Immunological studies on

Nippostrongylus brasiliensis

infection in the rat.

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by

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PREFACE

This work was carried out in the University of Glasgow Veterinary School during the tenure of a Horserace Betting Levy Board Veterinary Research Training Scholarship. The work presented in this thesis is original and where assistance was received from the work of others it is acknowledged in the usual way.

I should like to thank Professor W.I.M. McIntyre and Professor W. Mulligan under whose supervision the work was carried out. I am particularly indebted to Professor Mulligan who suggested the original problem, provided laboratory facilities and gave valuable advice and support throughout the work.

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CHAPTER I

General Introduction

The immune response in animals stimulated by infection with parasitic helminths has been the subject of reviews by several authors (Taliaferro, 1929, 1940; Culbertson, 1941; Chandler, 1953; Stewart, 1959; Tromba, 1962; Urquhart et al, 1962; Soulsby, 1963).

Acquired resistance to helminths can be manifest in a variety of ways, namely, inhibition of development, reduction in biotic potential, and expulsion of the parasitic worm. Over the past 30 years, immunity acquired by several species of experimental animals to more than a dozen nematode genera has been studied, however, little precise information has been gained as to the character of the functional antigens which give rise to protective antibodies. Furthermore, the role played by these antibodies in the various manifestations of acquired resistance remains an enigma.

It is known that certain species of animals will not become infected by certain parasitic worms. Such animals are said to possess a natural resistance against the parasite in question. The mechanism dictating host specificty of many parasites is not fully understood but it is thought that serum factors may play a significant role (Standen, 1952). A rapid foreign body cellular response to migrating larvae seems also to be a common feature in natural resistance (Lindquist, 1950).

When an animal suffers one or more infections with a species of parasitic worm, then this animal may develop an acquired immunity. Such an immunity to re-infection has been demonstrated in the case of Nippostrongylus brasiliensis (Taliaferro and Sarles, 1939), Trichinella spiralis (Roth, 1939; Oliver-Gonzalez, 1940), Ascaris lumbricoides (Sprent and Glen, 1949; Oliver-Gonzalez, 1956), Ascaridia galli (Sadum, 1948), Ancylostoma caninum (Otto and Kerr, 1939), Toxocara felis (Sarles and Stoll, 1935), Strongyloides ratti (Sheldon, 1937), Strongyloides papillosus (Turner, 1956), Haemonchus contortus (Stoll, 1929), Trichostrongylus calcaratus (Sarles, 1932), T. retortaeformis (Michel, 1953), T. colubriformis (Stewart, 1950), and T. axei (Gibson, 1953).

The capacity for self protection that an infected host develops is undoubtedly stimulated and brought about by substances originating from the parasitic worm. What is known concerning these helminth antigens?

Antigens

Chandler (1935) was first to suggest that worm enzymes, rather than worm tissue antigens, were responsible for stimulating protective antibodies. In this way it was thought that vital steps in the metabolic processes of the worm could be interfered with by the corresponding antibodies, so creating a situation where the worm's position in the host became untenable. With subsequent infections

a more rapid response could be elicited making the host environment even more unsuitable for the worms, which would be killed, expelled or retarded in development.

Taliaferro and Sarles (1939) working with N. brasiliensis found that both the larval and adult forms of this nematode when placed in serum from hyper-immunised rats, formed precipitates at their body apertures. Similar precipitates were found by these workers in close association with the adult worm, in the small intestine of the host, when immune expulsion was imminent. Similar phenomena have been noted with other nematodes and trematodes (Otto, 1940; Mauss, 1941; Smith, 1946: Sadun, 1949; Lin and Bang, 1950; Oliver-Gonzalez, 1950; and Soulsby, 1957). The appearance of such precipitates around the secretory openings of the worm in vitro did support the hypothesis that worms secreted substances which were antigenic and that antibodies directed against them were present in immune serum.

These findings were further confirmed when it was shown, using a fluorescent antibody technique, that antibody localises primarily in the precipitates formed at the oral openings of T. spiralis and N. brasiliensis (Jackson, 1959: 1960). In some cases fluorescein was also noted in the digestive tract and around the reproductive organs of these parasites on in vitro incubation in immune serum. Studies with Ascaris larvae in immune serum have given similar

results (Taffs and Voller, 1963; Crandall et al, 1963).

Thus the concept has arisen that antigens associated with metabolic secretions and excretions of the living parasite are the important ones. In general, the injection of dead worm materials (somatic antigens) has failed to produce a good immunity to parasitic infections. This has been shown to be the case with N. brasiliensis in rats (Chandler, 1937). However, it was demonstrated by Thorson (1953) that partial protection against a challenge infection of N. brasiliensis larvae could be induced in rats by injecting, intraperitoneally, 4 days before challenge, a preparation of 'excretions and secretions' collected from living larvae. Similar fluids collected whilst incubating live T. spiralis larvae in vitro were also capable of partially immunising the host against infection with that parasite (Campbell, 1955; Chute, 1956).

It has also been shown by Thorson (1954a) that the secretions and excretions of living N. brasiliensis larvae are capable of absorbing out most of the protective properties of hyperimmune rat serum, an effect not possessed by dead larval tissue. It is a reasonable assumption that the metabolic products secreted by living larvae could contain various enzymes which might be of importance in the penetration and feeding of infective larvae and adult worms. Immune rat serum was shown to have an inhibitory effect upon a component with lipase activity contained in the secretions and excretions of N. brasiliensis larvae

(Thorson, 1953). Thorson (1956a) also showed that antiserum from an immune dog inhibited proteolytic enzymes extracted from the oesophageal glands of Ancylostoma caninum. Dogs vaccinated with oesophageal extracts were partially immune to subsequent infection with this parasite. However, there is as yet no direct evidence to suggest that this inhibitory action by anti serum upon various enzymes produced by worms, takes place during an immune reaction in a host animal possessing an acquired resistance.

