has a lumen-dwelling adult stage (albeit lung rather than intestine) it may be that mechanisms similar to those involved in protection against Nippostrongylus are operating. However, it also has a tissue migrating phase which may make it vulnerable to cell-mediated killing such as is thought to occur in infections with Schistosoma mansoni.

The serological response to primary and secondary infections has been followed using complement fixation (CF) tests (Jarrett, Jennings, McIntyre, Mulligan & Urquhart 1955a; Weber 1958; Jarrett, Jennings, McIntyre, Mulligan, Thomas and Urquhart 1959b). Jarrett and his co-workers reported that complement fixing antibodies appeared in the serum around day 30-35 of a primary infection and rose to a peak around day 80 by which time the infection had been eliminated. In response to secondary and subsequent infections the peak level was higher and attained earlier, a situation typical of a secondary antibody response. Weber (1958), however, found no such secondary response. He obtained peak titres at day 18 in both primary and secondary infections and saw no significant increase in the levels of the peak titres.

Jarrett et al. (1955a) also showed that a globulin fraction of serum isolated from immune calves at the peak of their complement fixing antibody response could be used to protect

naive calves against challenge. Rubin & Weber (1955) also carried out serum transfer experiments. While both groups showed that protective substances had been transferred, the volumes of serum used were quite large. Thus, Jarrett et al. used 1,500 ml. of globulin fraction (equivalent to about 4.5 litres of whole serum) while Rubin & Weber injected approximately 750-800 ml. of whole serum.

Jarrett et al. (1955a) stated that "in parasitic bronchitis there are humoral antibodies which are detectable and measurable by a complement fixation test, and the serum containing these antibodies confers a considerable degree of protection against a single challenging infection of infective larvae of Dictyocaulus viviparus". Michel & Cornwell (1959) interpreted this as an assumption that the antibodies which were detected by the CF test and the antibodies which transferred protection were homologous and carried out a series of studies on the CF antibody response to lungworm infection (Michel & Cornwell, 1959; Cornwell & Michel, 1960; Cornwell, 1960a; Cornwell, 1960b). From these they finally concluded that there was little correlation between CF titres and protective immunity.

Attention then switched to the possibility of actively immunising calves using dead antigens. Jarrett, Jennings, McIntyre, Mulligan & Urquhart (1960b) performed experiments using an adjuvanted adult worm homogenate administered intramuscularly. While a reduction in worm burdens was noted in some of the experimental groups after challenge, it was concluded that protection was not great enough to be of any practical use. However, although little useful immunity was achieved the

vaccination produced an exaggerated response to the presence of adult parasites in the lung. This response was associated with eosinophil infiltration into the lobules and giant-cell phagocytosis of the worm material. This caused greater damage to the lung tissue than would normally be seen in a primary infection.

Wade, Swanson & Fox (1961) carried out active immunisation experiments on rabbits and guinea-pigs. They quote a 50% reduction in worm burdens in guinea-pigs given larval antigen over adjuvant only challenge controls; however, the significance of the paper is cast into doubt by a 58% worm burden reduction in a group vaccinated with saline alone. In a second paper (Wade, Swanson, Fox, Simpson & Malewitz, 1962) they give the results of vaccination experiments, conducted on calves, using a mixed larval and adult lungworm antigen. In this work eight month old calves showed a 80% reduction in worm burden following challenge. However, the significance here could also be questioned on the grounds that this reduction was from a 1% establishment in the controls to 0.2% in the vaccinates. One percent establishment in a control infection is extremely low and highlights a poor and possibly variable infectivity.

In 1957 Jarrett et al. (1957a) announced that experiments using x-irradiated lungworm larvae as a vaccine had shown sufficient promise to have reached the stage of a field trial. More full details were released in the following years (Jarrett, Jennings, Martin, McIntyre, Mulligan, Sharp & Urquhart 1958a; Jarrett, Jennings, McIntyre, Mulligan & Urquhart 1958b; Jarrett,

Jennings, McIntyre, Mulligan, Sharp & Urquhart 1959a; Sharp 1959; Jarrett, Jennings, McIntyre, Mulligan & Urquhart 1960c; Jarrett, Jennings, McIntyre, Mulligan & Sharp, 1961.

The discovery was based on the theory that the mesenteric lymph nodes were the site where larvae were killed in the immune animal. If lungworm larvae could be attenuated in such a way that they would remain infective yet die in the mesenteric lymph nodes then it was thought that immunity might be achieved without causing serious lung damage or clinical disease. Such a means of attenuation was found in irradiation with x-rays.

It was shown (Jarrett et al. 1960a) that a suitable degree of attenuation was produced at 40,000 roentgens. Oral administration of a single dose of 4,000 third-stage larvae irradiated at this level gave 98.5% protection against subsequent challenge. Jarrett et al. (1958a) conducted an experiment to assess the protection afforded by this form of vaccination under field conditions. In this trial the vaccine dose was reduced to 1,000 irradiated larvae. Following a very high pasture challenge, 3 out of 15 vaccinates died compared with 10 out of 12 controls. While this trial showed that a high level of protection could be given by a single dose of vaccine it was thought that two doses might provide a higher degree of immunity. Thus Jarrett et al. (1959a) performed an experiment in which calves were vaccinated with 1000 irradiated larvae on Day 0 as before and then given a second dose of vaccine on Day 42. The three doubly vaccinated groups received 4,000, 2,000 or 1,000 irradiated larvae respectively as the second dose. All were challenged with a single dose of 10,000 normal larvae on Day 93.

In none of the double vaccinates did the challenge reach patency and no adult worms were found at necropsy. In contrast, calves given a single dose of 1,000 irradiated larvae on Day 0 and challenged as above on Day 93 produced patent infections and a mean of 820 adult worms was found at post-mortem. No appreciable difference was observed between the different doubly vaccinated groups.

Again, a small-scale field trial was carried out (Jarrett et al., 1961) this time using the double vaccination procedure with both doses set at 1,000 irradiated larvae. The interval between doses was shortened to 30 days and the vaccinated calves were turned on to contaminated pasture along with challenge controls 20 days after the second vaccine dose. In none of the vaccinated calves could any mature lungworms be found at post-mortem examination 10 weeks later. In contrast, one of the control calves died of pre-patent husk and a mean of about 400 mature worms was found in the survivors.

A practical method had thus been devised whereby calves could be protected against clinical lungworm disease by the oral administration of two doses of 1,000 third-stage Dictyocaulus viviparus larvae irradiated by x-rays at 40,000 roentgens.

A commercial form of this vaccine was developed and launched by Allen & Hanburys on the 25th. February 1959 as "Dictol". It was widely hailed as the world's "first" commercial helminth vaccine as it was anticipated that many more such vaccines would follow. Poynter et al. (1960) reported experiments in calves and guinea-pigs conducted during the commercial development of the

vaccine. This group concluded that, contrary to the belief of Jarrett et al., there was no evidence to suggest that irradiated larvae died in the mesenteric lymph nodes. Nor did they find that this was the site where larvae were killed in the immune animal. Furthermore, they showed that, in calves as well as guinea-pigs, at least some larvae from an initial infecting dose were in the lungs by 36 hours i.e. some 5 days before Jarrett et al. had found their arrival in this organ and possibly indicative of a direct haematogenous route from the intestine to the lung.

However, Jarrett & Sharp (1963) remained unconvinced. In serial kills they could not find larvae in the lungs of calves before day 7. Even then, far fewer larvae were recovered from the lungs from a single dose of 1,000 irradiated larvae than from a similar dose of normal larvae. They argued that the larvae which Poynter recovered from the lungs had followed some aberrant migration and were only observed because of the higher challenge dose used. Also, considering the total numbers of larvae recovered from the lungs and lymph nodes, this still left the majority of larvae unaccounted for. Jarrett & Sharp considered that while it was possible that irradiated larvae were quickly expelled from the lung by ciliary/mucus movement, no histological evidence of their migration through the lung could be found. In contrast dead larvae could be found in the mesenteric lymph nodes although no figures were given for the numbers of such larvae. They concluded that the exact fate of irradiated larvae was uncertain, but reiterated their belief in the lymph node theory and also postulated a role for the bowel wall in preventing establishment in the hyperimmune animal.

Cornwell & Jones (1970a, 1970b, 1970c & 1971) used triethyl melamine to attenuate infective Dictyocaulus larvae and found the vaccine thus obtained to be as protective as irradiation-attenuated vaccine.

Others, (Bonazzi, Grimshaw, Bairden, Armour & Gettinby, 1983) have used gamma-irradiation instead of x-irradiation. The principal benefit of using gamma-rays from a Cobalt 60 source is the short time taken to attenuate the larvae. 40Krads can be achieved in less than an hour from a ^{60}Co source whereas the same irradiation dose takes 6 hours 40 minutes on the production line at Ware. However, uncertainty surrounds the comparative attenuation achieved by these two sources of irradiation. Kassai, Fitzpatrick & Mulligan (1966) calculated a "Relative Biological Effect" (RBE) based on the percentage establishment of Nippostrongylus brasiliensis in the rat. They found that ^{60}Co gamma-irradiation had only 70% of the RBE of x-irradiation at the same dose. It must be stressed, however, that this figure is based on the number of worms reaching adulthood in the rat's intestine and may therefore have a limited relevance to lungworm vaccination where the important factor is probably the invasiveness of the larvae and not their ability to become adult. Indeed, Bonazzi et al (1983) achieved effective vaccination against D.viviparus using 40Kr from ^{60}Co. where Kassai's figures would suggest 57 Kr as being the required dose. Other workers (Rodriguez, personal communication) oblivious of Kassai's RBE value have used 40Kr from a ^{60}Co source successfully.

Cornwell (1960a, 1960b) followed the complement-fixing

antibody response of calves during the vaccination period. He found that the first dose of vaccine produced a low level of CF antibody, at least as detected by a test using adult worm antigen. The second dose elicited a variable rise in titre although by the time the calves were ready to be put out to grass four weeks later, none of the titres could be described as high. It was suggested that the CF titre was in response to adult worm antigens and that the variable titre reflected the numbers of irradiated larvae which reached the lungs. When exposed to challenge (Cornwell, 1960b) those calves which had been vaccinated showed an immediate rise in CF titres whereas unvaccinated calves had a delay of 5 - 6 weeks before the titre began to rise. Even calves in which no detectable response to vaccination had occurred showed an early response to challenge. It was therefore clear that CF titres could not be used to estimate the immune status of an animal before challenge.

Cornwell (1962b), MacKenzie & Michel (1964) and Downey (1965) have examined the eosinophil response in cattle following challenge, vaccination and immune challenge.

Challenged naive calves show two peaks of circulating eosinophil numbers. The first response was at Days 9-15 and the second around day 40. Cornwell (1962b) found that in a primary infection the second eosinophil rise was the higher. After reinfection the response was quicker and this time the first response was the greater. Administration of irradiated larvae produced only the first rise in eosinophils and it was therefore suggested that the double peak in response to normal primary infection corresponded to an initial response to invading larvae

followed by a response to aspirated eggs once the worms reached patency. It follows then that irradiated larvae would only elicit the first response, i.e. that to the invading larvae.

An eosinophil response was shown in-vitro by Knapp & Oakley (1981). Third stage D.viviparus larvae incubated in the presence of whole blood were seen to become coated with cells, especially eosinophils and to a lesser extent neutrophils.

Wilson (1966) investigated further the role and nature of circulating antibodies to lungworm infection using the guinea-pig as a model. He found that protection was associated with gamma$_1$-globulin and fast-moving gamma$_2$-globulin and that these globulin fractions also showed anaphylactic activity. These antibodies are first detected in a primary infection during the elimination period at days 8-13 post infection. Guinea-pigs artificially immunised with Freund's adjuvanted adult worm homogenate produced anaphylactic gamma$_1$ and gamma$_2$ globulins but failed to protect against subsequent challenge.

It would appear to be possible that the strong immunity mounted in cattle against infection with Dictyocaulus viviparus is based on an eosinophil-mediated, antibody-dependant cellular cytotoxicity reaction. The great amount of eosinophil infiltrate and the lymphoid nodules found in the lungs of challenged immune calves would suggest that this killing is effected against the L_4 or L_5 in the lungs. Using man and asthma as a parameter it seems that the lung is well suited to mounting anaphylactic responses against airborn antigens. It is possible then to hypothesise that the immune response involves the recognition of lungworm

antigens by mast cells carrying specific anti-larval IgE. These would degranulate releasing, amongst other factors, ECF-A. Eosinophils would therefore be attracted to the site and could effect larval killing possibly by binding to complement on the surface of the parasite.

As mentioned earlier, the basic theory behind "Dictol" presumed that the mesenteric lymph nodes were the important site of larval killing and therefore for a vaccine to be effective the majority of the attenuated larvae would have to die in these tissues. In contrast, if the effector mechanisms are active in the lung, then vaccination with attenuated larvae by some parenteral route might prove possible. However, to be commercially acceptable, a parenterally administered vaccine would have to be sterile. The bulk of this thesis is therefore concerned with these two concepts, producing a sterile larval suspension and exploring the possibilities of parenteral vaccination.

In this context the work of Miller (reviewed in Miller 1971, Advances in Parasitology 9 and Miller 1978, Advances in Parasitology 16) is of note. This work centred around the problems of producing an irradiated vaccine against disease caused by Ancylostoma caninum in the dog. Eggs of Ancylostoma were washed in hypochlorite prior to hatching and the larvae were kept in sterile culture and fed on killed E.coli. Once infective, the larvae were irradiated and the resultant vaccine was administered subcutaneously. In the case of Ancylostoma, subcutaneous administration was the natural route of infection and therefore the obvious route for vaccination. Also, the fact that eggs are produced by A.caninum may perhaps be considered to have made the process of sterilisation less difficult compared with the problems of sterilising D.viviparus larvae passed in the faeces.

Chapter Two.

Materials and Methods.

Animals.

a. Calves.

In all experiments, male, 10 week old, weaned Ayrshire or Friesian cross calves weighing 80- 110 kg. were used. These were obtained from local sources where calves were known to be reared under conditions in which infection with helminth larvae was improbable. At the Parasitology Department the calves were bedded on clean straw which was replaced every second day. They were fed 1kg. of concentrate and 3kg. of hay daily. Water was available ad libitum.

b. Guinea-pigs.

Duncan-Hartley guinea-pigs were obtained from the Royal Hospital for Sick Children, Yorkhill, Glasgow. These were housed in plastic cages with wire mesh tops. They were fed on pelleted diet plus hay, lettuce leaves and water ad libitum. The animal house temperature was maintained at approximately 22°C.

Respiratory Rates.

Respiratory rates were estimated by counting the number of respirations in 15 seconds and multiplying that figure to give the number per minute.

Faecal Examinations.

Faecal samples were collected from the rectum and examined for helminth eggs or larvae by a modified M^{c}Master flotation technique (Gordon & Whitlock, 1939) or a modified Baermann method (Henrikson, 1965).

a. Modified M^{c}Master Method

Three grammes of fresh faeces was homogenised with 42 ml. of water and the resultant suspension passed through a coarse mesh

sieve of aperture size 250 microns (Endecotts Test Sieves Ltd., Morden, London.). After thorough mixing of the filtrate, 15 ml. was withdrawn into a flat-bottomed centrifuge tube (capacity 15 ml.) and centrifuged at 2000 revolutions per minute for 2 minutes. The supernatant was then discarded and the remaining faecal pellet broken up by rotary agitation (Whirlimixer, Griffin & George, Wembley, Middlesex). The tube was then filled to its former level with saturated salt solution and after inverting 6 times a volume of the suspension, sufficient to fill both chambers, was quickly transferred to a two-chambered M^{c}Master slide (Gelman Hawksley, Harrowden, Northampton.). The number of larvae under the total etched area of the slide was counted and the result multiplied by 50 to give the number of eggs or larvae per gramme of faeces.

b. Modified Baermann

Ten grammes of faeces was placed in gauze and suspended in warm saline in a plastic 250 ml. urine-glass for six hours, after which time both faeces and gauze were discarded. The suspension was allowed to sediment for three hours at 4^{o}C and the supernatant removed to give a final volume of 10 ml.. This was well mixed and the larvae present in a 1 ml. aliquot was counted and expressed as the number of larvae per gramme faeces.

Infective larvae.

Infective third stage larvae of Dictyocaulus viviparus were provided by Glaxovet, Ware, England. Larval inocula were prepared by counting the number of larvae present in 40 x 0.025 ml. aliquots. To facilitate counting the larval dilution was so

arranged that the number of L_3 per 0.025 ml. aliquot was approximately 30. Once the number of L_3 present in 1 ml. was known, the volume needed to provide the required inoculum was pipetted out and made up to the desired volume for infecting the animals. Throughout the whole counting procedure emphasis was placed on regular mixing of the larval suspension to prevent clumping.

Infection process.

a) Calves.

The required number of larvae were counted as above and diluted with distilled water to a final volume of 20 ml.. The dose was made up in 30 ml., glass, narrow-mouthed bottles with metal caps (used, washed Dictol bottles). Calves were held firmly and their mouths opened using the left hand, leaving the right hand free to mix the contents of the bottle and pour it down the animal's throat. Bottles were further washed out with distilled water and the washings poured into the animal's mouth as above.

b) Guinea-pigs.