Soulsby, Somerville and Stewart (1959) expressed the view that, while metabolic products of larvae are of value to the host in developing protection, the fluid released during exsheathment of H. contortus larvae provides the most important antigen in their system. No one has yet demonstrated a protective immunity resulting purely from the administration of exsheathing fluid, however, Soulsby and Stewart (1960) have presented evidence to show that in sheep infected with H. contortus the main stimulation at self cure appeared to be derived from substances released by larvae during the third ecdysis.

The complexity of the antigenic stimulus encountered by the host during an infection by a parasitic worm is recognised. Also it is known that the antigenic stimulus may vary quantitatively and qualitatively as the worm passes through various stages of development. It was shown by Soulsby (1957) and Douvres (1962) that no 'Sarles'

precipitates occurred when the infective larvae of A. lumbricoides and O. radiatum were incubated in homologous immune serum. However, after these larvae had undergone partial development precipitates were formed. These only occurred around O. radiatum larvae when the media contained saline extracts of intestinal tissues derived from immune calves. Since antiserum itself did not give precipitate formation it was postulated that antibodies might be tissue bound in this case.

With some species of nematodes it appears that host resistance can be stimulated by the larval stages alone. By suitable exposure of calves to infection with larvae of D. viviparous, attenuated by X-irradiation, so preventing development of most of the parasites beyond the final larval moult, it was possible to stimulate resistance (Jarrett et al, 1959). Acquired resistance to Haemonchus contortus (Jarret et al, 1961; Silverman and Patterson, 1960), Litomosoides carinii (Scott and Macdonald, 1958) and Dictyocaulus filaria (Michel and Sinclair, 1963) infections is thought to be primarily stimulated by the larval stages of these parasites. On the other hand it has been stated that the adult stage is the immunogenic one with N. brasiliensis (Chandler, 1936; Spindler, 1936), Haemonchus placei (Ross, 1963) and Trichinella spiralis (Kim, 1957).

Further evidence supporting the theory that worm enzymes may be important antigens in Schistosoma infections has been put forward by Bueding and MacKinnon (1955) and Mansour et al, (1954).

While the free amino acid and peptide composition of fluids obtained during the *in vitro* incubation of *T. spiralis*, *A. lumbricoides* and *N. brasiliensis* has been studied by Weinstein (1960), no information is available as to the chemistry of the parasite's excretory and secretory products of high molecular weight. It is known that poly-saccharides as well as proteins, derived from worms may be involved, e.g. a poly-saccharide present in both the secretions and excretions and extracts of *T. spiralis* and *A. lumbricoide* has been shown to be antigenic (Oliver-Gonzalez, 1954; 1958). Sprent (1950; 1951) has reported that non-dialysable protein free components derived from the incubation products of larval and adult *A. lumbricoides* are capable of producing an anaphylactic shock in guinea pigs sensitised by infection to that parasite.

A clearer understanding of the antigens involved in stimulating an acquired resistance in hosts infected with parasitic worms may result from successful collection and fractionation of metabolic products of living worms, cultured *in vitro* through each stage of their development from egg to adult. With successful axenic culture it may be possible to procure in sufficient quantities, the worm's secretory and excretory products, hence allowing fractionation and purification of the many components.

Antibodies

Bearing in mind the complexity of the antigenic stimulus resulting from an infection with parasitic worms, it is not surprising that the antibody response may prove to be equally complex. The results of various serological tests when applied to serum of animals with an acquired resistance to various helminths have emphasised the complexity of the antibody response.

Whilst a certain degree of resistance to re-infection can be passively transferred in some host-parasite systems using the serum of highly refractory hosts, the relationship between detectable antibodies (as shown by the various tests) and acquired resistance is not clear. Successful passive transfer of immunity, hence implicating humoral antibodies, has been demonstrated with N. brasiliensis, A. galli, Trichinella spiralis and D. viviparus (Sarles and Taliaferro, 1936; Sadun, 1949; Culbertson, 1942; and Jarrett et al, 1955).

The appearance of precipitate formation around larvae and adults of N. brasiliensis (Sarles and Taliaferro, 1936; Sarles, 1938) and T. spiralis (Oliver-Gonzalez, 1940) when incubated in serum from host animals possessing a strong acquired resistance to the particular parasite, coupled with the fact that similar sera could confer resistance passively, focussed attention on the possibility that these precipitates might interfere with the well being of the worms

in vivo. Jackson (1959; 1960) demonstrated that these precipitates did involve antibodies and although these precipitates did not seem to kill the parasites, they did appear to reduce infectivity (Mauss, 1940; Thorson, 1954b).

Although the appearance of Sarles' precipitates around the parasitic worm cannot be quantitative it does seem to be related to acquired resistance. Attempts have been made to correlate the precipitating antibodies involved in the formation of Sarles' precipitates with precipitating antibodies indicated by more conventional techniques. With *N. brasiliensis* it was found by Thorson (1953) that, following absorption of immune rat serum by worm extracts until no further precipitation took place, subsequent incubation in this serum produced precipitates around living larvae, hence suggesting the presence of different precipitating antibodies.

Despite the large volume of work on the various serological manifestations associated with nematode infections, it is not clear what kinds of antibody are responsible for immunity. Soulsby and Stewart (1960) studying *Trichostrongylus spp.* and *Haemonchus contortus* infections in sheep, found that serum antibody levels as measured by a complement fixation and a haemagglutination test gave no correlation with the ability of the host to resist infection. A similar lack of relationship was found by Michel and Cornwell (1959) between the complement fixing antibody titre of serum from

calves infected with D. viviparus. Kent (1963) has pointed out the complexity of the various antibody types (as indicated by the test procedure employed) produced in man as a result of infections with T. spiralis and A. lumbricoides and the difficulty in correlating antibody titres with resistance.

Immediate skin reactions to extracts of homogenised worms have been noted in man and animals infected with helminth parasites (Andrews, 1962). Recent evidence suggesting the production and participations of reagin-like antibodies in resistance shown to parasitic worms has been put forward by Ogilvie (1964a). Evidence implicating antibodies of this type has been obtained with infections of N. brasiliensis, T. spiralis and T. colubriformis. It is suggested that the presence of reagin-like antibodies could explain certain phenomena associated with helminth infections such as self cure, immediate skin reactions, mast cell disruption and eosinophilia.

Mechanism of acquired resistance

Having outlined briefly, the information available on the nature of the antigens produced by helminth parasites and the antibody response displayed by the infected host, it is necessary to discuss the mechanism of acquired resistance in so far as it affects the well being of the parasite.

Host resistance can lead to numerous adverse effects upon the parasitic worm. With gastro-intestinal parasites following an initial infection it is possible to find expulsion of mature worms, often preceded by a cessation of egg production. In immune hosts larval development is often inhibited, leading to fewer mature worms becoming established, often, such adult worms are stunted and incapable of egg production. N. brasiliensis larvae are killed in the lungs and expelled from the intestine of resistant rats (Sarles and Taliaferro, 1936; Taliaferro and Sarles, 1939). In a previously infected rat, the infective larvae were trapped in the skin and lungs of the host. Precipitates formed around the larvae and they were enclosed by an inflammatory reaction. These workers concluded that humoral antibodies produced the initial effect and that the cellular reaction was a foreign body response taking place after immobilisation of the larvae. That humoral antibodies are implicated, was strengthened by the fact that similar precipitates with the associated inflammatory response occurred in passively immunised hosts. Weinstein (1955) found that by the suitable administration of cortisone to a resistant rat, the characteristic cellular response to the invading larvae, in the skin of the host was depressed, but nevertheless, the larvae were surrounded by precipitates and trapped by the cellular reaction in the lungs, liver and peritoneum. It was concluded that cortisone may have blocked the cellular response

in the skin but after immobilisation on exposure to antibodies in the lungs, liver and peritoneum of the host, the trapped larvae stimulated a foreign body response at these sites. By giving large doses of cortisone to an immune rat, resistance to further infection by N. brasiliensis larvae can be totally abolished (Ogilvie, 1963). How the corticosteroid acts when such high levels of the drug are used is not clear.

Cooperia spp. larvae showed no development beyond the third stage in resistant cattle (Stewart, 1958; Soulsby, 1959) and Haemonchus contortus larvae can be inhibited at the third moult in resistant sheep (Soulsby, Sommerville and Stewart, 1959). However, it has been shown that this inhibition is not irreversible and unless the parasites are expelled from the host, they may develop to maturity, probably at a time when the host's immunity is waning. This has also been shown to be the case with Ostertagia and Trichostrongylus spp. (Soulsby, 1957; Michel, 1963; Armour et al, 1965).

Exactly how migrating larvae are inhibited or destroyed in immune hosts is not fully understood. This lack of knowledge is also true as regards the immune expulsion of a population of mature worms from the gastro-intestinal tract.

Stoll (1929) followed the faecal egg count of two grazing lambs infected with H. contortus. 10 weeks after being placed in

a limited grazing area, the faecal egg count which had gradually increased over this period suddenly dropped, and the lambs were subsequently resistant to re-infection. The term self cure was introduced to describe this phenomenon, which was more critically examined by Stewart (1950 a, b, c). It was shown by these later studies that self cure was initiated by the intake of large numbers of infective larvae. This resulted in expulsion of all or part of the adult worm population present in the abomasum, which brought with it the dramatic fall in faecal egg count.

Gordon (1948) and Stewart (1950) found that the immunity described by Stoll (1929) after self cure was not absolute, as the larvae which stimulated the reaction subsequently developed to maturity themselves.

The mechanism causing self cure was investigated by Stewart (1953; 1955). It was found that the level of blood histamine of sheep undergoing self cure increased around 2 to 4 days after re-infection and that the dramatic fall in faecal egg count was in some cases abolished by administration of an anti histaminic Anthisan (mepryamine maleate). This implied that a local anaphylactic reaction might be occurring in the abomasum. On injecting very large doses of exsheathed infective larvae directly into the exposed abomasum of sensitised sheep, peristalsis with marked oedema resulted, the reaction subsiding within 2 hours.