The necessary number of larvae, usually at a concentration of c1200 L_3/ml. was put into conical centrifuge tubes and allowed to sediment at $4^{o}C$ for 4 hours. Excess supernatant was then removed to give a final volume of 1 ml.. Oral infection was by use of a shortened intravenous cannula (Red Luer, 5 FG O/D 1.65 mm., Portex Ltd., Hythe, Kent.) connected to a plastic 2ml. syringe. Guinea-pigs were lightly anaesthetised using trichloroethylene (Trilene, BDH Chemicals Ltd., Poole, Dorset.)

and the cannula gently introduced into the animal's oesophagus. Sub-cutaneous inoculation was performed using a 2 ml. syringe and a 20 gauge, 1 inch needle (Becton-Dickinson, Wembley, England). A fold of skin in the guinea-pig's neck was chosen as the site of inoculation.

Exsheathing process.

Where exsheathed larvae were used the exsheathment was achieved using 5% Milton's Fluid (Milton 2, Richardson-Vicks Ltd., England) equivalent to 0.001% w/v sodium hypochlorite. A known number of larvae was suspended in this solution for 15 minutes then centrifuged lightly (500 rpm for 5 minutes) 3 times, replacing the supernatant with distilled water each time.

Vaccine.

"Dictol" oral lungworm vaccine was provided by Glaxovet, Ware, England. This was pooled and used as a stock solution of 40 Kr, x-irradiated larvae.

In experiment 4 in Chapter 6, normal infective larvae were irradiated in the chamber of a Cobalt 60 source (Nuclear Engineering, Berkshire, England). The output of the irradiation source at the time of preparation was 695 rads/min. Therefore to achieve the required dosage of 70 kr, the larvae were left in the chamber for 101 minutes.

Irradiated larvae were counted as before and the required number suspended in 2ml. distilled water for injection.

Vaccination.

Doses of vaccine were administered by one of three routes:

1) Orally.

As described in the "Dictol" data sheets.

2) Intra-peritoneally.

Intra-peritoneal injections were performed using a 5 ml. sterile plastic syringe and 18 gauge 1.5 inch needles (Becton-Dickinson, Wembley, England). The site of injection was chosen as the lower portion of the iliac triangle of the calf's right flank.

3) Sub-cutaneously.

Sub-cutaneous injections were carried out using the same type of syringes as above and 20 gauge, 1 inch needles. The site chosen was a fold of skin on the right shoulder.

Post-mortem technique.

a) Calves.

Calves were stunned by a captive-bolt pistol and immediately pithed and bled out. The lungs were then removed together with the trachea, oesophagus, heart and pericardial sac.

The exterior of the lungs was washed in running water and the lungs laid out on plastic trays. Any consolidation present was drawn on to lung "maps" for later estimation of the lesion scores. The bronchi were then cut open using round-ended scissors and lungworms removed to normal saline with forceps. Worms were stored in saline at $4^{o}C$ and counted within a few days of collection. Lesion scores were calculated by assigning each of the lung maps described above a value between 0 and 5 where 0

represented no consolidation and 5, 50% consolidation.

b) Guinea-pigs.

Guinea-pigs were killed by deep anaesthesia in Trilene, followed by dislocation of the neck. The chest was opened with fine scissors and the lungs and heart removed. The lungs were then separated from the heart, chopped finely and suspended in gauze in warm saline overnight. After incubation, the lung tissue and gauze were discarded and the saline centrifuged to concentrate the lungworm larvae. The total number of larvae collected was then counted under a dissecting microscope.

Larval Viability.

The viability of Dictyocaulus infective larvae was estimated microscopically. Aliquots of larvae were taken from well-mixed samples, placed on clean, glass microscope slides and examined under a dissecting microscope. Larvae were considered to be alive if they were motile or capable of maintaining a tightly coiled posture. Results were expressed as the number alive as a percentage of the total. Normally a minimum of 100 larvae was counted.

Bottles Testing.

The number of larvae per ml. of vaccine in the bottles examined in Chapter Four were counted in the following way :
Bottles were inverted seven times to ensure adequate mixing, the caps were removed and the contents were emptied into a clean 100 ml. glass beaker. The total volume was poured from one beaker to another six times to ensure thorough mixing prior to the removal of sufficient fluid to fill the chamber of an eelworm

counting slide (Gelman-Hawksley, England). The number of larvae in the etched area was counted. This corresponded to 1ml. of vaccine. Five such aliquots were counted per bottle.

Sterilisation.

Bottles of nutrient agar or water, screw-capped glass tubes and cellulose acetate filters were sterilised in an automatic pressure cooker (Tower, England). Pressure was raised to 1.05 kg. per cm^2 (15 lbs./in^2) and maintained for 15 minutes.

Filtration apparatus was sterilised in a bench autoclave (Denley, Billingshurst, England) again at 1.05 kg/cm^2 for 15 min.

Culture Media.

Nutrient agar (Oxoid) was made up following the manufacturer's instructions. Batches of agar were dispensed into 30 ml. glass universals and sterilised as above. These were stored at 4°C until use. Prior to use the agar was melted in an instrument steriliser and poured on to sterile plastic petri dishes (Sterilin, Teddington, Middlesex, England). In certain instances this was enriched with 10% bovine blood freshly collected by sterile means.

Incubation.

Agar plates were incubated either at 37°C or room temperature under aerobic or anaerobic conditions. Aerobic conditions were achieved simply by placing the agar plates in a 37°C incubator or by leaving them in a plastic box on the bench. Anaerobic conditions were maintained using anaerobic jars which were gassed using Gaspak disposable hydrogen and carbon dioxide

generator envelopes (Becton-Dickinson, Wembley, England). Anaerobic jars were placed in a 37°C incubator or left on the bench.

Examination of agar plates.

After the incubation period had elapsed the plates were examined by eye and the number of discrete colonies counted. The colonies were presumed to have arisen from individual bacteria and their number was calculated back to give the bacterial concentration in the original preparation.

Filtration.

For determination of bacteriological sterility, preparations were passed through 0.22 um. pore size, 47 mm diameter cellulose acetate filters (Millipore, Germany) in a standard bacteriological filtration apparatus.

Virology.

The materials and methods used in the virus experiments are outlined in the appendix to Chapter Seven.

Chapter Three.

The preparation of a bacteriologically sterile irradiated larval vaccine.

The larval suspension used by Glaxo Operations Ltd. at Ware (Herts.) in the commercial production of Dictol, is isolated from faecal culture by a process of migration, sieving and washing. It could therefore be expected that Dictol would contain contaminating enteric bacteria. If adequate precautions were not taken, pathogens present in the culture calves could very quickly be spread *via* Dictol to calves all over Britain and indeed to many European countries. Obviously precautions such as screening the culture calves for the presence of *Salmonella spp.* are taken to prevent this. However, at present these are restricted to scrupulous cleanliness and prophylactic measures at Bury Green Farm and Lady Hayes Farm. While up to now these safety measures have proved adequate, and it could be argued that an oral vaccine does not need to be sterile, the time could arise when a sterile Dictol would be necessary in order to pass more stringent licencing requirements in the future or to sell Dictol in countries that are unwilling to import the current non-sterile product. It would also be of prime importance if a live injectable vaccine was to prove possible. The need for sterility in an injectable system is borne out by the perhaps apocryphal story related by Nelson (1984), where in the early days of the vaccine, one farmer complained to Allen and Hanbury's of the abscesses which formed when he injected his calves with Dictol.

Most of the usual methods of rendering substances sterile are not applicable to Dictol. Obviously heat treatment or autoclaving would kill the larvae. Filtration is equally impossible. Irradiating the vaccine with gamma rays would

possibly require up to four megarads - one hundred times the dose which is used to attenuate Dictol. The inclusion of an antibiotic in the larval suspension would be feasible but is undesirable on the grounds that it might select for antibiotic resistant strains. The only method remaining is the use of chemical disinfectants. Even here the choice of compound is limited. The use of disinfectants which incorporate heavy metals is considered undesirable. Also substances which are likely to exsheath the larvae have to be avoided. The ideal chemical (or commercial disinfectant) would have a wide spectrum of activity at very low concentrations, have no effect on the larvae, be easily incorporated into the production process and be available cheaply.

Determination of Contamination level.

A previously unopened bottle of normal D.viviparus third stage larvae from Ware (23,400 larvae/ml.) was examined for the presence of bacteria. Two 0.5ml. samples were removed from the bottle and diluted with sterile water to give final dilutions of 1/20, 1/200 and 1/2000. 4.5ml. samples of these dilutions were passed through 47mm. cellulose acetate filters with a pore size of 22um.. The filters were then incubated overnight at 37^{o}C on nutrient agar (N.A.) and blood agar (B.A.) under aerobic and anaerobic conditions.

The results of the incubation in aerobic conditions are shown in Table 1. Only minimal growth was observed on the plates incubated in anaerobic conditions.

Table 1.

The number of bacterial colonies observed after aerobic incubation of diluted larval suspensions.

Dilution :	1/20	1/200	1/2000
Blood agar	tmtc	2	10
Nutrient agar	tmtc	122	2

tmtc = too many to count.

From the 1/200 N.A. plate the contamination level was estimated to be 5.4×10^3 bacteria per ml. of the larval suspension.

The above procedure was repeated using an unopened bottle of Dictol. 20ml. was diluted to give 18ml. each of a 100%, 10% and 1% concentration. A sterile water control was also filtered. All were incubated on B.A. at $37^{o}C$ for 18 hours and the results are shown in Table 2.

Table 2.

The degree of bacterial contamination of Dictol.

Dictol Dilution	Number of colonies
Undiluted	tmtc
10% Dictol	1160
1% Dictol	170
Sterile water	0

The bacterial level in the Dictol bottle therefore was between 644 and 944 bacteria/ml. This is equivalent to between 15,500 and 22,700 bacteria per dose of Dictol.

Identification of Contaminants.

Selected colonies from the first filtration experiment outlined above were replated on B.A. These were sent to Dr Birkbeck, Microbiology Department, Glasgow University, for identification. This was done by grams stain and API strips for the identification of enteric bacteria. Of 9 samples examined, 3 appeared to be E.coli, 3 Pseudomonas spp. and 3 were unidentifiable. One of the E.coli isolates was maintained in culture for use in the subsequent bactericidal tests.

Disinfectant Screening.

A number of potentially bactericidal compounds were added to larval suspensions to determine their toxicity to Dictyocaulus L_3. The larvae were then examined microscopically for viability. A 10% formaldehyde (HCHO) solution killed the larvae within 30 minutes as did 1% HCHO and 1% Savlon. 0.8% Milton's Fluid exsheathed the larvae. The larvae survived 30 minutes in 1% Pevidene and 1% Jeyes Ibocol. They also survived 90 minutes in 1/10 N HCl at pH2 and 2% $K_2Cr_2O_7$.

Doubling dilutions of Cetavlon, Ibocol and Pevidene were made up ranging from 25% down to 0.8%. All dilutions of Cetavlon and Ibocol killed the larvae by 2 hours. Larvae survived slightly better in Pevidene in that a few larvae survived 3 hours in the

25% solution but even at the lower dilutions dead larvae were present.

37 ppm HCHO was tried next and larvae survived this for more than 2 hours. A 37ppm solution of HCHO was inoculated with bacteria, incubated for an hour and the fluid plated on to agar. Heavy growth was apparent on this and on a water control. This procedure was repeated with concentrations of HCHO ranging from 370ppm to 25ppm. Moderately heavy growth was found from all dilutions becoming heavier in the lower concentrations of HCHO.

A suspension of bacteria was made such that it had an optical density (O.D.) at 600nm. of 0.92 (approximately 10^9 bacteria/ml.). This was diluted 1:10 with sterile water then 0.1ml was added to 9.9ml. of the following solutions : 370ppm HCHO; 37ppm HCHO; 1% Pevidene; 2% $K_2Cr_2O_7$ and sterile water. After 1 hour at room temperature these were diluted 1:100 and 0.1ml was plated on to B.A. The results are shown in Table 3.

Table 3.

The bactericidal activity of some disinfectants.

Disinfectant	Number of colonies
370ppm HCHO	4
37ppm HCHO	tmtc
1% Pevidene	1
2% $K_2Cr_2O_7$	0
H_2O control	38

This above test was repeated with dilutions of $K_2Cr_2O_7$ and the results are shown in Table 4.

Table 4.

The bactericidal activity of dichromate dilutions.

Disinfectant dilution	Number of colonies
2% $K_2Cr_2O_7$	1
1% "	2
0.5% "	0
0.2% "	1
H_2O control	554

The percentage kill therefore was between 99.6% and 100% for all dilutions tested and at this point Potassium dichromate appeared to be the best solution examined.

Panacide.

At this stage a disinfectant marketed by BDH Chemicals (Poole, Dorset) called Panacide was investigated. This product claimed to be a good bactericide, algicide and fungicide at concentrations as low as 0.006% w/v of active ingredient. Panacide is the trade name for 5,5'-Dichloro-2,2'-dihydroxydiphenylmethane which has the common name dichlorophen. Panacide is approved by the M.A.F.F. for animal husbandry and the storage of wheat, potatoes and bananas. It is also permitted by the E.E.C. as a preservative for cosmetics. The pharmaceutical grade of dichlorophen is used as a Taeniacide in animals and man. The product investigated was a 40% w/w solution of the technical

grade monosodium salt of dichlorophen. All the subsequent tests were performed using dilutions of this stock solution and quoted as percentage w/v solutions of sodium dichlorophen.

Dichlorophen Results.

Dichlorophen was tested in a similar manner to that used for the previous compounds under investigation. Larvae were suspended in doubling dilutions of dichlorophen from 10% w/v solution to 0.004% w/v. After 3 hours no harmful effects could be seen below a 1.25% w/v solution.

Again a bacterial culture was made at an O.D. of 0.90. This was added in the ratio of 1:1000 to various dilutions of dichlorophen. After one hour incubation this was diluted a further 1:100 and 0.1ml. plated on to agar. The results are shown in Table 5.

Table 5.

The bactericidal activity of dichlorophen dilutions.

dichlorophen dilution	No. of Colonies
0.4%	0
0.24%	0
0.024%	0
0.0024%	0
sterile water	780

This represents 100% bactericidal activity at all the dilutions tested.

Following these promising results from dichlorophen a further viability test was performed.

A series of sterile stoppered tubes was set up each containing c730 L_3 in 1ml.. To this was added 3ml. dichlorophen (to a final dilution of 0.0024%) or 3ml. distilled water. The dichlorophen was left in the tubes for 1 hour then replaced with two changes of sterile water. The tubes were stoppered and stored at 4°C. On selected days thereafter, percentage viability counts were performed by microscope. These are shown in Table 6.

Table 6.

The percentage long-term viability of larvae washed in 0.0024% w/v dichlorophen or in distilled water.

Day	0	7	15	22	31	55
dichlorophen	98.8	98.3	99.1	98.4	98.2	96.0
Control	99.1	98.7	98.4	95.7	98.3	97.1

This shows that dichlorophen washed larvae remained as viable as the controls over a 55 day period (13 days beyond the 42 day shelf-life for which Dictol is licenced).

At day 15 in the above trial 0.1ml. from two control tubes and two dichlorophen washed tubes was plated on to nutrient agar. The results are shown in Table 7.

Table 7.

A comparison of numbers of bacteria in dichlorophen-washed or water-washed larval suspensions after 15 days storage.

Treatment	Number of colonies	
dichlorophen wash	0	0
water wash	75	90

At day 23, 20ml. of a 1/10 dilution from control and dichlorophen treated tubes was filtered and the filters incubated on nutrient agar. The results are shown in Table 8.

Table 8.

A comparison of numbers of bacteria in dichlorophen-washed or water-washed larval suspensions after 23 days storage.

Treatment	Number of colonies
dichlorophen wash	0
water wash	tmtc

Infectivity.

While the viability as determined microscopically may have remained high in the samples of washed larvae the relevance of this to actual infectivity is questionable. An experiment was therefore performed to infect calves with dichlorophen washed larvae. Six doses of 5,000 larvae were made up. Two were made to

20ml. with distilled water and used as controls. The remaining four were made to 20ml. as 0.0024% w/v dichlorophen. These were left for 90 minutes then the supernatant was replaced with sterile water 3 times - allowing 30 minutes for sedimentation between each wash. All six doses were stored at 4°C until used. The day the doses were made up was taken as day 0. Three calves were infected on day 14 and three on day 42, two dichlorophen treated and one control at each occasion. Each calf was killed 28 days after it was infected and the total number of worms present in the lungs was counted. The results are shown in Table 9.

Table 9.

Post-mortem worm burdens of calves infected with dichlorophen washed or unwashed larvae after 14 or 42 days storage.

	day 14 infection	day 42 infection
dichlorophen wash	1090	495
dichlorophen wash	1405	840
Control	1780	645

All calves displayed clinical symptoms of husk and at necropsy showed typical lungworm lesions.

Larval Filtration.

A final filtration experiment was performed to investigate the internal cleanliness of dichlorophen washed larvae. It was

thought possible that while the exterior of the larval sheath may have been freed from bacteria the inside of the sheath may still be able to harbour pathogens.

Larvae were suspended at 10,000 L_3/ml. and washed in dichlorophen followed by sterile water as before. Controls were set up using sterile water in place of dichlorophen and also with sterile water alone. One washed aliquot was subjected to grinding in a "Griffiths" tissue grinder. All the aliquots were filtered as before and the filters placed on nutrient agar. The results are shown in Table 10.

Table 10.

Numbers of bacteria in dichlorophen washed or unwashed larval suspensions and in washed larvae ground in Griffiths tube.

Treatment	Suspension Dilution 1/10	1/100	1/1000
larvae + Panacide	0	0	0
larvae + water	tmtc	62	4
larvae + Panacide + grinding	0	0	1*
water alone	0	0	0
water + grinding	0	0	0

* Single colony - regarded as extraneous contamination.