This provided supporting evidence for the idea of a local anaphylactic reation. It has been pointed out by Soulsby et al (1959) that the rise in blood histamine and the eosinophilia found in the mucosa of the abomasum which occurred 2 to 4 days after re-infection of infected sheep, coincided with the third parasitic moult. This suggested that exsheathing fluid from this moult might contain antigenic components important in the self cure reaction. This was in some part confirmed by the finding that injection of this moulting fluid into the abomasum of an infected sheep caused expulsion of adult H. contortus. Injection of this fluid at other sites on the sheep or the parenteral introduction of developing larvae, did not induce self cure (Stewart, 1961). This has led to the suggestion that self cure of an infected sheep is the result of a hypersensitivity reaction of the abomasum provoked by antigens produced when H. contortus larvae moult from the 3rd to the 4th stage. The subsequent effects of this reaction upon the mucosa of the abomasum or upon the worms causes expulsion of the adult parasites. It has been postulated that self cure might be mediated by spasms of smooth muscle in the abomasum coupled with lowered local O_2 tension due to the inflammatory reaction (Soulsby, 1957).

The hypersensitivity reaction to H. contortus not only results

in the expulsion of that parasitic worm from the abomasum but also causes expulsion of other abomasal parasites, T. axei and O. circumcincta. It was also found that during self cure T. colubriformis which parasitises the small intestine of sheep was also expelled. This "cross-expulsion" along with the immediate skin response to H. contortus antigens elicited by sheep capable of self cure has emphasised the systemic nature of the associated hypersensitivity (Stewart, 1953). It is probable that the immune response leading to self cure is of a systemic nature though it becomes manifest locally at the point of antigen release. Self cure of the abomasal parasites could not be stimulated by T. colubriformis, and it was suggested that the cross reaction caused by the self cure of H. contortus was due to the passage of antigens from that worm into the small intestine.

The antibodies produced by sheep capable of self cure have been investigated by Soulsby and Stewart (1960) and Soulsby (1960). It was found that the titres of complement fixing, precipitating and haemagglutinating antibodies increased dramatically in sheep from the time of self cure. Precipitating and haemagglutinating antibodies were not present in extracts of abomasal mucosa, hence no correlation seemed to exist between these kinds of antibody and resistance.

The *in vitro* respiratory rate of *H. contortus* larvae when suspended in homologous antiserum was not inhibited, however, antiserum to *T. colubriformis* did inhibit the respiration of worms of this species (Stewart, 1959).

With *H. contortus* in sheep, self cure is thought to be initiated by the antigenic stimulation associated with the intake of a large number of infective larvae. The termination of other nematode infections not involving the acquisition of a new infection may be a fundamentally similar process and it is often referred to as "self cure".

When mice which have had no previous experience of *T. spiralis*, were infected with that parasite, a mild inflammation occurred in the wall of the small intestine around 4 days post infection. This response to infection became acute after 8 days and resulted in the expulsion of the worms. In immune animals after re-infection, the inflammatory response in the gut reached a peak after only 4 days, hence resulting in a more rapid elimination. Larsh ~~et al~~ (1963) reviewing the mechanisms of acquired resistance to *T. spiralis* infections in the small intestine of the mouse, postulated that the worms were expelled by the combined effect of the creation of an unsuitable environment, due to the host tissue inflammation, and the direct action of antibody upon the worm, reducing its metabolic activit and causing it to be stunted. It was found that the expression of

immunity was blocked on the administration of cortisone, which greatly suppressed the cellular reactions in immune animals (Coker, 1956). Precipitating antibodies were present before the cellular reactions appeared, and cortisone did not apparently suppress the titres of these antibodies.

Acquired resistance shown by rats to a large initial infection of N. brasiliensis larvae results in the rapid elimination of the adult worm population from the small intestine of the infected host. This expulsion takes place between thirteen to fifteen days post infection (Haley and Parker, 1961). Sarles and Taliaferro (1936) and Taliaferro and Sarles (1939) have made a close study of the histological changes in the gut before and during this expulsion. Adult worms are located with their anterior ends buried in the crypts of the mucosa, often eroding the epithelium of the intestinal wall. Some inflammatory reaction was noted in the submucosa as early as 3 days after infection. It appears that this effect of the host upon the parasite becomes critical around 7 days post infection and manifests itself as an inflammatory reaction at the site at which the adult worm is feeding. Granular precipitates associated with the worms were also becoming evident at this point. This inflammatory response increases in intensity, the gut becoming dilated to two to three times its normal size by the tenth day of the infection. Expulsion takes place soon afterwards.

The timing and extent of the host reaction leading to 'self cure' or the termination of the infection depends upon the size of the initial infection. With large infections of N. brasiliensis larvae, egg production by the adult worm stops around day 12 (Haley and Parker, 1961) while moderate infections display a cessation of egg production by day 18 or 19 (Africa, 1931; Schwatrz et al, 1931; Graham, 1934; Porter, 1935). The small infections i.e. <80 larvae employed by Hurley (1959) gave prolonged initial infections.

That the expulsion of adult N. brasiliensis derived from an initial infection, is an immune phenomenon, is strengthened by the finding that if an adult worm population is removed from the host rat just before the onset of expulsion and placed surgically within the duodemun of a previously uninfected rat, then this population will successfully become established in its new host and continue egg laying for a further 9 to 10 days before resistance develops in the new host with ensuing expulsion (Chandler, 1936; Spindler, 1936). This suggest that the termination of an initial infection involving loss of the adult worms preceded by cessation of egg production is not due to some physiological factor in the parasite, but is due to an immune response on the part of the host. This is in some way confirmed by the fact that daily administration of large doses of cortisone abolishes the host's immune response. Soon

after cortisone treatment is stopped, expulsion of the worm population ensues (Ogilvie, 1963).