These results show that larvae treated with dichlorophen are bacteriologically sterile internally as well as externally.

Discussion.

As predicted Dictol has been shown to contain contaminating bacteria. These include bacteria of enteric origin such as E.coli. The possibility that transmission of pathogenic bacteria could occur via Dictol undoubtedly exists. A system based on a one hour immersion of the larval suspension in a 0.0024% w/v solution of the sodium dichlorophen followed by washing with sterile water has proved to free the larval culture from bacteria. Larvae so treated have been shown to retain their infectivity. It therefore seems that dichlorophen treatment could be incorporated into the production process and possibly prevent Dictol being implicated in any outbreaks of bacterial disease.

In light of the results of the parenteral vaccination experiments outlined in chapter six, further sterility experiments were carried out and these are described in chapter seven.

Chapter Four.

Investigations into the usefulness of plastics for packaging lungworm vaccine.

Since the introduction of Dictol by Allen and Hanbury's in 1959 the presentation of the vaccine has remained virtually unchanged. About 24ml. of Dictol is bottled in 33ml. glass bottles with metal screw caps. This allows for a large bubble of air which aids in mixing the vaccine immediately prior to use. The bottles are of a convenient design for use. They are sturdy enough to not break easily. Also they have a small enough opening to enable the farmer to hold the bottle in one hand with his thumb across the mouth to prevent spillage while the other hand is free to open the calf's mouth.

The main drawbacks to using glass bottles are their weight and their relative fragility. To overcome the risk of breakage in transit Dictol is well packaged. Twelve glass bottles are placed in a plastic tray in a cardboard box. Batches of these small boxes are put into larger boxes and sealed with nylon-web tape. The individual 12-bottle boxes when full weigh about 950 grammes of which 70% is made up of packaging (bottles plus box). This packaging obviously adds to the cost of producing the vaccine. The volume that packaged Dictol takes up when stored in the domestic refrigerator can also prove to be a nuisance.

If Dictol was packaged in bottles of a less fragile nature then the amount of cardboard and plastic protection could be reduced and production costs cut. For this reason replacing the glass bottles with unbreakable plastic bottles has been an attractive proposition. In the past, research at Ware has been carried out to examine the possibility of using polythene bottles or polypropylene bottles.

A study in 1968 of low density polythene bottles showed that

it was impossible to get as many live larvae out of the bottles as were put in. Even soon after the bottles were filled, the delivery of live larvae from the polythene bottles was lower than that from glass. This drop in numbers of live larvae was not mirrored by an increase in the numbers of dead larvae but was found to be due to large numbers of larvae adhering to the sides of the bottles. It was reported at the time that these larvae were very difficult to remove from the plastic by brushing or shaking.

A further trial was performed in 1981 using polypropylene bottles. This trial gave more promising results than the 1968 polythene bottles trial. On the basis of the 1981 results a larger trial was carried out in 1982. Also calf protection experiments were planned and tooling costs were projected. However, this repeat trial was not as successful as the 1981 trial. A large percentage of batches tested were deemed to be failures (i.e. delivered less than 40 larvae/ml.) and further investigation of polypropylene was suspended.

In the current work seven types of bottle were considered :

1) Glass - as currently used.
2) Polyvinyl chloride (P.V.C.) 30 ml.. Meason Freer Ltd.
3) P.V.C. 50 ml.. Meason Freer Ltd.
4) Polyethylene tetraphthalate (P.E.T.) Glaxo Operations Ltd.
5) Polypropylene ATO 3020. Rockware Plastics Ltd.
6) Polypropylene ATO 3020 + Clarifying Agent. Rockware Plastics
7) High Density Polythene. Rockware Plastics.

Before any experimental work was considered the high density polythene bottle was discarded as it was considered too opaque

and too slippy to be of any practical use. The 50ml. P.V.C. bottle was considered too large and was also removed from further investigation.

The remaining five bottle types were used in the following trial. Five bottles of each type were filled by pipette with 25ml. of a well mixed suspension of D.viviparus larvae. The bottles were stored at $4^{o}C$ for one week. 1ml. was removed from each of the bottles after thorough mixing and the number of larvae in that 1ml. sample counted.

Results.

The results of this preliminary experiment are shown in Table 11. These are expressed as the number of larvae recovered per ml. from each of the five bottles followed by the mean and standard error for the group.

Table 11.

The number of live larvae recovered per ml. from each of five bottles of each bottle type.

1) Glass.					
48.0	43.4	44.0	44.4	45.6	mean = 46.2 ± 1.5
2) P.V.C.					
47.0	47.8	43.6	41.2	44.2	mean = 44.8 ± 1.2
3) P.E.T.					
46.2	43.4	45.6	47.0	46.8	mean = 45.8 ± 0.7
4) Polypropylene.					
38.2	38.2	39.6	39.2	46.2	mean = 40.3 ± 1.5
5) Polypropylene + clarifying agent.					
46.8	42.2	44.0	41.6	50.0	mean = 44.9 ± 1.6

Following this preliminary trial it was decided that mimicking the production line fill process in the laboratory was not satisfactory. The previous work was therefore repeated using bottles filled on the Dictol production line at Ware.

Results.

The results of this trial are presented in Table 12. Again the mean number of larvae per ml. from each of four bottles of each type is listed along with the overall group mean and standard error.

Table 12.

The mean number of larvae recovered per ml. from each of four bottles of the five bottle types under investigation.

1) Glass				
76.0	64.2	84.4	67.0	mean = 72.9 ± 4.6
2) P.V.C.				
73.8	81.8	63.0	62.2	mean = 70.2 ± 4.7
3) P.E.T.				
67.8	89.2	71.8	92.4	mean = 80.3 ± 6.2
4) Polypropylene.				
60.8	78.2	59.0	68.6	mean = 66.7 ± 4.4
5) Polypropylene + clarifying agent.				
84.0	58.0	58.4	57.6	mean = 64.5 ± 6.5

From these preliminary trials the most useful bottle type appeared to be the P.E.T.. A larger scale trial was performed to compare glass and P.E.T. bottles in greater detail. Both bottle types were filled on the production line and sent to Glasgow where the number of live larvae in five 1 ml. samples from each bottle was counted. 100 P.E.T. and 50 glass bottles were tested at 2 weeks after filling and 25 of each type tested at 5 weeks after filling.

Results.

The results of this trial are summarised in Table 13.

Table 13.

A summary of the results of the comparison study on P.E.T. and glass bottles.

	2 weeks		5 weeks	
	glass	P.E.T.	glass	P.E.T.
number of bottles	50	100	25	25
mean larvae/ml.	67.5	69.9	67.2	61.5
standard error	0.67	0.47	0.67	1.91
mean dead/ml.	-	-	3.3	3.6
std. error dead	-	-	0.22	0.21

The individual aliquot counts for these 200 bottles are listed in the appendix along with their means.

The total volume of vaccine contained in each of 25 glass and 25 P.E.T. bottles was calculated using a 25 ml. glass pipette. The individual values are listed in the appendix but are summarised in Table 14.

Table 14.

The mean volume of vaccine contained in 25 glass and 25 P.E.T. bottles.

	glass	P.E.T.
mean volume (ml)	24.1	21.3
standard error	0.07	0.39
range of values (ml)	23.4 - 24.6	18.8 - 24.0

Ten of the P.E.T. bottles which gave the lowest number of larvae/ml. at week 5 above were emptied and cut open. The internal surfaces were examined microscopically for the presence of larvae adhering to the plastic. No such larvae could be found.

The data thus gathered were subjected to standard statistical analyses. Firstly it was necessary to show that all the sets of figures displayed normal distributions. This was essential if any parametric tests were to be subsequently employed. In a normal distribution >95% of the counts should lie in a range given by the mean $\pm$ "t" times the standard deviation for the group. "t" was obtained by use of "t" tables at a probability of 0.05 and n-1 degrees of freedom (where n is the number of counts in the group). The calculations for the eight groups are given in the appendix. All the groups displayed normal distributions about their means.

The significance of differences between pairs of group means were calculated using students t-tests. The formula used was :

$$t = \frac{X_1 - X_2}{Sp\sqrt{1/n_1 + 1/n_2}}$$

where : x = the group mean

n = the number in the group

Sp = the pooled standard deviation

Values for t were thus calculated and where t observed was greater than t at p = 0.05 and $n_1 + n_2 - 2$ degrees of freedom from t-tables, the difference was deemed to be significant. The

individual calculations are in the appendix and are summarised in Table 15.

Table 15.

Summary of significance tests on means.

Glass 2 wks. v P.E.T. 2 wks. - P.E.T. significantly greater.

Glass 2 wks. v Glass 5 wks. - No significant difference.

P.E.T. 2 wks v P.E.T. 5 wks. - No significant difference.

Glass 5 wks v P.E.T. 5 wks. - Glass significantly greater.

There was no significant difference between the numbers of dead larvae in the bottles at 5 weeks.

When interpreting data such as that presented above, it is essential not to lose sight of the practical implications especially where significance tests have been applied. The difference between the means of the Glass and P.E.T. bottles at 2 weeks is statistically significant showing P.E.T. to deliver more larvae per ml. In reality, however, the difference between the two means is only 2.4 larvae per ml. which would be of little significance in practice.

In contrast, the 8.4 larvae/ml. difference between the P.E.T. at 2 weeks and 5 weeks would at first glance appear to be important. Yet this is not significant statistically and is due to the very large range of values in the 5 week P.E.T. bottles. Since this variability is not caused by dead or adhering larvae it can only be accounted for by a greater variability in the

number of larvae injected into the P.E.T. bottles at filling. The figures for the total volumes of vaccine in the two bottle types show a wide variability in the P.E.T. bottles which is not present in the glass bottles. This directly reflects uneven filling on the production line. The filling machine at Ware has been designed to accommodate round, glass bottles. This it does very well, as is shown by the very small variation in the fill levels of the glass bottles. It does not, however, cope well with the P.E.T. bottles. Obviously if production was to switch to P.E.T. bottles the machine could easily be converted to produce consistent filling with these bottles. As it stands, the conclusion must be reached that the variability in results is due to the inconsistency at filling.

However, the fact remains that the P.E.T. bottles have performed well. There is no evidence to suggest that larvae die quicker in P.E.T. bottles than in glass. Nor is there any reason to suppose that fewer larvae can be recovered from these bottles than are originally put in. Indeed none of the bottles examined fell below the mean delivery of 40 larvae/ml. which Glaxo have in the past used as the criterion for success. Further testing would be necessary before retooling for P.E.T. could be recommended. Before any more work is done, however, some way will need to be found to eliminate the filling inconsistencies which undoubtedly exist.

Chapter Five.

A Freeze-dried Larval Vaccine.

The cost of postage is one of the factors influencing the retail price of Dictol. It has been suggested that if both doses were despatched together then costs would be reduced. However, this would mean that the second dose would be at least 31 days old before it was used. While still within the licenced shelf-life, such storage would be undesirable. Even if the delay in usage could be reduced by shortening the time interval between the first and second doses, the presence of a large amount of Dictol in the domestic refrigerator for any length of time could prove unpopular.

One possible solution to this problem might be the use of an injectable dead vaccine to replace the second dose. It is known from earlier experiments (Jarrett et al. 1960) that a single intramuscular injection of a freeze-dried adult worm preparation confers a certain degree of immunity. While this immunity on its own is not sufficient to give calves a satisfactory degree of immunity to subsequent infection, it is possible that such an antigenic preparation superimposed on the partial immunity gained from a single oral dose of Dictol may boost the protection to adequate levels.

However, the use of adult worm extract as antigen is probably not a good commercial proposition. The collection of such material would necessitate the slaughter of infected calves, the cost of which would probably outweigh any savings made on postage and packaging. For this reason it was decided in the current experiment that any source of antigen would have to be the normal infective L_3.

If an L_3 extract proved possible as a second dose an added

benefit would be that such dead material could be manufactured during the summer months when Dictol is not being produced. At present, production is concentrated within a few months with the larval production capacity only ticking over in the remaining part of the year.

In deciding the amount of material to use per dose, it was considered that the benefits would be lost if the 1,000 larvae oral dose had to be replaced by an extract from a massive number of larvae. For this reason a dose of 1,000 larvae or their equivalent was chosen.

It was further decided that freeze-drying was a convenient and very simple method of preparing the antigen and that as this was essentially a preliminary experiment no adjuvant should be included.

The possible injection routes were: intramuscular, subcutaneous, or intra-peritoneal. The intra-peritoneal route was considered undesirable. The other two routes seemed both equally easy and practicable and the subcutaneous was chosen.

Freeze-dried vaccine.

Aliquots of 1000 normal D.viviparus L_3 in 1ml. distilled water were dispensed into 2ml. glass tubes and frozen in a -20°C freezer. Tubes of frozen larval suspension were then placed in a freeze-drier (Edwards, Crawley, England) overnight. Tubes were then stoppered and stored at 4°C until use. Immediately prior to use the doses of vaccine were reconstituted with 1ml. sterile water and each was administered subcutaneously into the animal's right shoulder.

Experimental Design.

15 male Friesian-cross calves were divided into 3 groups.

Group One. Each of 5 calves were given normal oral doses of Dictol on Days 0 and 28.

Group Two. Each of 6 calves was given an oral dose of Dictol on Day 0 and a subcutaneous injection of 1,000 reconstituted freeze-dried larvae on Day 28.

Group Three. This group of 4 calves received no treatments on Days 0 or 28.

All calves were challenged with 30 L_3/Kg on Day 56 and killed on Day 84.

Results.

At post-mortem, worm burdens and lung lesion scores were assessed. These are shown in Tables 16 and 17 respectively. The mean respiratory rates of the three groups are presented in Fig. 5.1.

Table 16.

The post-mortem worm burdens of individual animals following challenge with 30 L_3/Kg. in groups given : 1) two doses of Dictol, and 2) one dose of Dictol followed by one dose of larval antigen, compared with 3) challenge only controls.

	Group One	Group Two	Group Three
	0	736	1110
	0	385	1256
	0	502	902
	59	410	1844
	413	820	
		*	
mean	94.4	570.6	1278
protection	92.6%	55.4%	

* This animal inhaled ruminal contents at after stunning, rendering estimation of worm burden unreliable.

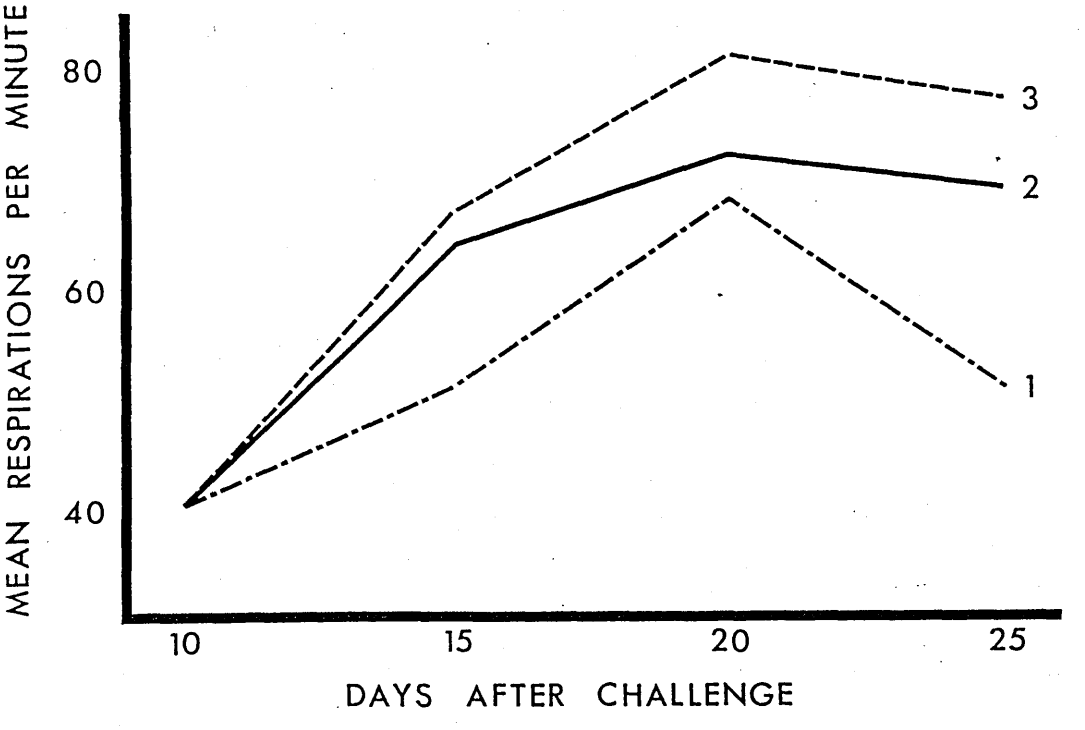

Fig.5.1. The mean respiratory rates, following challenge with 30 D.viviparus L_3/Kg. in groups of calves vaccinated with 1) two doses of Dictol or 2) one dose of Dictol followed by one dose of freeze-dried larvae, compared with 3) challenge only controls.

Table 17.

The post-mortem lung lesion scores of individual animals following challenge with $30L_3$/Kg. in groups given : 1) two doses of Dictol, and 2) one dose of Dictol followed by one dose of larval antigen compared with 3) challenge only controls.

	Group One	Group Two	Group Three
	0	2	*
	0	2	2
	1	3	2
	1	3	3
	3	4	
		4	
	----	----	----
mean score	1	3	2.3

* This animal died prior to necropsy. Post-mortem changes in lung condition prohibited calculation of a lesion score.