Delay in expulsion of N. brasiliensis adults was found with rats fed on a low protein diet (Wells, 1962). Such rats showed a reduced eosinophilic response, in the small intestine, however the mast cell and histamine content of the small intestine were found to be higher than in rats fed on a normal diet.

General summary of the work undertaken

The object of the work described in this thesis was to study certain aspects of the 'self cure' of N. brasiliensis infections in the rat, with a view to throwing some light on the mechanism of immunity to gastro-intestinal parasites generally.

The starting point for the investigations was the immune expulsion of the adult parasites at the terminal phase of a primary infection. This was investigated in a more quantitative fashion than formerly and its rapidity and extent clearly demonstrated. In order to study this reaction between host and adult parasite in an uncomplicated way, a reliable quantitative method had to be developed for the introduction of adult worms into the test animal. A surgical technique was evolved which was safe and reliable and gave "takes" which were nearer the theoretical and no less uniform than those resulting from infections with larvae.

Using the adult transfer method, the fate of parasites transferred to rats of different immunological status was studied and the "half lifes" of these introduced populations measured. Significant differences in the rate of immune expulsion were observed.

The most important question to be answered for this system is the mechanism of the immune expulsion. Parallel work by other colleagues in the department had indicated that local anaphylactic reactions in the gut might give rise to conditions which were "unsuitable" for the worms and thus lead to their elimination. It seemed to the author that the local anaphylaxis might only be one component in the expulsion mechanism and that the associated increased capillary permeability might allow plasma and, therefore, antibody, in quantity to come in contact with the parasite, and that the main effect might be due to "antibody v parasite".

In order to try and assess the importance of "antibody v parasite" in this system, two parallel studies were conducted. The first was aimed at examining some of the ways in which antibody might come in contact with the worm. Experiments with red cells labelled with ^{51}Chromium showed the extent to which this might occur due to blood sucking by the parasite or haemorrhage caused by it. Experiments with ^{131}Iodine labelled polyvinyl pyrrolidone were carried out to study the increased permeability to macromolecules and possibly therefore antibody which might occur in infected as compared to normal rats.

The second part of the "antibody v parasite" approach depends upon demonstrating that, if antibody does come in contact with the worm in quantity, it can do some harm. Again this aspect was investigated in two ways. Firstly, by incubating adult worms in immune serum before introducing them to the host, and secondly, by studying the effects of immune serum on the *in vitro* metabolism of the worms. A variety of different ways for producing immune serum was included in this study.

The possible significance of cell-fixed or reagin-like antibodies in immunity to *N. brasiliensis* and the participation and importance of such antibodies in the passive transfer of resistance was studied. Attempts were made to demonstrate the presence of cell bound antibody in the mucosa of the small intestine and other tissues of resistant rats by *in vitro* incubation of viable cell suspensions of these tissues with ^{131}Iodine labelled crude adult worm extract.

CHAPTER II

Life cycle of N. brasiliensis

Nippostrongylus brasiliensis, formerly called N. muris (Haley, 1961) is a small trichostrongyloid parasite of rats and has proved a convenient organism for the study of immunity to gastro-intestinal helminths (Chandler, 1937; Taliaferro and Sarles, 1939; Haley, 1962). The life cycle of this parasitic worm was first described by Yokagawa in 1922 and this has been followed by more detailed studies (Twohy, 1956; Haley, 1962).

Faeces from infected hosts contain eggs which under suitable conditions develop through 2 larval stages to the third stage within 3 to 4 days (Haley, 1962). The third stage larvae are the infective larvae and normally infect the host by percutaneous migration, however, it is possible for a few of the larvae taken by mouth to migrate from the stomach and complete the normal life cycle (Yokagawa, 1922; Africa, 1931; Schwatrz and Alicata, 1934).

The migration through the epidermis and dermis of the skin may be direct or via a hair follicle and is accomplished within 30 minutes to 2 hours. The larvae appear to remain in the hypodermis, for several hours, feeding (Taliaferro and Sarles, 1939; Twohy, 1956). After this period they migrate via both the blood stream

(Yokagawa, 1922) and the lymphatic system (Gharib, 1961) arriving in the lungs 11 to 15 hours post infection (Yokagawa, 1922; Twohy, 1956).

Little growth of the larvae has taken place until they reach the lungs where they feed on blood and tissue cells (Taliaferro and Sarles, 1939). During this period 18 to 32 hours post infection, the larvae grow rapidly (Twohy, 1956) and between 32 to 46 hours, the first parasitic moult yielding fourth stage larvae takes place (Yokagawa, 1922) whereupon the larvae migrate up the trachea, are swallowed, and begin to appear in the duodenum between 50 to 60 hours after infection (Yokagawa, 1922; Sarles and Taliaferro, 1936; Twohy, 1956). Rapid growth of the worms is found at this stage (Twohy, 1956).

The second parasitic moult takes place in the small intestine between 90 to 108 hours after infection (Yokagawa, 1922) and these immature adult worms quickly reach maturation with associated sexual maturity. Fertilisation occurs and eggs can be detected in the faeces of the infected host by the sixth day of the infection (Weinstein and Jones, 1959).

The adult worm is found in the mucosa of the duodenum, jejenum and upper ileum, where it burrows among the villi and crypts. On the basis of histological evidence Taliaferro and Sarles (1939) stated that the adult worms feed on tissue cells and blood, but the

presence of intestinal flagellates in the intestine of worms suggests that they ingest some gut contents. This has been confirmed by Weinstein and Jones (1956), however, other workers (Rogers and Lazarus, 1949a) using radioactive phosphate concluded that the adult worm feeds primarily on host tissue.