Discussion.

In terms of worm burden, respiratory rate and lung lesions, **Group One** showed a response typical of double vaccination with Dictol. In contrast **Group Three** displayed the normal reaction of naive calves experiencing a large challenge infection. When worm burdens are expressed as a percentage reduction in "take" in the vaccinates relative to the controls, the Dictol group has performed well, the protection being 92.6%.

Group Two, the experimental group, did not perform so well. In terms of worm burden this group showed only a 55.4% reduction over the controls. The respiratory rates of this group did not reach the levels of the control group but neither did they show the decrease from around day 20 after challenge shown by the Dictol group. Also the degree of pulmonary consolidation was greater than that of the calves in Group One.

Obviously this vaccination regime could not be recommended as a replacement for the current vaccine. Indeed this experiment shows the necessity of the second vaccine dose. However, this work by no means exhausts the possibilities of a killed injectable second dose since only one means of antigen preparation was investigated and only one dose level tried. Possibly also, the incorporation of a suitable adjuvant would improve the degree of protection against challenge.

Chapter Six.

Parenteral Vaccination Experiments.

Parenteral vaccination against infection with D.viviparus may have various advantages over oral vaccination not least of which is the greater ease of handling when larger calves are to be vaccinated. Three factors had now combined to make parenteral vaccination with live larvae worthy of closer investigation :

1) The evidence of Wade & Swanson (1958) that patent infections could be initiated by the parenteral injection of normal larvae.

2) The fact that farmers are now accustomed to using parenteral preparations themselves without veterinary supervision.

3) The techniques outlined earlier in this thesis which enable larvae to be made bacteriologically sterile and thus more acceptable for parenteral administration.

Guinea-pig Experiments.

Introduction.

Guinea-pigs have been widely used as experimental hosts for Dictyocaulus viviparus (Soliman 1953; Poynter et al. 1960; Wade, Fox and Swanson 1960 a; 1960b; Wade, Swanson and Fox 1961; Wilson 1966). This species is routinely used by Glaxo Animal Health at Ware to assess the infectivity and attenuation of batches of Dictol. In the experiment outlined here, guinea-pigs were used as model hosts to investigate the infectivity of larvae when administered by the sub-cutaneous route.

Experimental Design.

Nine Duncan-Hartley guinea-pigs were divided into three groups.

Group One consisted of four animals. These each received one dose of 5,000 exsheathed third-stage larvae subcutaneously.

Group Two was of two animals each receiving 5,000 normal L_3 subcutaneously.

Group Three had three animals. These were infected orally with 5,000 normal larvae and acted as controls.

Results.

The post-mortem worm recoveries are shown in Table 18.

Table 18.

The post-mortem worm burdens of guinea-pigs infected with :

1) 5,000 exsheathed D.viviparus L_3 subcutaneously,

2) 5,000 normal L_3 subcutaneously or

3) 5,000 normal L_3 orally.

	Group One	Group Two	Group Three
	1	127	199
	74	135	211
	179		222
	321		
	---	---	---
mean	144	131	211
recovery	2.9%	2.6%	4.2%

Discussion.

From the above experiment it was apparent that in guinea-pigs larvae were capable of migrating to the lungs after subcutaneous administration. Also this development was not dependent on prior exsheathment of the larvae.

Calf Experiments.

Introduction.

The work of Wade & Swanson (1958) was carried out to investigate the possibility that lungworm disease in calves could be initiated by infection through the skin, either through wounds and insect bites or by the active penetration of larvae. In these experiments patent infections were achieved. However, several hundred thousand larvae were administered and it could be argued that the larvae which reached the lungs did so only by chance. It was therefore essential to attempt subcutaneous infection in calves using a modest number of larvae.

Experiment One.

In this preliminary experiment, one Friesian cross calf was injected in its right shoulder with 3,200 normal D.viviparus L_3 (30 L_3/Kg).

Results.

A normal infection established in this calf. Faecal samples became positive for lungworm larvae on Day 28 and remained positive until Day 50.

Experiment Two.

Since this preliminary experiment suggested that a patent infection could be satisfactorily achieved with a moderate number of larvae, then it seemed possible that vaccination by the same route might also be possible.

Experimental Design.

Nine Friesian cross calves were divided into 5 groups and treated with either one or two injections of irradiated third-stage larvae sub-cutaneously or intra-peritoneally as summarised in Table 19.

Table 19.

An outline of the design of Experiment Two.

Group	No.of calves	Day 0	Day 28	Day 56	Day 84
i	2	-	s/c	C	K
ii	2	s/c	s/c	C	K
iii	2	-	i/p	C	K
iv	2	i/p	i/p	C	K
v	1	-	-	C	K

s/c = injection of 2,400 irradiated L_3 sub-cutaneously.

i/p = " " " " " intra-peritoneally.

C = Challenge with 30 L_3/Kg.

K = Kill

Results.

The post-mortem worm burdens of the five groups are shown in Table 20.

Table 20.

The post-mortem worm burdens of calves vaccinated with one or two doses of irradiated larvae subcutaneously or intraperitoneally.

Group :	i	ii	iii	iv	v
	80	18	3	2	1393
	264	109	300	790	
mean	172	63.5	151.5	396	1393
protection	87.7%	95.4%	89.1%	71.6%	-

Discussion.

From these results it appeared that double sub-cutaneous vaccination gave comparable protection to that which could be expected from oral Dictol. However, since little significance can be attached to the results of two calves, this vaccination regime was repeated on a larger number of animals.

Experiment Three.

In this experiment fourteen Friesian cross calves were divided into two groups.

Ten calves were given a sub-cutaneous injection of 1,000 irradiated larvae on Days 0 and 28. These calves and 4 challenge controls were challenged with 30 L_3/Kg. on Day 56 and killed on Day 84.

Results.

The post-mortem worm burdens are shown in Table 21, the lung lesion scores in Table 22 and the respiratory rates in Fig. 6.1.

Table 21.

The post-mortem worm burdens of subcutaneously vaccinated calves challenged with $30L_3$/Kg. compared with unvaccinated challenge controls.

	Vaccinates	Controls
	0	514
	0	946
	0	1025
	2	2250
	2	
	9	
	34	
	38	
	150	
	290	
mean	52.5	1184.0
protection	95.5%	

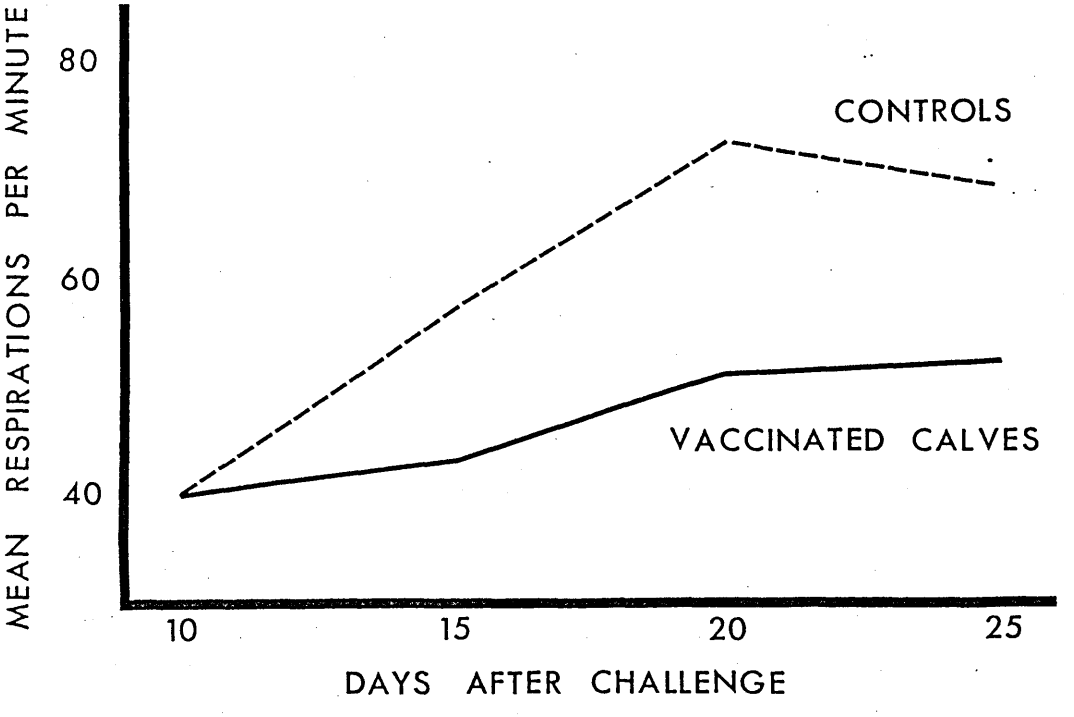

Fig. 6.1. The mean respiratory rates following challenge with 30 D.viviparus L_3/Kg. of a group of calves vaccinated subcutaneously with two doses of irradiated larvae, compared with challenge-only controls.

Table 22.

The lung-lesion scores of subcutaneously vaccinated calves challenged with 30 L_3/Kg. compared with unvaccinated challenge controls.

Vaccinates : 1,1,1,1,1,1,2,2,3,3 mean = 1.6

Controls : 2,2,3,4 mean = 2.75

All controls showed a positive faecal larval count at necropsy compared with none in the vaccinates.

Discussion.

In terms of respiratory rate, worm burden, lung lesion score and faecal larval output, vaccination with two doses of 1000 irradiated D.viviparus L_3 subcutaneously would appear to have been successful and gave a mean protection over controls of 95.5%. Results from use of oral Dictol (quoted in Poynter, 1970) range from 88.6% to 99.6% protection. It is therefore reasonable to conclude that oral and subcutaneous administrations of live attenuated vaccine are equally effective.

Experiment Four.

While double sub-cutaneous vaccination had proved successful it was considered that a single dose vaccine would be advantageous. The original theory underlying Dictol was that the larvae should be attenuated in such a way that the majority died

in the lymph nodes. Clearly though, significant numbers of the 40Kr irradiated larvae reach the lungs (Poynter et al 1960). Perhaps if the dose of irradiation was increased, a greater percentage of larvae would die at an earlier stage of development in the lymph nodes and consequently be more immunogenic allowing a single dose vaccine to be effective. With oral vaccination, however, increasing the irradiation dose runs the risk of reducing the invasiveness of the larvae. With subcutaneous vaccination, such problems may be reduced.

It was decided, therefore, to carry out an experiment to further investigate the degree of protection afforded by a single subcutaneous dose of 40Kr irradiated larvae when compared with a single subcutaneous dose of larvae irradiated at a higher than normal level.

The simplest method of irradiation available was gamma-irradiation from a ^{60}Co source. A radiation dose of 70Kr was chosen which by the RBE value of Kassai, Fitzpatrick & Mulligan (1966) would be equivalent to 49Kr from an x-ray source.

Experimental Design.

Twelve calves were divided into three groups.

Group One. Five calves were each given a single dose of Dictol sub-cutaneously. This was adjusted to 1500 L_3 per dose.

Group Two. The four calves in this group were each given a single subcutaneous dose of 2000 L_3 irradiated at 70Kr from a Cobalt source.

Group Three. Three calves served as challenge controls.

Groups One and Two were vaccinated on Day 0. All the calves

were challenged with 5,000 L_3 on Day 38 and killed on Days 63 - 65.

Results.

The post-mortem worm burdens, faecal larval counts and lung lesion scores are shown in Tables 23, 24 and 25 respectively. The mean repiratory rates of the groups of calves are shown in Fig. 6.2.

Table 23.

The post-mortem worm burdens of calves challenged with 5,000 D.viviparus larvae following subcutaneous vaccination with a single dose of 40 Kr. irradiated larvae (Group One) or a single dose of 70 Kr. (cobalt) irradiated larvae (Group Two) compared with challenge-only controls (Group Three).

	Group One	Group Two	Group Three
	0	299	63
	2	400	170
	19	518	372
	25	785	
	320		
mean	73	501	202

Table 24.

The number of D.viviparus larvae per gramme of faeces at post-mortem of calves challenged with 5,000 larvae following subcutaneous immunisation with a single dose of 40 Kr. irradiated vaccine (Group One) or a single dose of 70 Kr. (Cobalt) irradiated vaccine (Group Two) compared with challenge controls (Group Three).

	Group One	Group Two	Group Three
	0	*	6
	0	38	16
	0	48	25
	0	103	
	10		
mean	2	63	16

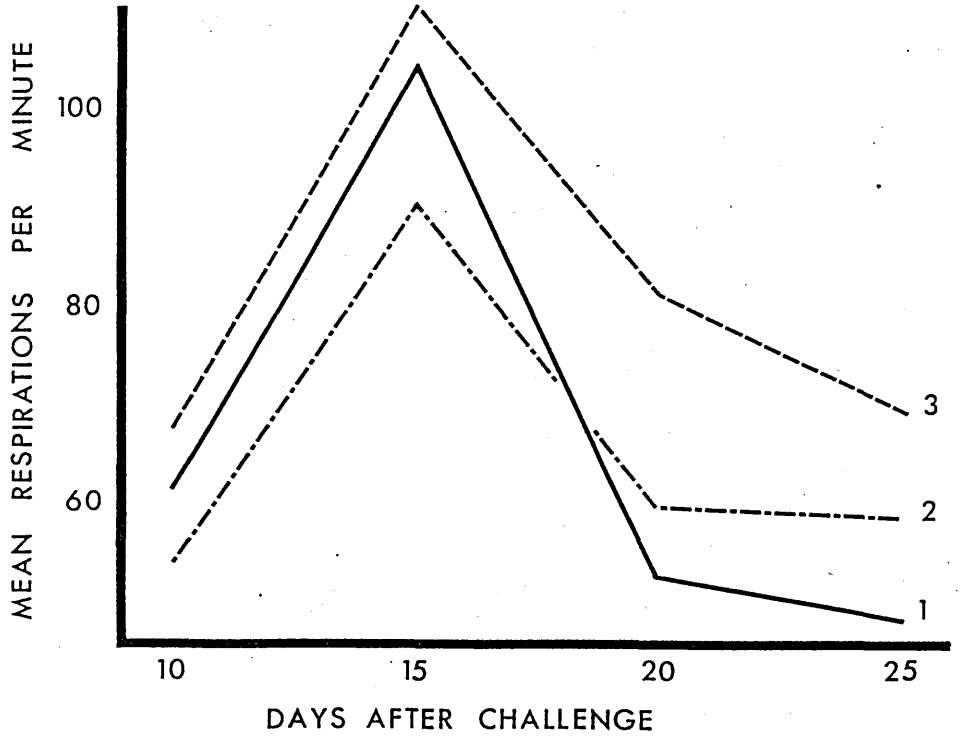

Fig. 6.2 The mean respiratory rates, following challenge with 5,000 D.viviparus L_3/Kg. of groups of calves vaccinated with 1) a single dose of 40Kr. irradiated vaccine or 2) a single dose of 70Kr. (Cobalt) irradiated vaccine compared with 3) challenge-only controls.

Table 25.

The post-mortem lung-lesion scores of calves vaccinated with a single dose of 40 Kr. irradiated larvae (Group One) or a single dose of 70 Kr. (Cobalt) irradiated larvae (Group Two) and subsequently challenged with 5,000 D.viviparus L_3 compared with challenge only controls.

Group One	Group Two	Group Three
1	*	2
1	3	2
1	3	3
2	4	
3		
1.6	3.3	2.3

Discussion.

Caution has to be exercised in the interpretation of the results of this experiment. The challenge controls, **Group 3,** showed poor worm burdens at post-mortem and this fact overshadows the results of the experimental groups. What can be definitely concluded is that single subcutaneous vaccination with 70Kr ^{60}Co irradiated larvae afforded the calves no protection. This possibly suggests that the larvae were no longer sufficiently invasive to leave the injection site.

In **Group One,** four out of the five animals would appear to

have been well protected against challenge. One can only speculate on the reason for the apparent failure of vaccination in the fifth calf; perhaps the most likely explanation is that it responded poorly due to innate unresponsiveness or to an infectious pneumonia. Overall, the percentage protection of **Group One** over the challenge controls was 64%. This is obviously a long way short of a satisfactory level of protection although this figure could be artificially low due to the very low "take" in the challenge controls. It is interesting that if the 4 animals with the lowest worm burdens are compared with the control group then the protection could be described as 94%.

From this experiment there is no firm evidence that a single subcutaneous injection of 40Kr x-irradiated larvae gives sufficient protection against subsequent challenge. However, a repeat of this experiment would be desirable before the use of a single dose of injectable vaccine is ruled out.

Chapter Seven.

The development of a sterile subcutaneous irradiated larval vaccine.

The experiments described so far have shown that it is possible to produce a lungworm vaccine which is bacteriologically sterile and that live vaccine is equally effective whether administered orally or subcutaneously.

Although these conclusions indicate that a sterile, parenteral larval vaccine is feasible, the more stringent requirements of commercial production require the investigation of a number of further parameters.

For example, the sterilisation procedure would be considerably simpler if dichlorophen could be incorporated into the final vaccine, rather than using a system whereby the larvae are washed in dichlorophen and resuspended in sterile water. If this is possible it is obviously necessary to investigate the effect on D.viviparus larvae of prolonged exposure to dichlorophen.

This treated vaccine can at present only be described as "clean". In order to be marketed as a truly sterile preparation it would have to be shown to be free from all extraneous bacteria, fungi, mycoplasmas and viruses.