From the sixth day to the 10th day post infection, the egg production rises rapidly remaining at a high level for 3 to 5 days before falling to a low or zero level 15 to 20 days after infection (Africa, 1931; Schwartz and Alicata, 1934; Graham, 1934).

Very soon after the dramatic drop in oogenesis, most of the adult worms are expelled from the host's gut. The timing of onset and the rate of this expulsion, varies with the size of the initial infection. Small infections give a gradual loss extending over as much as 30 days, whereas with large or intermediate infections, almost total expulsion takes place rapidly, between the 10th and 20th day post infection (Haley and Parker, 1961). It appears that female worms are expelled more rapidly than males (Africa, 1931; Porter, 1935).

CHAPTER III

General methods and materials

Experimental animals

Rats of the hooded Lister type were used in all experiments. These animals were obtained from two different sources. Initial supplies were bred and reared until 6 to 8 weeks old at the animal breeding station of the London School of Tropical Medicine and Hygiene. Later supplies were obtained from Animal Suppliers Ltd., London, again when the rats were around 6 to 8 weeks old. Both strains were similar and were free from helminth parasites with the exception of *Hymenolepis spp.* which were occasionally noticed at autopsy.

The rats were kept in wire cages in an animal house maintained at 65°F. The cages were suspended in racks such that the wire mesh floor of each cage was three inches above a sawdust covered metal tray placed beneath. The rats' faeces and urine could easily pass through this wire mesh floor of the cage on to the tray below. This system of maintainance of the rats precluded any possible accidental cross infections. Occasionally non-infected rats, suitably marked for identification purposes, were placed in a cage along with other rats infected with *N. brasiliensis*. This situation did not at any time result in the non-infected rats

becoming infected through accident. All rats were fed on a pelleted diet (Diet 41, supplied by W. Primrose & Son, Glasgow), this and water being supplied ad libitum.

Both male and female rats were used in all experiments, unless otherwise stated, and they each weighed approximately 160-190 gms. at the time of experiment.

Parasite

The strain of Nippostrongylus brasiliensis used in all experiments was obtained initially from Dr. Hopkins of the Zoology Department, Glasgow University, and was maintained by regular passage through rats similar to those used for experimental purposes.

Culture of infective larvae

Faeces were collected from rats with a patent infection, usually over days 8 and 9 post infection. This was accomplished by placing a sheet of newspaper on the metal tray beneath the cage containing the infected rats. The faecal pellets so collected, were broken up and mixed to a paste with a little water in a mortar. Using a spatula, a portion of the faecal paste was spread on to the centre of a circle of Whatman's No. 1 filter paper, diameter 7 cm., the faecal smear being about 3 cm. diameter, such that the periphery

of the filter paper was kept clear. The paper was then dipped briefly in water and placed on a circle of plastic foam material, 1 cm. thick, saturated with water, in a disposable plastic petri dish (produced by Oxoid Ltd.). Care was taken to ensure that the outer fringe of the filter paper was kept clear. Lids were placed on the petri dishes and they were stored in a humid incubator at about 27°C.

Normally after 5 days the larvae could be seen collecting in a fringe around the periphery of the filter paper. The larvae were always harvested between 5 and 10 days after setting up the faecal culture, and were normally used to infect rats on the same day. Harvesting of the infective larvae was accomplished by flooding the petri dishes with water at about 35°C, thus permitting the larvae to swim off the filter paper. After lifting out and discarding the plastic foam pad and the filter paper, the warm water containing the larvae and some faecal material was filtered, under suction, in a large Buchner funnel, through strong filter paper. (Green's Hyduro 904, 18.5 cms. diameter). This filter paper was then removed, inverted, and placed on an Endecott sieve (mesh 400) in a Baermann apparatus filled with water at 35°C. The larvae swam downward through the fine mesh of the sieve, whilst the faecal debris was held back. The larvae collected at the bottom of the funnel and were run off into a suitable container.

Counts were made by a dilution technique. A 1.0 ml. sample was diluted to 100 ml. with water and 0.1 ml. samples of this suspension, made homogeneous by thorough mixing, were placed on a slide and the larvae counted with the aid of a binocular microscope. Usually about ten such counts were carried out.

Infection of rats with larvae

The original larval suspension was then diluted so that the required number of larvae for infection purposes was contained in 1.0 ml. of suspension. Antibiotics were added to the suspension giving a final concentration of 100 units Penicillin and 10 micro gm. Streptomycin per millilitre. The rats, while lightly anaethsetised with ether, were injected subcutaneously in the groin region using a 1.0 ml. syringe fitted with a No. 15 needle (B.S.W.G.).

Due to errors involved in the dilution counting procedure and in the injection itself, it was not possible to determine the number of larvae given to each rat with great accuracy, and as a result, where thousands of larvae were administered, the infection dose is given to the nearest hundred e.g. circa(c) 3,000.

Recovery of adult worms from the small intestine

In order to reduce to a minimum, the amount of food material present in the gut, the rats were starved overnight before killing. Each rat was killed by a hard blow to the head and the small intestine removed immediately after death, and carefully slit longitudinally.

When it was desired to gain an accurate estimation of the number of adult worms present in the gut of each individual rat, the small intestine, after being slit longitudinally, was cut into approximately 5 cms. lengths and placed in a muslin bag which was suspended in a 250 ml. beaker half filled with warm saline. The beaker was placed in a constant temperature water bath set at 37°C. for about an hour, during which time virtually all the adult worms swam out and collected at the bottom of the beaker. They were then collected by means of a broad mouthed Pasteur pipette.