The final experiments outlined in this thesis were performed with four aims.

1) To provide further evidence of the degree of sterility of dichlorophen-treated vaccine in order to satisfy licencing requirements.

2) To prove the efficacy of dichlorophen-treated vaccine.

3) To test the safety of dichlorophen-treated vaccine.

4) To compare the shelf-life of dichlorophen-treated and

untreated larvae.

STERILITY.

New veterinary products must conform to the guidlines laid down by the British Pharmacopoeia, the British Veterinary Pharmacopoeia and the European Pharmacopoeia. In this respect a prescribed number of samples from each batch of a particular product must be tested and shown to be free from any contaminating organisms. Dictol was first marketed in 1959, before the Veterinary Products Committee (VPC) came into being. It, therefore, has a "licence of right" in that it has never been assessed by the VPC and has consequently never been asked to conform to the sterility guidlines. If, however, the product licensing authorities were to reassess Dictol it seems likely that they might insist on sterility. Furthermore, while the pharmacopoeiae only require a product to be demonstrably free from contamination, it is possible that the authorities would like it proven that pathogens accidentally introduced into the vaccine are removed by the production process. To this end therefore, experiments were carried out where vaccine was artificially contaminated with potential pathogens, subjected to the usual treatment process as would occur in the factory and then assayed for the pathogen. Experiments with virus contamination were undertaken in conjunction with Professor O. Jarrett of the Department of Veterinary Pathology, Glasgow University and experiments on bacterial contamination with Dr. H. Birkbeck of the Microbiology Department, Glasgow University.

BACTERIOLOGY.

The experiments in Chapter 3 showed that a one hour wash in 0.0024% w/v dichlorophen rendered third stage Dictyocaulus larvae sterile.

Following discussions with the manufacturers of Panacide, BDH, (Purkiss, personal communication) it became clear that a higher dichlorophen dilution might be desirable. Purkiss suggested that the most difficult organism to kill with dichlorophen would be Pseudomonas spp. and recommended a 0.009% w/v dichlorophen solution. However it was thought that the lowest effective concentration should be used in order to minimise the risk of reducing the infectivity of the larvae. A 0.004% w/v solution was therefore chosen.

Initially dichlorophen was added to three bottles of Dictol to a final concentration of 0.004% w/v. These were left at $4^{o}C$ for 24 hours and then the total contents of each bottle filtered on to separate cellulose acetate filters; this process of filtration was repeated with three control bottles of Dictol. The filters were incubated in thioglycolate medium for seven days as recommended by the British Pharmacopoeia. Bacterial growth was observed in the three control flasks but no growth occurred from the three treated bottles. This showed that incorporation of 0.004% w/v dichlorophen rendered Dictol bacteriologically sterile after 24 hours at $4^{o}C$.

Following this, it was decided to show if such treatment would kill 100% of deliberately added bacteria. Suspensions of E.coli were made up at 10^4, 10^3 and 10^2 organisms per ml. of

sterile distilled water. To aliquots of each bacterial suspension was added dichlorophen to give a final concentration of 0.004% w/v dichlorophen. All bacterial suspensions were stored at $4^{o}C$ for 24 hours. Duplicate bacterial suspensions in sterile water acted as controls. Again these were filtered and grown in thioglycollate medium for seven days.

No growth was apparent in the dichlorophen treated aliquots whereas growth occurred in all the controls.

This above procedure was repeated using Pseudomonas aeruginosa added to Dictol giving final concentrations of 10^4, 10^3 and 10^2 bacteria per ml. of vaccine in the presence or absence of 0.004% w/v dichlorophen. Again incubation at $4^{o}C$ for 24 hours was followed by filtration and incubation. Growth was seen in all the cultures with the exception of the 10^2 bacteria per ml dichlorophen treated vaccine. Clearly then, 24 hours at $4^{o}C$ was insufficient to kill large quantities of artificially introduced Pseudomonas.

It seemed probable that the bactericidal action of dichlorophen could be improved by increasing the temperature or by lengthening the incubation period prior to filtration. Again Pseudomonas was added to Dictol at 10^4, 10^3 and 10^2 organisms/ml. in the presence or absence of dichlorophen. Duplicates were incubated at $20^{o}C$ for 24 hours and $4^{o}C$ for 40 hours. As before, following incubation, these were filtered and the filters incubated in thioglycollate. Bacterial growth was observed in all the controls and in the highest bacterial concentration (10^4) of the $4^{o}C$ incubated group.

It was becoming evident that to kill efficiently

large numbers of added Pseudomonas, vaccine would either have to be left at room temperature for a period of 24 hours or remain at 4°C for a prolonged period.

In the normal course of events, Dictol is left at ambient temperature from the time that the bottles are filled until the day's bottling is completed and the pallets of boxed vaccine are put into a 4°C store. Vaccine is despatched from this store after anything from 18 hours to 7 days. It can be safely assumed that no vaccine is used before four or five days after filling.

A sterilising regime which relied on vaccine remaining at 20°C for 24 hours could not be recommended as it may have an adverse effect on the longevity of the larvae. Similarly a prolonged treatment at 4°C might mean that a heavily contaminated batch of Dictol would be at the farm before it could be guaranteed sterile. Increasing the dichlorophen concentration was also thought undesirable and would have necessitated repeating the safety/efficacy work which was already in progress. A compromise was therefore investigated whereby 0.004% w/v dichlorophen treated vaccine would be incubated at room temperature for 6 hours followed by three days storage at 4°C.

Initially an unopened box of Dictol was selected and to ten of the bottles was added 240 ul of 0.4% w/v dichlorophen giving a final dichlorophen concentration of 0.004% w/v. The remaining two bottles were untreated and acted as controls. All the bottles were left at room temperature for 6 hours and then placed in a 4°C fridge for 3 days. At the end of this time all the bottles were filtered and the filters incubated as before. There

was no growth in the dichlorophen treated bottles compared with heavy growth in the two controls.

This procedure was repeated with Pseudomonas aeruginosa added at 10^4 organisms/ml vaccine. As before the ten dichlorophen treated and two control bottles were left at room temperature for 6 hours followed by $4^{o}C$ for 3 days. Again, following incubation of filters in thioglycollate broth, bacterial growth was only observed in the controls.

This last experiment showed that a technique had been devised which could kill 100% of a large volume of an artificially added contaminant which is known to be relatively highly resistant to the bactericidal action of dichlorophen. It now seemed sensible to test this protocol against a range of potential pathogens chosen to represent the major groupings of bacteria. Since the results of these experiments would be likely to be submitted to the Ministry of Agriculture it was thought essential to use specified strains of bacteria obtained from the Central Public Health Laboratory's National Collection of Type Cultures.

To this end therefore, the following organisms were selected:

Escherichia coli.....NCTC 9002

Salmonella abony.....NCTC 6017

Pseudomonas aeruginosa.....NCTC 6750

Clostridium perfringens.....NCTC 8237

As before the organisms were added to unopened bottles of vaccine to a final concentration of 10^4 organisms per ml. Twelve bottles were inocculated with each species of bacterium. To ten

of the bottles was added dichlorophen to a final concentration of 0.004% w/v. The remaining two bottles acted as controls.

The results of this test are shown in Table 26.

Table 26.

The sterility of dichlorophen treated vaccine following the addition of 10^4 bacteria per ml. and incubation at $20^{\circ}C$ for 6 hours and $4^{\circ}C$ for 72 hours.

Bacterium added	number of bottles sterile
E.coli	10/10
S.abony	10/10
Ps.aeruginosa	10/10
Cl.perfringens	10/10

The dichlorophen-treated vaccine was found to be free from contamination in every case. All the controls gave heavy bacterial growth.

VIRUS EXPERIMENTS.

The problem of virus contamination in Dictol is in a sense less serious than that of bacterial contamination. Unlike many bacteria, viruses are unable to replicate in a cell-free medium. Their numbers are therefore unlikely to increase during storage of Dictol. In addition, certain viruses quickly lose infectivity when suspended in water. However, it must be appreciated that these viruses which can survive long periods outwith the host are extremely resistant to killing by chemical means.

The initial isolation of larvae from faeces depends on migration of larvae to the surface of the faecal pat. This stage alone ensures that larvae are selected out of the vast bulk of any pathogens in the faeces. After this there follows a process of various stages of filtration, migration and sedimentation. The amount of water used in this process is unknown (Jenkins, personal communication.) but represents a quite considerable dilution of the faecal matter. The final dilution from the irradiation vial to bottling is, however, quantifiable and at peak production is typically about 1:40,000. It is considered that this final dilution would effectively reduce any viral contamination to below detectable and infective levels.

There were two principal objectives to the virus experiments which are outlined here. Firstly it was decided to determine the amount of viricidal action which dichlorophen itself possesses. The second objective was to examine the combined effect of washing the larval suspension with a large volume of water followed by the addition of dichlorophen to the final suspension. To do this, selected viruses would be required to be added to a

suspension of irradiated larvae; the suspension would then be washed with a large volume of water and after sedimentation to the original volume, dichlorophen added in order to mimic the likely production process. At the end of this process the vaccine would be examined for presence of virus.

Experiment One.

Dichlorophen is reported to have a certain amount of action against lipophilic viruses such as Newcastle disease virus (Purkiss, BDH - personal communication). It was therefore decided to assess the activity of dichlorophen against the lipophilic IBR virus.

Three vials were prepared. One contained cell-culture medium, one had 0.005% w/v dichlorophen in water and the remaining vial contained 0.01% w/v dichlorophen in water. To each was added c2.0 x 10^6 infectious units of IBR virus per ml. These were stored at 22^{o}C and assayed for virus at 0, 3 and 24 hours.

Results.

The level of virus at the three time intervals in the three groups are shown in Table 27.

Table 27.

The virucidal activity of dichlorophen against IBR virus.

Group	plaque forming units virus per ml. at time.		
	0 hours	3 hours	24hours
Control	2.2×10^6	8.0×10^5	1.3×10^6
0.005% w/v	1.4×10^6	1.8×10^5	4.0×10^4
0.01% w/v	1.2×10^6	3.0×10^4	7.0×10^2

Discussion.

These results show that at 24 hours dichlorophen reduced the level of virus by 10^4 in the higher dichlorophen concentration and by 10^2 in the lower dichlorophen concentration. While this activity is quite considerable it is probably insufficient alone to render an artificially contaminated bottle of Dictol sterile.

Experiment Two.

The second virus experiment was carried out to examine the combined effect of dichlorophen treatment and the dilution factor in the production process. While the estimated dilution factor between irradiation and bottling was about 1:40,000 for oral vaccine this would fall to about 1:2,000 for an injectable preparation due to the smaller final volume. This dilution factor was therefore chosen in the following experiment.

Design.

Approximately 10^6 infectious units of IBR virus were added

to two standard bottles of Dictol. One of these was diluted 1:2,000 with distilled water by a process of centrifugation and supernatant replacement. 240ul of 0.4% w/v dichlorophen was added to give a final concentration of 0.004% w/v. Both bottles remained on the bench for 6 hours followed by three days at $4^{o}C$. After this time both bottles were assayed for the presence of virus.

Results.

No virus was isolated from either bottle.

Discussion.

This experiment showed that the viricidal effect of low temperature storage in water was sufficient to reduce contamination to below detectable levels. It would also suggest that the margin for safety in the diluted and dichlorophen treated bottle must be extremely large.

Experiment Three.

The above experiment was repeated using Bovine Virus Diarrhoea virus (Mucosal Disease Virus) in place of IBR virus. Again, 10^{6} infectious units of virus were added to two bottles of Dictol. One of these was diluted and treated with dichlorophen as above. Both were incubated for 6 hours at $22^{o}C$ and three days at $4^{o}C$.

Results.

Again no virus could be isolated from either bottle.

Discussion.

The experiments outlined above show that if IBR or BVD virus was present in the larval suspension, dilution in water alone without the addition of dichlorophen will prevent the virus from being present in the final vaccine. However, this work will need to be repeated with such other viruses as are suggested by the Ministry of Agriculture.

One possibility that has yet to be investigated is that viruses could be present within the larvae. It is known that Metastrongylus apri larvae harbour the virus which causes swine influenza and act as a method of transmission for this virus from pig to pig (Shope,R.E., 1943, J. Exp. Med. 77 111-38). Experiments to rule out the presence of viruses in Dictyocaulus larvae would be extremely difficult to carry out and were considered beyond the scope of the current work.

EFFICACY EXPERIMENT.

The commercial success of oral Dictol has been mainly founded on its known high efficacy in protecting against clinical parasitic bronchitis. It now appeared that a parenteral preparation could be as effective as the oral product. However, no experiments had been carried out to test that dichlorophen-treated vaccine was as effective as untreated vaccine. To this end, therefore, the following experiment was carried out to determine the level of protection conferred by sterile injectable and sterile oral vaccination.

Design.

Eighteen cross calves were divided into three groups of six calves.

The calves in **Group One** were each vaccinated orally with 1,500 dichlorophen-treated, irradiated larvae on days 0 and 28.

The calves in **Group Two** were each vaccinated subcutaneously with 1,500 dichlorophen-treated, irradiated larvae on days 0 and 28.

The calves in **Group Three** remained unvaccinated and acted as challenge controls.

All three groups of calves were challenged with 3,000 normal lungworm larvae on day 56 and killed on days 80 and 81.

Results

i) Vaccination.

No adverse reactions were noted at the sites of subcutaneous vaccination.

Transient periods of coughing were noted amongst both groups of vaccinated calves. An independent assessment of the degree of

coughing suggested that this was greatest in the orally vaccinated group.

ii) Respiratory Rates.

The mean respiratory rates of the groups of calves in this experiment are shown in Fig. 7.1. Following challenge, the respiratory rates of the calves in **Group Three** rose earlier, reached a higher peak and decreased more slowly than those of the two vaccinated groups. No difference was observable between the two vaccinated groups.

iii) Post-mortem Worm Counts.

The worm burdens of the 18 calves are listed in Table 28.

Table 28.

The post-mortem worm burdens of calves challenged with 3,000 lungworm larvae following oral vaccination (Group One) or subcutaneous vaccination (Group Two) compared with unvaccinated controls (Group Three).

	Group One (Oral vacc'n)	Group Two (s/c vacc'n)	Group Three (controls)
	0	0	864
	0	3	1062
	0	4	1140
	16	18	1149
	100	25	1275
	130	110	1655
mean	41	26.7	1190.8
protection	96.6%	97.8%	

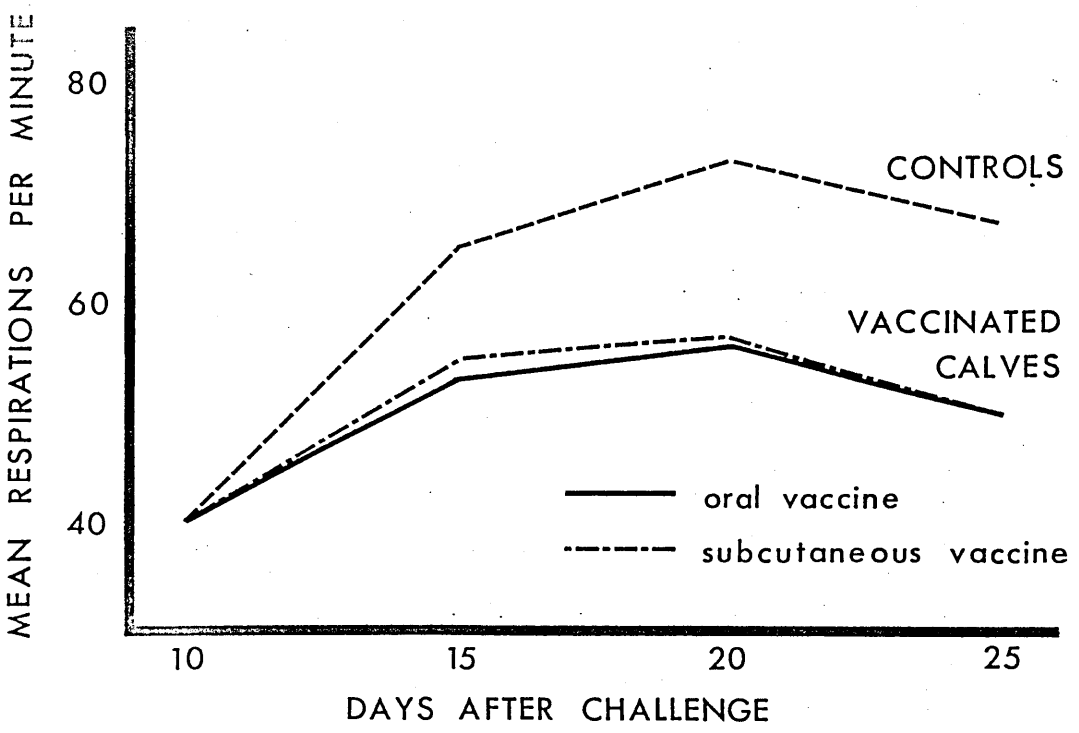

Fig. 7.1. The mean respiratory rates, following challenge with 3,000 D.viviparus L_3 of groups of calves vaccinated with 1) sterile oral vaccine or 2) sterile subcutaneous vaccine compared with 3) challenge-only controls.

iv) Faecal Larval Output

The mean output of lungworm larvae per gramme (lpg) of faeces at post-mortem in each group was as follows :

Group One (oral vacc'n).....1 lpg

Group Two (s/c Vacc'n).....0 lpg

Group Three (controls).....87.4 lpg

v) Lung Lesion Scores

The degree of lung damage based on a lesion score of 0-5 is shown in Table 29.

Table 29.

The estimated lung-lesion scores of calves challenged with 3,000 lungworm larvae following oral vaccination (Group One) or subcutaneous vaccination (Group Two) compared with unvaccinated controls (Group Three).

	Group One (oral vacc'n)	Group Two (s/c vacc'n)	Group Three (controls)
	1	1	3
	1	1	3
	1	2	4
	1	2	4
	3	2	4
	5	3	5
	---	---	---
mean	2.0	1.8	3.8

---

vi) Calf 88

One calf from the orally vaccinated group was killed in-

extremis on day 77 (21 days after challenge). At necropsy very few adult worms were found in the lungs. The lungs were oedematous and showed a very severe degree of consolidation although this was diffuse (rather than lobular as might be expected). No vaccination/challenge lymphoid nodules were observed.

Discussion.

In terms of respiratory rate, post-mortem worm burdens, faecal larval output and lung lesion score, the two vaccinated groups showed a considerable degree of protection against challenge. It can therefore be concluded that dichlorophen-treated vaccine is as immunogenic as the current product and that subcutaneous vaccination is as effective as oral vaccination.

While in terms of worm burden, larval output, lesion score and degree of post-vaccination coughing, the subcutaneously vaccinated group appeared to have performed better than the oral group, it is unlikely that the differences between these two groups are significant.

The reason for the adverse reaction in calf 88 is obscure. It would appear possible, based on the histological findings of alveolar epithelialisation and worm debris, that this was a response similar to that seen in the reinfection syndrome and was a reaction to worm material in the lungs.

SAFETY EXPERIMENT.

This experiment was designed to investigate the safety of the sterile injectable lungworm vaccine should the occasion arise where calves were inadvertently exposed to more than the stated dose of vaccine.

Design.

Four Friesian-cross calves were divided into two groups of two calves.

The calves in **Group One** were injected subcutaneously in their right shoulders with 3,000 dichlorophen-treated, irradiated D.viviparus L_3.

The calves in **Group Two** were injected subcutaneously with 4,500 dichlorophen-treated, irradiated larvae.

All animals were killed on Day 27.

Results.

i) Injection site.

The injection itself did not appear to cause the animals any pain nor was any irritation noticeable immediately following vaccination.

No adverse reactions were noted at the injection sites during the course of the experiment or at post-mortem.

ii) Respiratory rates.

The mean respiratory rates of the groups of animals in this experiment are shown in Fig. 7.2. The respiratory rates of the animals in both groups rose from day 10 after vaccination and

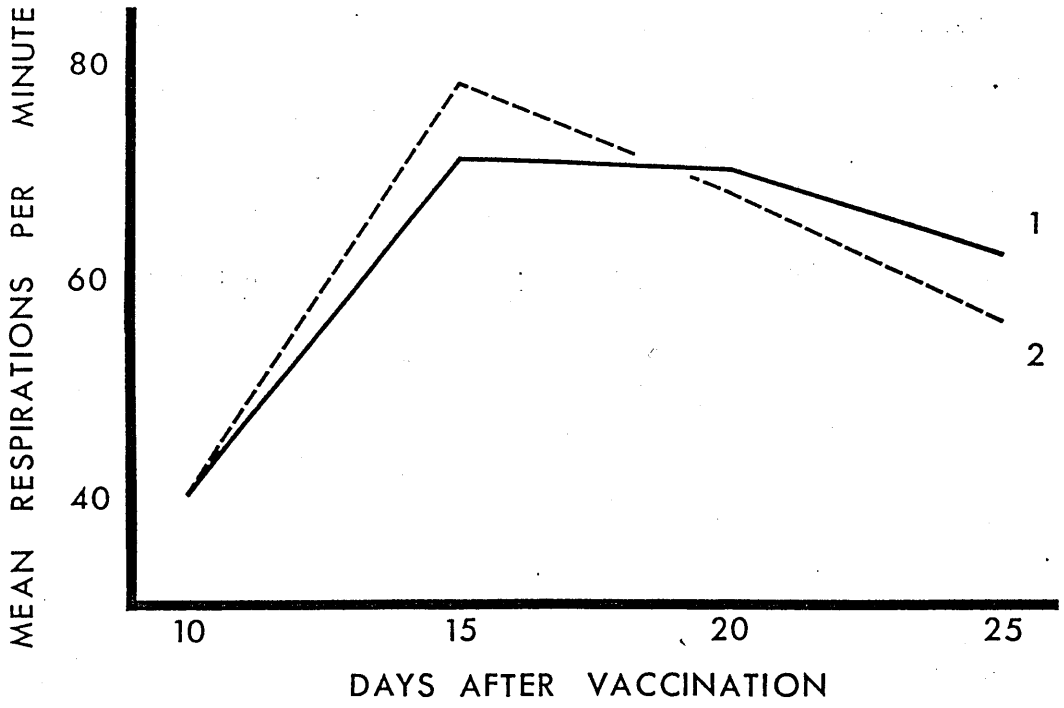

Fig. 7.2. The mean respiratory rates, following vaccination of groups of calves given 1) 3,000 irradiated larvae subcutaneously or 2) 4,500 irradiated larvae subcutaneously.

reached a peak at day 15. Thereafter the respiratory rates gradually declined.

iii) Worm counts.

The post-mortem worm burdens of the 4 animals were as follows :

Group One	0	1
Group Two	1	16

All the worms recovered were extremely small (< 1 cm in length) and were all sterile females.

iv) Lung condition at necropsy.

The lungs of all the calves showed small patches of consolidation in the diaphragmatic lobes. The lesion scores (on a scale of 0 - 5 depending on degree of consolidation) were as follows :

Group One	1	1
Group Two	1	2

Discussion.

The two doses of vaccine used in this experiment were chosen to represent 2 times and 3 times the normal vaccine dose. Both these doses of vaccine were sufficient to cause a marked increase in respiratory rate. However, neither proved fatal and at no time were the animals in great distress. The condition of the lungs of these animals at necropsy was reasonably good and it could be concluded that these excessive doses of vaccine would not cause any permanent harm to the animals.

VIABILITY EXPERIMENTS.

Finally, having found a system by which vaccine could be made sterile and a new means of administration for this vaccine, it was essential to prove that neither the sterilant nor the necessarily increased larval concentration in an injectable preparation had any detrimental effects on the long term viability or infectivity of the larvae.

Design.

On Day 0 suspensions of larvae were made up and bottled as follows :

1) 1500 L_3 in 2ml distilled water.

2) 1500 L_3 in 2ml 0.004% w/v dichlorophen.

3) 1500 L_3 in 25ml distilled water.

4) 1500 L_3 in 25ml 0.004% w/v dichlorophen.

Twelve duplicates of each was made up and these were stored at $4^{o}C$ until use.

On Days 8, 18, 26 and 42 one bottle from each group was examined microscopically and an estimate of the viability of the larvae was made.

On day 7, six calves were infected as follows:

1) One calf received two doses of the larvae stored in 2ml distilled water.

2) Two calves each received two doses of the larvae stored in 2ml 0.004% w/v dichlorophen.

3) One calf received two doses of the larvae stored in 25ml distilled water.

4) Two calves each received two doses of the larvae stored in

25ml 0.004% w/v dichlorophen.

Each calf therefore received 3000 larvae.

The above procedure was repeated with a further six calves on day 42 of the experiment. All calves were slaughtered 28 days after infection.

Results.

i) Microscopic viability estimation.

The percentage viability of the larvae at the various sampling dates is shown in Table 30.

Table 30.

The percentage viability of dichlorophen-treated or untreated larvae stored in 2ml vials or 25ml bottles.

Day	Group 2ml untreated	2ml treated	25ml untreated	25ml treated
8	94.4	95.2	98.6	94.8
18	90.8	94.6	93.0	96.8
26	95.4	98.1	84.0	94.1
42	91.2	94.6	87.7	90.7

These figures show no appreciable loss of viability of larvae stored in a 0.004% w/v. solution of dichlorophen for 42

days as compared to the controls stored in water. Also, storage in 2ml vials rather than 25ml bottles would not appear to have had any adverse effect on the estimated viability.

ii) Infectivity results.

Respiratory Rates.

The respiratory rates of the individual animals all showed increases over the experimental period typical of lungworm infection and displayed little variation between groups or periods of storage. See Figures 7.3 and 7.4.

Post-mortem worm counts.

The post-mortem worm burdens of the calves in this experiment are shown in Table 31.

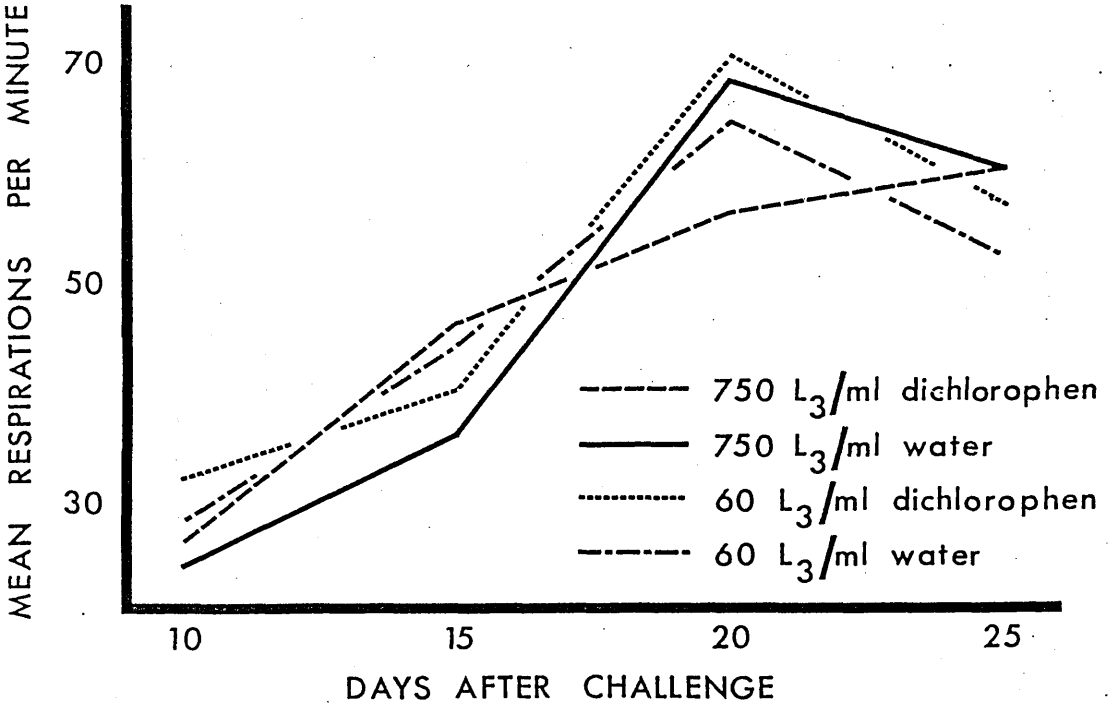

Fig. 7.3. The mean respiratory rates of groups of unvaccinated calves following challenge with 3,000 D.viviparus L_3 stored for 7 days 1) as 750 L_3/ml in distilled water, 2) as 750 L_3/ml in 0.004% w/v dichlorophen, 3) as 60 L_3/ml in distilled water or 4) as 60 L_3/ml in 0.004% w/v dichlorophen.

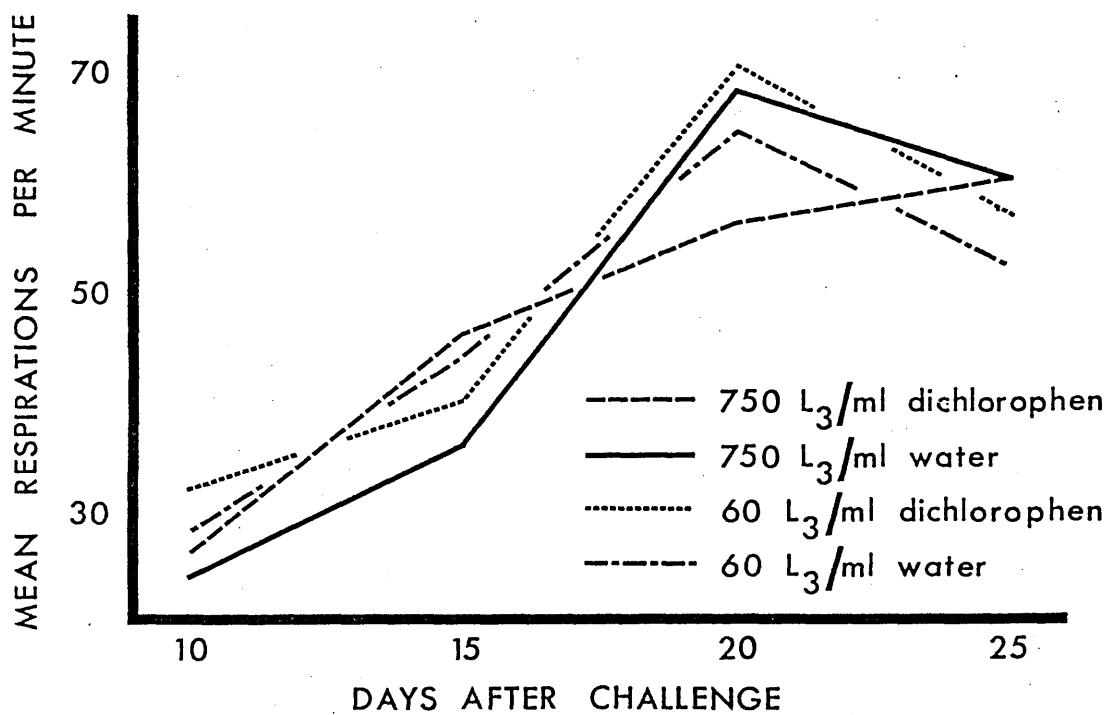

Fig. 7.3. The mean respiratory rates of groups of unvaccinated calves following challenge with 3,000 D.viviparus L_3 stored for 7 days 1) as 750 L_3/ml in distilled water, 2) as 750 L_3/ml in 0.004% w/v dichlorophen, 3) as 60 L_3/ml in distilled water or 4) as 60 L_3/ml in 0.004% w/v dichlorophen.

Table 31.

The post-mortem worm burdens of calves infected with 3000 lungworm larvae which had been stored for 7 or 42 days in 2ml vials or 25ml bottles of dichlorophen or water.

Group	Day 7 Inf'n.	Day 42 Inf'n.
1) 2ml untreated	236	*1000
2) 2ml treated	440	395
	*629	300
3) 25ml untreated	694	383
4) 25ml treated	562	32
	457	3

* These calves were slaughtered in extremis during pre-patency and worm recoveries were performed using a different technique to that employed for the other calves whereby the lungs were floated in warm saline to recover the smaller worms.

All calves exept those killed early showed positive faecal samples at post-mortem.

The worm numbers recovered from **Groups 1** and **2** at day 7 and day 42 infections were all within the range that might be expected and showed no significant reduction over the six week shelf-life. The two highest counts were from animals which were killed in pre-patency and the larger number of worms present probably reflect the technique required to recover worms at this

stage.

The worm recoveries from **Groups 3** and **4** showed no difference at day 7 but a considerable reduction in infectivity at day 42 in the dichlorophen treated group. This reduction in infectivity was not mirrored by a reduction in the microscopically estimated viability for this group. The reasons for this are obscure.

This experiment showed that larvae prepared for use as a sterile injectable vaccine did not suffer any shortening of shelf-life. However, the inexplicable drop in the infectivity of larvae prepared as for a sterile oral vaccine required further investigation and to this end the following experiment was carried out.

Design.

25ml. aliquots of a 60 L_3/ml. suspension of normal lungworm larvae were made up on day 0. To these was added either 250 ul. distilled water or 250 ul. 0.4% w/v dichlorophen.

ii) On day 35 two calves were each given two dichlorophen-treated aliquots **(Group One).**

iii) On day 44 two calves were each given two dichlorophen-treated aliquots **(Group Two).**

Also on day 44 two calves were each given two of the untreated aliquots **(Group Three).**

Each calf therefore received 3,000 larvae.

iv) All calves were killed on day 64.

Results.

i) Respiratory Rates.

The respiratory rates of the groups of calves in this experiment are shown in Figure 7.5. All calves showed increases in respiratory rates typical of lungworm infection.

ii) Post-mortem Worm Counts.

The post-mortem worm burdens are shown in Table 32.

Table 32.

The post-mortem worm burdens of calves infected on day 35 or 44 with treated or untreated larvae.

	Group One Day35(treated)	Group Two Day 44(treated)	Group Three Day 44 (untreated)
	527	320	360
	901	470	369
	---	---	---
mean	714	395	365

---

iii) Microscopically determined viability.

The viability of the larvae were assessed microscopically at intervals throughout the experiment. These results are shown in Table 33.