Where a quantity of adult worms was merely being collected for some other experiment, the guts after being slit longitudinally, were put in an Endecott sieve of mesh 50. This was placed in a Baermann apparatus filled with saline and set up in a deep water bath at 37°C. Within 30 minutes the majority of the adult worms had migrated downwards and had become concentrated at the bottom of the filter funnel. These could then be run off into a suitable container for counting. This proved an efficient and rapid method of obtaining a large number of adult worms, free of debris.

The number of adult worms present in a given suspension was determined by removing 5 ml. aliquots by means of a pipette with the tip sawn off. Each aliquot was diluted to 50 ml. with warm saline and the number of worms present in a 1 ml. sample was counted with the aid of a binocular microscope. The mean number of worms from ten such counts was taken.

Faecal egg counts

Faeces from the infected rats were collected over a 24 hour period. From this a 1.0 gm. sample was chosen at random, homogenised in 10 ml. water, passed through a sieve (mesh 50) and the filtrate centrifuged (1,500 r.p.m.) The supernatant was discarded while the precipitate was resuspended in 15 ml. of saturated sodium chloride solution. Both chambers of a McMaster slide were filled with this suspension by means of a fairly broad mouthed Pasteur pipette. Both chambers, each representing a volume of 0.15 ml. were examined under the microscope for parasite eggs. The mean value from both chambers was obtained and this figure times 100 gave the number of eggs present in the original gram of faeces (e.p.g.) This assay was done in duplicate for each 24 hour faecal sample.

In some experiments, the total weight of faeces making up the 24 hour sample was recorded and the results expressed as total eggs per 24 hour faecal sample per rat. Chandler (1936) claimed

that this was a better method to use for the comparison of the parasite egg content of the faeces between two groups of experimental animals than the concentration measure of eggs per gram.

Nitrogen determination

In some experiments the results were expressed in terms of worm nitrogen. This was determined by the micro-Kjeldahl method as described in Kabat and Mayer (1960).

The whole worm sample or the measured aliquot of worm homogenate as the case might be, was placed in a 25 ml. micro-Kjeldahl flask. To this was added a boiling stone, about 1 gm. of mixed K_2SO_4 and $CuSO_4$ catalyst, and 2.0 ml. H_2SO_4, all chemical reagents being "low N" analytical reagents. Digestion was carried out in a special micro-Kjeldahl digestion rack. Boiling was continued for 30 minutes after the solution had become colourless. The digested sample was removed from the digestion flask and made up to a convenient known volume. A measured aliquot was removed and placed in a Markham Kjeldahl distillation apparatus. A 50 ml. Erlenmeyer flask containing 5 ml. of boric acid-indicator mixture and 5 ml. water was placed under the condenser. 9.0 ml. of saturated Na OH solution was then added to the digested sample and steam distillation carried out until the volume in the Erlenmeyer flask had increased to approximately 25 ml.

The distillate was then titrated with exactly N/70 H.Cl. from a micro-burette. Blank runs on the reagents used for digestion and distillation were performed and any nitrogen detected was deducted from the worm sample values. As a check on the method, frequent runs with standard ammonium sulphate solution were carried out.

CHAPTER IV

Studies on the termination of a primary larval infection

Introduction

It has been recognised with N. brasiliensis infections in the rat, that host resistance to a primary infection brings about a reduction in the adult worm burden. It was first noted that the faecal egg count of rats infected with N. brasiliensis fell to a very low count 18 to 21 days after infection (Africa, 1931; Schwatrz and Alicata, 1934; Graham, 1934). It was shown by Taliaferro and Sarles (1939) that this drop in egg production just precedes expulsion of the adult worm population which begins around day 16. With small initial infections there is either no immune expulsion or a very gradual loss of adult worms (Hurley, 1959; Haley and Parker, 1961) however large or intermediate infections resulted in an abrupt loss between day 10 and day 20.

Most of these previous studies have been of a semi-quantitative nature. In order to rigorously examine the terminal phase of an initial infection of N. brasiliensis in rats, the following experiment was carried out. The number of infective larvae used to give the primary infection was similar to that used in other experiments in this thesis.