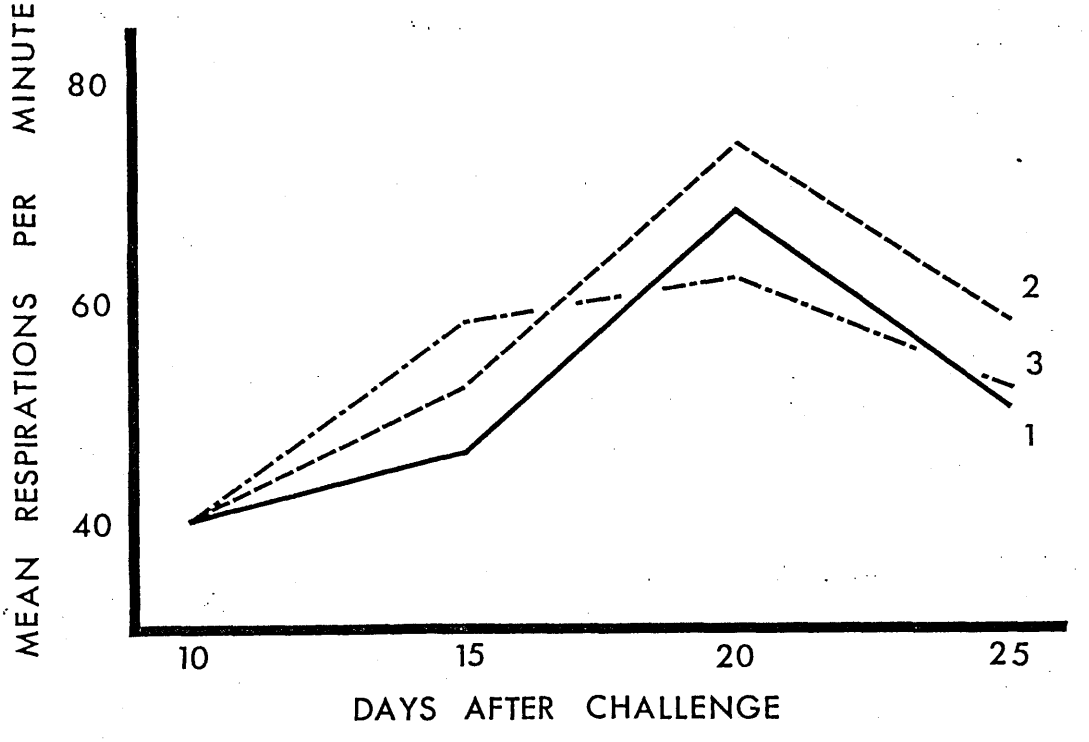

Fig. 7.5. The mean respiratory rates of groups of unvaccinated calves challenged with 3,000 D.viviparus L_3 which had been stored 1) for 35 days at 60 L_3/ml in 0.004% w/v dichlorophen, 2) for 42 days at 60 L_3/ml in 0.004% w/v dichlorophen or 3) for 42 days in distilled water.

Table 33.

The percentage viability of dichlorophen treated or untreated larvae stored at 60 L_3/ml.

Day	Treated	Untreated
0	97.3	94.1
13	78.8	90.4
26	74.2	91.1
35	69.6	84.2
44	68.4	81.6

Discussion

The infectivity and viability of Dictyocaulus larvae are reduced following prolonged storage. The worm burdens of the animals challenged with day 44 larvae are approximately 30% of the burdens that might be expected from a challenge of 3,000 fresh larvae (c.f. challenge controls in the efficacy experiment where mean = 1191 worms). In terms of worm burden, however, it would appear that the infectivity of the larvae is not adversely affected by the presence of dichlorophen.

In contrast, the viability as determined microscopically is reduced to a greater degree in the dichlorophen-treated group than in the untreated group. The fact that this is not reflected in the infectivity results possibly highlights the unreliability of viability testing. However, even at day 44 in the dichlorophen-treated larvae the 68.4% viability still leaves

>1,000 live larvae per bottle and is above the 40 viable larvae per ml. cut-off point used in the past by Glaxo as a criterion of success (R.Peacock, Glaxo internal report 7/5/82).

It would appear that the dichlorophen incorporation does not greatly harm the larvae even after 44 days. Clearly the real test of this is to examine the efficacy of treated vaccine after various periods of storage.

Chapter Eight.

Final Discussion and Conclusions.

The bulk of this thesis has been concerned with the development of a sterile irradiated lungworm vaccine for administration by the oral or subcutaneous route.

Originally, sterility was achieved by means of a one hour wash in a 0.0024% w/v solution of sodium dichlorophen followed by suspension in sterile water and bottling in sterile containers by aseptic means. While this system had the desired effect of producing vaccine from which no extraneous bacteria could be isolated, the extra washing involved made this technique cumbersome. In addition, further information from BDH, the makers of Panacide, suggested that a higher concentration of dichlorophen would be required if it was necessary to cope with a greater degree of contamination. An improved technique was therefore devised whereby a higher concentration of dichlorophen (0.004% w/v) was added and this remained in the vaccine until use. In this way the dichlorophen had a longer time in which to effect the sterilisation of the vaccine. In theory this technique would also reduce the need for sterile bottling facilities although in practice it is possible that this will be required in order to gain a product licence.

The efficacy of this sterilisation regime was tested by adding organisms to vaccine, adding dichlorophen and then assaying for the organisms after a period of incubation. However, the bactericidal effect of a predetermined concentration of dichlorophen varies with the period of contact with the organisms, the temperature and the number of organisms present. In order to subject the procedure to a realistic level of

contamination, 10^4 bacteria were added per ml. of final vaccine. Taking into account the dilution factor between irradiation and bottling, this level of contamination is the equivalent of at least 80 million bacteria per ml. of larval suspension (3,000 times greater than the actual contamination level determined in Chapter Three).

The incubation temperature was chosen to mimic the production process. Vaccine storage is at $4^{o}C$, a temperature at which dichlorophen is slowly bactericidal. If dichlorophen is added at the point where the irradiated larval suspension is diluted to form the final vaccine, it is estimated that the vaccine/dichlorophen mix will have 6 hours at ambient temperature prior to storage in a room held at $4^{o}C$. On the basis of this 6 hour room temperature incubation followed by $4^{o}C$ storage, it was found that this regime successfully kills 100% of added bacterial contaminants.

In addition, it was shown that this regime also kills artificially added Infectious Bovine Rhinotracheitis virus and Mucosal Disease virus. The evidence so far suggests that this treatment does not significantly impair the shelf-life of the vaccine.

While it is envisaged that dichlorophen would only be introduced at the final stage where larvae are diluted following irradiation, it may be necessary for licensing reasons to add dichlorophen at an earlier stage. For example, dichlorophen could be present in all water used in the production process from the time when the larvae are first washed off the surface of the faeces right through to bottling. This would result in the use

of large volumes of dichlorophen and inevitably increase the cost of production.

Perhaps, a suitable compromise would be that dichlorophen could be used in all the water from the 2nd migration stage onwards. This is the final washing stage by which time production has moved to Ware, whereas the earlier stages are at Bury Green and Lady Hayes farms. In addition 0.22um pore size cartridge filters could be built in to the water supply systems at all 3 locations to ensure that all the water used in the production process was itself sterile.

The experiments described in **Chapters 6** and **7** have shown that a live subcutaneously administered vaccine is as protective as the existing oral preparation. Initially, having confirmed that D.viviparus L_3 are infective when injected subcutaneously, the relative protection afforded by one or two doses of 2,400 irradiated larvae administered subcutaneously or intra-peritoneally was investigated in groups of two animals per treatment. While all regimes gave protection (the minimum being 72% for the double intra-peritoneal injection) it was found that double subcutaneous vaccination gave 95.4% protection. A second experiment with 10 calves vaccinated subcutaneously with two doses of 1,000 irradiated larvae gave 95.5% protection against challenge.

Further investigation of single dose subcutaneous vaccination with 40Kr x-irradiated or 70Kr gamma-irradiated larvae gave inconclusive results due to a poor establishment in the challenge only controls.

A final protection experiment was carried out in **Chapter 7** comparing dichlorophen-treated subcutaneous vaccine with dichlorophen-treated oral vaccine and challenge only controls. Groups of six calves showed that subcutaneous vaccination gave 97.8% protection and oral vaccination 96.6%.

A total of 18 calves in three experiments have therefore been vaccinated subcutaneously and in these the groups have given mean levels of protection between 95.4% and 97.8%.

The principal advantages of such an injectable vaccine are twofold. Firstly there is the ease of handling. With the current product the calf must be restrained while its head is held up, its mouth opened and the vaccine poured down the throat. While this is normally quite easy with pail fed, 10 week old dairy replacement calves, it is more difficult to perform the same task on autumn born suckler calves which are perhaps 5 or 6 months old at vaccination and not used to being handled. This is especially difficult if no crush is available. In contrast such calves can be tightly penned and injected with much less fuss.

The second advantage of an injectable vaccine is the certainty that the whole dose of vaccine has been administered. With oral vaccination, there is the risk of the animal not swallowing the whole dose or of spillage by the person performing the vaccination.

If both oral and injectable products were available the farmer would be able to choose the system best suited to his needs.

There are also several purely commercial, perhaps unscientific, advantages in having a sterile oral/injectable

vaccine. The very fact of having a "new" vaccine lends a marketing advantage over any competition. In addition, if it is known to be free from bacterial contamination a further advantage over a competitor is gained from the fear that the other product could be harbouring pathogens. Finally the injectability lends a more scientific aura to the vaccine. While the majority of farmers tend to have a much better understanding of agricultural science than their counterparts of 25 years ago, there is still a certain belief in the "witchcraft of the needle" which farmers find more impressive than oral dosing.

Chapter Four describes experiments to investigate the usefulness of some plastics as materials for packaging lungworm vaccine.

The advantages of using plastic are the lighter weight of packaged vaccine for postage and the reduced need for protective outer packaging to prevent breakage in transit. The drawbacks are the fear of toxic residues from the plastic, the variability of successive batches of plastic bottles and the danger that static forces built up at filling can lead to larvae adhering to the plastic and not being delivered at dosing.

Of various plastics investigated, P.E.T. (polyethylene-tetraphthalate) appeared to give the best results. However, problems arose with the evenness of filling these bottles on the unadapted Dictol production line and further investigation would be required before any conclusive recommendation of these bottles could be made.

Another suggested method of reducing production and delivery

costs was that the initial oral dose could be boosted by an injection of lungworm antigen in place of the second oral dose. Such antigen could be produced at times of the year when Dictol is not in production and could be delivered to the farm along with the first oral dose thus reducing the postage costs.

The experiment described in **Chapter 5** investigated such a regime where the second vaccine dose consisted of an injection of freeze-dried L_3 antigen. This regime gave poor protection against challenge (55.4%) and served to highlight the importance of the second oral dose.

As is often the case with research, while a certain amount of progress has been made a greater amount remains to be done. In particular much remains to be done in order to gain a Ministry of Agriculture product licence for the sterile oral/injectable vaccine. The actual experiments and amount of data needed will be a matter for discussion and negotiation with the licencing authorities but the general areas of work can be foreseen.

Firstly it is likely that the Ministry will ask for a greater amount of sterility data especially with regard to viruses. Before this is done it would be useful to decide at what point dichlorophen is going to be introduced into the production process. This would enable experiments to be performed which directly reflect conditions on the production line.

Further protection trials should also be performed. To date, only a modest number of calves have been vaccinated with the sterile oral or sterile injectable vaccine. The actual number of calves required to satisfy the Ministry will need to be

assessed in order that experiments can be planned that will meet their approval. It is also probable that the vaccine will have to be tested under conditions of field challenge. Field trials will have two aims. Firstly to prove efficacy against a known level of field challenge. This will probably be on experimental paddocks artificially seeded with lungworm larvae. The second aim will be to have the vaccines used on a large number of calves by farmers on their own farms. This will determine the safety and acceptability of the sterile injectable vaccine under normal farming conditions.

In addition further protection trials should be performed with various ages of vaccine in order to determine the shelf-life of the sterile products.

With regards to the use of the injectable vaccine, in all these experiments it will be essential that the vaccine be packaged in as near as possible to its final form.

The question of packaging is one of the major problems still to be solved. A multidose package is an attractive idea but probably not realistic due to the speed at which larvae in suspension sediment. It is also likely that a system whereby the same needle can be used for more than one animal is unlikely to find favour with the licensing authorities since it could be implicated in the spread of diseases such as Infectious Bovine Leucosis.

This leads to the conclusion that the best answer is single dose, prefilled, syringes packed with needles already fitted. However, this reintroduces the questions posed in Chapter Four of

the advantages of glass versus plastic as a packaging material. It is only fairly recently that the technology has been available to make small P.E.T. bottles (Lovatt, Glaxo Operations, personal communication) and it is currently unlikely that P.E.T. syringes could be readily obtained.

Single dose glass prepacked syringes are already used for many human viral vaccines (e.g. Fluvin, Evans) although these tend to be small (0.5ml.) and are probably not sturdy enough for farm use.

A study similar to that of Chapter Four will have to be undertaken on various types of syringe and needle in order to determine a suitable combination.

The costs of introducing and marketing a new form of vaccine will inevitably be high and obviously commercial considerations will need to be taken into account in the final decision whether the introduction of a sterile injectable vaccine is desirable.

One such consideration is the recent finding that ivermectin is a very efficient drug for treating lungworm infections and that anthelmintic activity against invading D.viviparus larvae is retained for up to 28 days after dosing (Armour, Bairden, Batty, Davison & Ross, 1985). This has led to the suggestion (Taylor, Mallon & Kenny, 1985) that prophylactic use of ivermectin could make lungworm vaccination obsolete and eventually eradicate husk. However, this has been questioned (Bain & Urquhart, 1986) on the grounds that it is not yet known if calves treated prophylactically in this way are immune to challenge in their second grazing season. In addition it is probably ill advised to use one drug repeatedly to the exclusion of all others thus

increasing the risk of anthelmintic resistance arising in the worm population.

It also seems unlikely that husk can ever be totally eradicated. Low levels of pasture challenge can quickly cause clinical husk and there is ample evidence to suggest that the windborne spores of the fungus Pilobolus can spread the larvae efficiently from field to field and farm to farm. Furthermore, if eradication of husk was achieved by repeated use of ivermectin, then the subsequent generations of calves would be completely susceptible to husk should they be moved onto an infected pasture. It would seem, therefore, that, in the foreseeable future there will still be a considerable need for an effective lungworm vaccine.

Looking further ahead, it has always been appreciated that a sterile, live, injectable vaccine can only be a stop-gap on the way to an improved vaccine.

Possibly the next step in this process should be to investigate the continuous in-vitro cultivation of Dictyocaulus viviparus. This was attempted by Silverman in the late 1960's (reviewed in Silverman, 1965) but recent advances, especially in cell culture techniques now make this a more realistic goal. The advantages of in-vitro cultivation are three-fold. Firstly, it eliminates the need for the numerous culture calves at Bury Green and Lady Hayes Farms. Secondly, it would enable the larvae to be produced aseptically and could make the use of dichlorophen redundant. Thirdly, and most importantly for future vaccine development, it would make all stages of the life cycle available

for research towards a killed vaccine, since at present only adult and L_3 antigens can be used in any quantity as experimental antigen for immunological studies.

It has always been presumed that lungworm vaccine works by presenting the right antigen to the host, in other words, the right stage of the worms' parasitic life. Presenting the wrong antigen is at best ineffective or at worst causes an allergic reaction as was suggested when Jarrett et al. (1960b) used adult D.viviparus homogenate to vaccinate calves. The animals in this case were probably immune only to adult antigen and when this was presented in large quantities in the lung in the challenge infection, a very potent immune response was elicited. While this did in fact kill the worms, the nature of the response was such that severe respiratory distress resulted.

Perhaps, then, in order to stimulate protective immunity without causing any adverse allergic response it would be possible to use an antigen derived from the L_4 or L_5. If such an antigen was isolated, it could then be synthesised in large quantities using recombinant DNA technology and used as a vaccine. However, the production of an effective synthetic vaccine could possibly involve the incorporation of several different antigens in order to stimulate sufficent immunity.

More importantly, the antigen may have to be presented to a particular site. At present the larvae efficiently carry the antigen to the mesenteric lymph nodes and on to the lungs. It is possible that antigen is taken up by antigen presenting cells (APC) and that plasma cells at a particular site need to recognise the antigen in conjunction with the cell surface

antigens of the APC. With the knowledge that lymphocytes recirculate to their own particular location it may be that to elicit an efficient immune response to developing worms in the mesenteric lymph nodes or lung it is necessary to present the antigen at these sites. At present, the art of antigen targeting is not advanced enough to make this a feasible proposition. However, the experiment of Jarrett et al. (1960b) reported above suggests that parenterally introduced antigen can in fact elicit a very effective immune reaction in the lung.

The immune response to lungworm infection would appear to involve a humoral and a cell mediated component, but until recently, studies on vaccination against helminth parasites have been preoccupied with humoral responses, the emphasis being on producing a clearly defined antigen and eliciting a specific antibody response. It is probably necessary to look in greater detail at the cell mediated component of the response and at ways in which this can be enhanced or initiated to coincide with the adminstration of the antigen.

The understanding of immunology has come a long way since the concept of an irradiated larval lungworm vaccine was first envisaged thirty years ago. The techniques and knowledge are probably now available to study the immune response to vaccination in sufficient detail to determine the protective mechanisms involved. Once these are known it may be possible to manufacture a synthetic vaccine. In addition, the understanding of this very effective immune response could shed light on ways in which less efficient responses (for example that to

Ostertagia) could be enhanced.

It now seems that a live injectable lungworm vaccine is a feasible proposition. However, should such a vaccine be produced and no subsequent follow-up research performed, a great opportunity would be lost to make a breakthrough in nematode vaccination.

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Appendix to chapter 4.

Glass Bottles (2 weeks)

Values for the number of larvae in each 1 ml. aliquot.

Bottle no.	Aliquot number 1	2	3	4	5	mean
1	59	65	72	47	66	61.8
2	75	71	45	54	71	63.2
3	66	78	65	79	85	74.6
4	63	66	68	69	56	64.4
5	55	54	73	56	82	64.0
6	72	68	68	76	59	68.6
7	67	61	63	53	75	63.8
8	65	74	48	61	66	62.8
9	71	52	72	63	77	67.0
10	74	72	62	60	72	68.0
11	60	64	74	64	63	65.0
12	62	76	60	77	69	68.8
13	81	56	65	71	68	68.2
14	74	63	61	73	69	68.0
15	67	62	75	53	84	68.2
16	64	66	64	77	60	66.2
17	60	78	73	61	73	69.0
18	79	72	74	78	55	71.6
19	74	72	97	94	98	87.0
20	66	74	77	79	61	71.4

Glass bottles (aliquot counts continued)

Bottle no.	Aliquot number 1	2	3	4	5	mean
21	78	60	85	63	69	71.0
22	62	69	62	69	65	65.4
23	62	73	79	78	65	71.4
24	81	56	74	69	89	73.8
25	81	76	62	51	80	70.0
26	70	70	67	70	69	69.2
27	85	63	69	68	67	70.4
28	70	66	71	63	63	66.6
29	43	62	65	51	72	58.6
30	72	79	71	54	58	66.8
31	55	54	57	77	66	61.8
32	82	69	65	58	60	66.8
33	69	86	66	73	78	74.4
34	81	51	65	72	59	65.6
35	57	66	57	73	57	62.0
36	75	59	71	70	66	68.2
37	66	58	61	75	75	67.0
38	57	66	65	78	71	67.4
39	63	70	61	50	72	63.2
40	55	71	57	73	85	68.2
41	48	61	68	59	61	59.4
42	82	55	84	71	78	74.0
43	59	88	54	66	49	63.2

Glass Bottles (aliquot counts continued)

Bottle no.	Aliquot number 1	2	3	4	5	mean
44	57	57	78	72	69	66.6
45	66	64	59	62	84	67.0
46	76	67	67	52	72	66.8
47	71	49	82	72	65	67.8
48	62	62	68	71	54	63.4
49	61	54	70	62	67	62.8
50	73	70	71	74	79	73.4

P.E.T. Bottles at 2 weeks.

Values for the number of larvae in each 1 ml. aliquot.

Bottle no.	Aliquot number 1	2	3	4	5	mean
1	57	65	59	65	79	65.0
2	87	83	67	70	72	75.8
3	76	62	42	71	86	67.4
4	56	72	68	60	67	64.6
5	70	74	62	90	76	74.4
6	65	61	66	62	61	63.0
7	78	62	64	78	62	68.8
8	70	74	79	76	70	73.8
9	80	76	78	87	66	77.4
10	76	91	70	71	75	76.6
11	70	62	72	66	72	68.4
12	84	67	77	72	70	74.0
13	68	62	61	64	66	64.2
14	84	63	59	68	70	68.8
15	61	54	81	69	73	67.6
16	76	80	70	62	77	73.0
17	77	63	73	77	64	70.8
18	74	75	70	85	75	75.8
19	83	55	66	57	68	65.8
20	67	57	49	68	60	60.2
27	72	69	65	86	68	72.0
28	67	73	61	65	75	68.2

P.E.T.Bottles 2 weeks (aliquot counts continued).

Bottle no.	Aliquot number 1	2	3	4	5	mean
29	59	80	67	56	75	67.4
30	69	79	76	75	88	77.4
31	71	57	75	79	87	73.8
32	63	68	63	79	72	69.0
33	70	64	73	71	57	67.0
34	61	69	74	55	74	66.6
35	60	67	62	61	54	60.8
36	65	57	51	55	72	60.0
37	64	62	69	64	65	64.8
38	62	74	86	72	64	71.6
39	74	68	67	79	69	71.4
40	66	75	81	78	56	71.2
41	75	69	59	78	72	70.6
42	64	67	74	56	60	64.2
43	79	75	71	80	52	71.4
44	96	85	66	60	73	76.0
45	88	74	66	75	81	76.8
46	58	59	58	64	67	61.2
47	86	66	77	72	64	73.0
48	73	62	78	71	56	68.0
49	82	73	55	51	61	64.4
50	64	72	65	68	68	67.4
51	79	83	89	80	72	80.6

P.E.T. Bottles 2 weeks (aliquot counts continued).

Bottle no.	Aliquot number 1	2	3	4	5	mean
52	54	79	63	61	74	66.2
53	79	55	71	65	79	69.8
54	64	79	84	71	70	73.6
55	77	64	75	71	67	70.8
56	80	65	69	57	59	66.0
57	72	63	58	75	73	68.2
58	59	61	73	59	65	63.4
59	91	68	56	91	74	76.0
60	75	65	74	79	58	70.2
61	73	65	63	65	79	69.0
62	92	80	78	57	69	75.2
63	75	66	83	77	76	75.4
64	71	78	91	66	64	74.0
65	71	58	67	90	83	73.8
66	72	69	74	90	65	74.0
67	75	55	74	65	59	65.6
68	63	73	70	68	66	68.0
69	71	67	71	74	69	70.4
70	57	74	83	73	80	73.4
71	66	51	78	66	92	70.6
72	79	76	84	65	66	74.0
73	57	77	72	87	73	73.2
74	81	75	58	76	79	73.8

P.E.T.Bottles 2 weeks (aliquot counts continued).

Bottle no.	Aliquot number 1	2	3	4	5	mean
75	73	64	78	72	54	68.2
76	72	53	58	64	63	62.0
77	72	64	67	68	56	65.4
78	51	64	64	79	75	66.6
79	61	54	85	72	79	70.2
80	62	67	69	89	62	69.8
81	55	68	62	65	76	65.2
82	81	78	86	64	80	77.8
83	70	77	93	74	80	78.8
84	69	51	72	69	72	66.6
85	69	65	68	74	56	66.4
86	61	86	69	71	58	69.0
87	76	74	82	93	60	77.0
88	52	64	70	71	67	64.8
89	82	72	64	81	62	72.2
90	67	67	74	72	62	68.4
91	77	65	77	55	83	71.4
92	61	84	75	70	76	73.2
93	83	71	72	69	83	75.6
94	70	62	72	64	66	66.8
95	83	72	66	70	50	68.2
96	62	76	74	61	81	70.8
97	67	66	63	72	69	67.4

P.E.T. Bottles 2 weeks (aliquot counts continued).

Bottle no.	Aliquot number 1	2	3	4	5	mean
98	64	65	68	69	74	68.0
99	68	84	67	65	59	68.6
100	53	66	75	77	51	64.4
101	71	66	61	81	52	66.2
102	64	73	81	66	67	70.2
103	91	80	66	66	61	72.8
104	79	64	64	54	49	62.0
105	75	77	58	85	83	75.6
106	77	88	91	74	70	80.0

Glass Bottles at 5 weeks.

Values for the number of live larvae in each 1 ml. aliquot.

Bottle no.	Aliquot number 1	2	3	4	5	mean
201	75	53	62	72	62	64.8
202	63	66	66	79	62	67.2
203	71	75	77	66	62	70.2
204	68	73	68	70	64	68.6
205	68	48	61	68	64	61.8
206	59	68	64	76	60	65.4
207	68	80	70	59	60	67.4
208	62	65	63	72	59	64.2
209	67	66	77	68	57	67.0
210	69	62	67	81	58	67.4
211	72	66	67	67	66	67.6
212	72	58	66	77	74	69.4
213	70	69	64	73	66	68.4
214	67	70	85	70	81	74.6
215	72	59	68	62	73	66.8
216	70	66	61	58	66	64.2
217	70	51	77	64	62	64.8
218	67	65	71	64	80	69.4
219	58	56	67	59	64	60.8
220	69	84	71	68	65	71.4
221	53	70	67	63	79	66.4
222	73	73	76	79	57	71.6

Glass Bottles 5 weeks (aliquot counts continued)

Bottle no.	Aliquot number 1	2	3	4	5	mean
223	76	71	66	71	73	71.4
224	51	64	68	64	59	61.2
225	60	66	79	65	68	67.6

P.E.T. Bottles at 5 weeks.

Values for the number of live larvae in each 1 ml. aliquot.

Bottle no.	Aliquot Number 1	2	3	4	5	mean
201	41	42	46	37	41	41.4
202	55	66	44	62	67	58.8
203	42	45	38	48	43	43.2
204	60	55	62	73	72	64.4
205	59	63	61	65	67	63.0
206	36	49	43	49	54	46.2
207	54	65	62	67	61	61.8
208	75	78	75	71	68	73.4
209	88	62	60	80	65	71.0
210	61	69	61	74	71	67.2
211	57	71	71	64	76	67.8
212	48	38	63	46	32	45.4
213	55	57	60	53	64	57.8
214	53	60	52	64	82	62.2
215	73	44	73	54	65	61.8
216	70	62	67	72	85	71.2
217	71	75	58	56	60	64.0
218	85	63	54	69	69	68.0
219	55	49	59	50	61	54.8
220	63	67	73	44	66	62.6
221	68	53	46	55	58	56.0
222	58	55	68	65	58	60.8

P.E.T. Bottles 5 weeks (aliquot counts continued)

Bottle no.	Aliquot number 1	2	3	4	5	mean
223	71	76	69	78	55	69.8
224	86	74	72	80	81	78.6
225	73	60	57	70	74	66.8

Glass Bottles at 5 Weeks.

Values for the number of dead larvae in each 1 ml. aliquot.

Bottle no.	Aliquot Number 1	2	3	4	5	Mean
201	3	4	3	1	3	2.8
202	4	5	7	2	3	4.2
203	5	6	7	4	3	5.0
204	3	1	5	2	1	2.4
205	1	3	4	2	2	2.4
206	3	1	2	1	1	1.6
207	2	7	3	5	8	5.0
208	4	3	6	1	7	4.2
209	1	3	2	5	1	2.4
210	3	2	3	4	2	2.8
211	4	5	1	7	3	4.0
212	3	3	4	4	2	3.2
213	4	1	3	1	3	2.4
214	2	6	1	1	1	2.2
215	2	3	2	0	5	2.4
216	3	5	6	7	6	5.4
217	8	6	6	4	0	4.8
218	2	1	4	1	2	2.0
219	3	5	8	1	3	4.0
220	3	1	5	2	4	3.0
221	3	3	3	4	5	3.6
222	5	6	1	0	0	2.4

Glass Bottles at 5 weeks (aliquot counts continued).

Bottle no.	Aliquot Number 1	2	3	4	5	mean
223	2	3	8	4	2	3.8
224	3	2	6	3	8	4.4
225	3	2	1	2	1	1.8

P.E.T. Bottles at 5 weeks.

Values for the number of dead larvae in each 1 ml. aliquot.

Bottle no.	Aliquot Number 1	2	3	4	5	mean
207	6	2	8	3	2	4.2
208	3	7	7	6	4	5.4
209	6	3	6	6	0	4.2
210	5	4	2	1	2	2.8
211	3	0	5	2	1	2.2
212	3	1	4	2	4	2.8
213	7	2	3	4	3	3.8
214	3	2	2	2	3	2.4
215	3	1	4	1	4	2.6
216	4	6	3	1	2	3.2
217	2	5	4	3	4	3.6
218	4	6	3	2	3	3.6
219	4	4	2	5	4	3.8
220	6	6	3	5	4	4.8
221	4	3	3	1	3	2.8
222	4	4	7	4	3	4.4
223	2	3	2	6	4	3.4
224	6	5	5	1	7	4.8
225	3	3	4	2	2	2.8

Total Volume of vaccine contained in each of 25 glass bottles (ml.).

23.4	23.6	23.8	23.8	23.8
23.8	23.8	24.0	24.0	24.0
24.0	24.0	24.0	24.2	24.2
24.2	24.2	24.4	24.4	24.4
24.4	24.6	24.6	24.6	24.6

Total volume of vaccine contained in each of 25 P.E.T. bottles (ml.).

18.8	19.2	19.2	19.2	19.2
19.4	19.4	19.4	19.6	19.6
20.2	21.2	21.4	21.6	21.6
21.8	21.8	22.0	23.8	23.8
24.0	24.0	24.0	24.0	24.0

Proof that all above data display normal distributions.

1. Glass bottles 2 weeks

> 95% must lie in mean $\pm$ 2.01 x standard deviation

= 67.48 $\pm$ (2.01 x 4.72)

= 67.48 $\pm$ 9.49

= 57.99 to 76.97

1 sample out of 50 lies outwith this range = 98% within.

Therefore these figures display a normal distribution.

2. P.E.T. bottles at 2 weeks

> 95% must lie in mean $\pm$ 1.99 x standard deviation

= 69.94 $\pm$ (69.94 x 4.68)

= 69.94 $\pm$ 9.31

= 60.6 to 79.25

4 samples out of 100 lie outwith this range = 96% within

Therefore these figures display a normal distribution.

3. Glass bottles, live larvae at 5 weeks.

> 95% must lie in mean $\pm$ 2.06 x standard deviation

= 67.2 $\pm$ (2.06 x 3.37)

= 67.2 $\pm$ 6.94

= 60.3 to 74.1

1 sample out of 25 lies outwith this range = 96% within.

Therefore these figures display a normal distribution.

4. P.E.T. bottles, live larvae at 5 weeks.

> 95% must lie in mean $\pm$ 2.06 x standard deviation.

$= 61.5 \pm (2.06 \times 9.53)$

$= 61.5 \pm 19.63$

$= 41.9$ to 81.2

1 sample out of 25 lie outwith this range = 96% within.

Therefore these figures display a normal distribution.

5. Glass bottles, dead larvae at 5 weeks.

> 95% must lie in mean $\pm$ 2.06 x standard deviation

$= 3.3 \pm (2.06 \times 1.12)$

$= 3.3 \pm 2.31$

$= 1.0$ to 5.6

No samples lie outwith this range = 100% within.

Therefore these figures display a normal distribution.

6. P.E.T. bottles, dead larvae at 5 weeks.

> 95% must lie in mean $\pm$ 2.11 x standard deviation

$= 3.6 \pm (2.11 \times 0.90)$

$= 3.6 \pm 1.90$

$= 1.7$ to 5.5

No samples lie outwith this range = 100% within.

Therefore these figures display a normal distribution.

7. Glass bottles, total volumes per bottle.

> 95% must lie in mean $\pm$ 2.06 x standard deviation

= 24.1 $\pm$ (2.06 x 0.33)

= 24.1 $\pm$ 0.68

= 23.4 to 24.8

No samples lie outwith this range = 100% within.

Therefore these figures display a normal distribution.

8. P.E.T. bottles, total volumes per bottle

> 95% must lie in mean $\pm$ 2.06 x standard deviation

= 21.3 $\pm$ (2.06 x 1.96)

= 21.3 $\pm$ 4.04

= 17.3 to 25.3

No samples lie outwith this range = 100% within.

Therefore these figures display a normal distribution.

Appendix to Chapter Seven.

Virology Materials & Methods.

Samples of bovine herpesvirus (BHV) type-1 (infectious bovine rhinotracheitis virus) and bovine viral diarrhoea virus (BVDV) were added to the Dictyocaulus preparation and processed as for vaccine production. A sample of each was then taken and assayed for viral infectivity together with samples of the original, unprocessed virus stock. To a volume of 0.4ml of the vaccine was added 1ml. of 10x concentrated phosphate buffered salin to restore the sample to isotonicity. The samples were stored at -70^{o}C before assay.

Bovine herpesvirus.

BHV infectivity was titrated in a plaque assay using a line of bovine conjunctival cells in 6-well cluster plates (Nunc). The plates were seeded with 1 x 10^{6} cells per well 24 hours previously. The medium was then replaced with 1.0ml of the sample in a range of dilutions from undiluted to 1:5. The untreated sample was diluted 10^{-2} to 10^{-7} in medium. The samples were incubated at 37^{o}C for 3 hours. The inocula were then removed, the cell sheet was washed with medium and an overlay of medium with 1% agarose was added. The plates were incubated at 37^{o}C for 3 days when the overlay was removed and the cells were stained with 1% crystal violet in 5% methanol and 10% formalin. The resulting plaques were counted.

The titre of the untreated sample was 2.0 x 10^{7} plaque forming units (pfu) per ml. No virus was detected in the vaccine at a dilution of 1:5 which was the lowest dilution which did not lyse the cells.

Bovine viral diarrhoea virus.

Test virus was the cytopathic NADL strain of BVD virus (Gutekunst and Malmquist, 1963) derived from the American type culture collection (ATCC VR-543), and received from Dr. P. Roeder CVL Weybridge at the 7th passage level in bovine cells (BEK and bovine turbinate). After two further passages in BEK cells the virus was purified three times and a stock was established at the 13th passage level.

The plaque assay procedure was based on that described by Lande and Gelfi (1979). Confluent monolayers of bovine embryonic testis cells in 12 well Linbro plastic plates (Flow) were washed three times with Hanks BSS and inoculated with 0.2ml of dilution of virus. After two hours adsorption at 37^{o}C the inocula were removed and all wells received 2ml of "199" medium supplemented with BSA (0.5%), lactalbumin hydrolysate (0.1%), yeast extract (0.1%) and heat-inactivated horse serum (3%), and containing 100 iu/ml penicillin, 100ug/ml streptomycin, 2ug/ml amphotericin B deoxycholate and 0.6% agarose. Four days later, cells were fixed with 10% formol saline and stained with 1% (w/v) crystal violet in 20% (v/v) ethanol in distilled water.

The titre of the untreated sample was 1.3 x 10^{6} pfu/ml. No virus was detected in a 1:5 dilution of the vaccine.

References

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