Figure 1

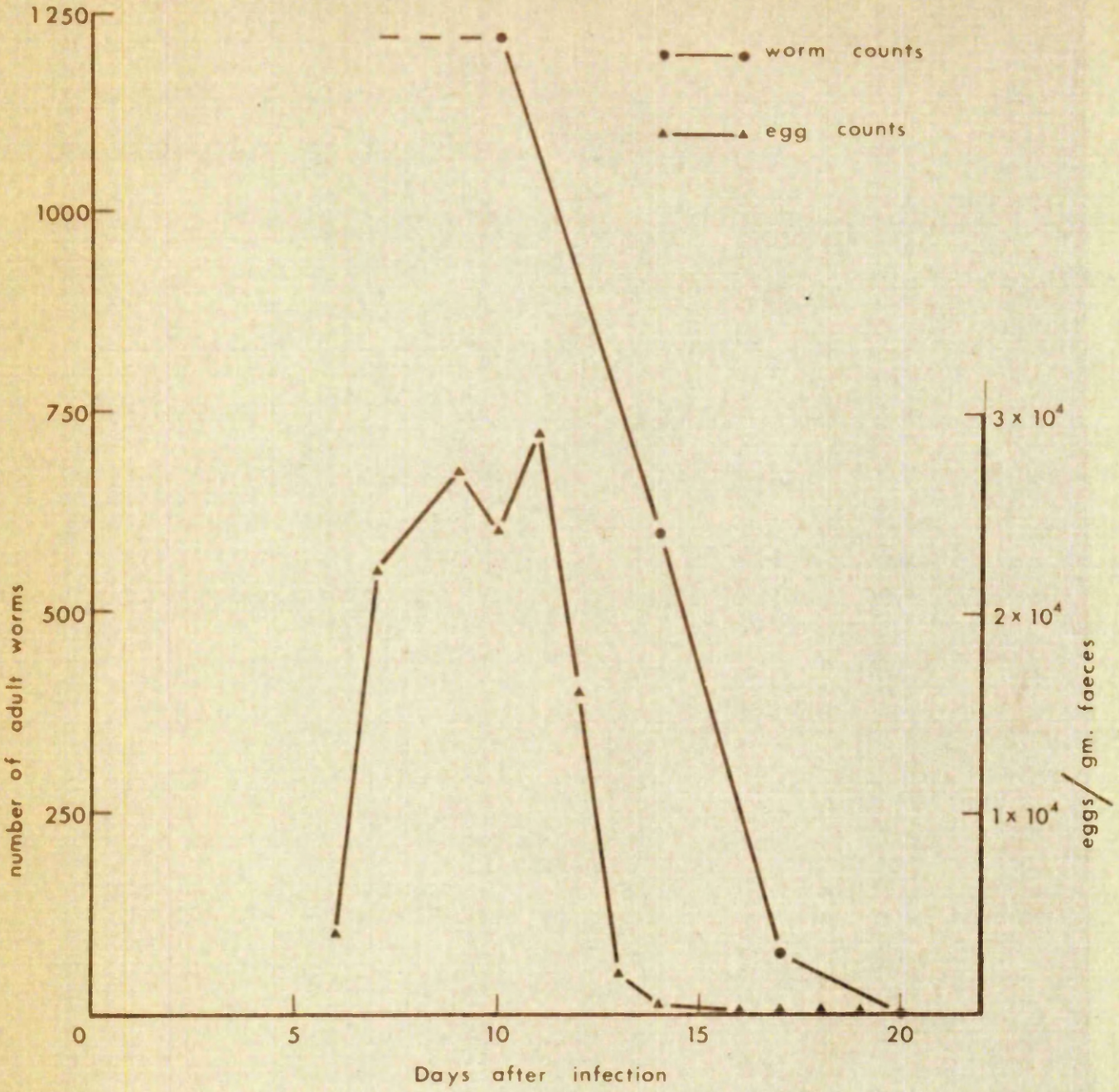


Figure 1. Eggs per gram of faeces and number of adult N. brasiliensis isolated from the small intestine of rats each infected with 5,000 larvae on day 0.

Methods and materials

A group of 60 hooded rats, comprising of approximately equal numbers of both sexes, each about 150-175 gms. live weight, were each infected, subcutaneously, with 5,000 N. brasiliensis larvae. Details concerning the isolation, preparation and injection of the dose of larvae are given in Chapter III.

The infected rats were sub-divided into 4 groups, each of 15 rats. One group was killed on each of the following days:- the 10th, 14th, 17th and 20th day post infection. The faecal egg counts were followed throughout the experiment and the number of adult worms found in the small intestine at autopsy was recorded.

Results and discussion

The mean number of adult worms recovered from the small intestines and the mean faecal egg count of the rats constituting each group are shown in fig. 1.

It can be seen that complete elimination of the adult worm burden took place between day 10 and day 20 following infection. The greater part of the worm population was expelled between day 10 and day 17. Hence it is clear that, the comparatively large infection used in this experiment resulted in the rapid and complete elimination of the adult worm population.

Since the fall in egg output preceded the drop in worm burden, it is apparent that the egg output curve reflected not only a fall in the number of adult worms but also some interference with reproduction due to a suppression of egg production or to a disproportionate elimination of female worms. Earlier workers have indicated that adult female worms are in the majority at the beginning of an infection, but are expelled before the males, with the result that toward the end of an infection there are more males than females (Africa, 1931; Porter, 1935).

It seemed appropriate to try and elucidate the mechanism of this sudden loss of adult worms and most of the experiments described below are directed towards this end.

Summary

(1) A study was made of the extent and rate of the expulsion of adult N. brasiliensis from the small intestines of rats following an initial larval infection.

(2) Almost complete expulsion, preceded by a fall in egg production, took place between day 10 and day 20 post infection.

CHAPTER V

Development of a technique for the introduction of adult worms directly into the duodenum

Introduction

All the evidence suggests that the expulsion of adult *N. brasiliensis* demonstrated in the previous experiment is the result of an immune response on the part of the host. In order to make an uncomplicated study of this immunological attack on the adult worms, it was necessary to challenge rats by introducing adult worms directly to the duodenum rather than by infecting them subcutaneously with larvae. Earlier workers have shown that it is possible to transfer adult worms directly to the small intestine of a recipient rat (Chandler, 1936; Spindler, 1936; Sarles and Taliaferro, 1936).

To see if this method of infection could be consistently achieved in a quantitative fashion an experiment involving the transfer of different numbers of adult worms was carried out.

Methods and materials

Adult *N. brasiliensis* worms were carefully isolated from donor rats, which had been infected with larvae 10 days previously. The concentration of the freshly obtained worm suspension was

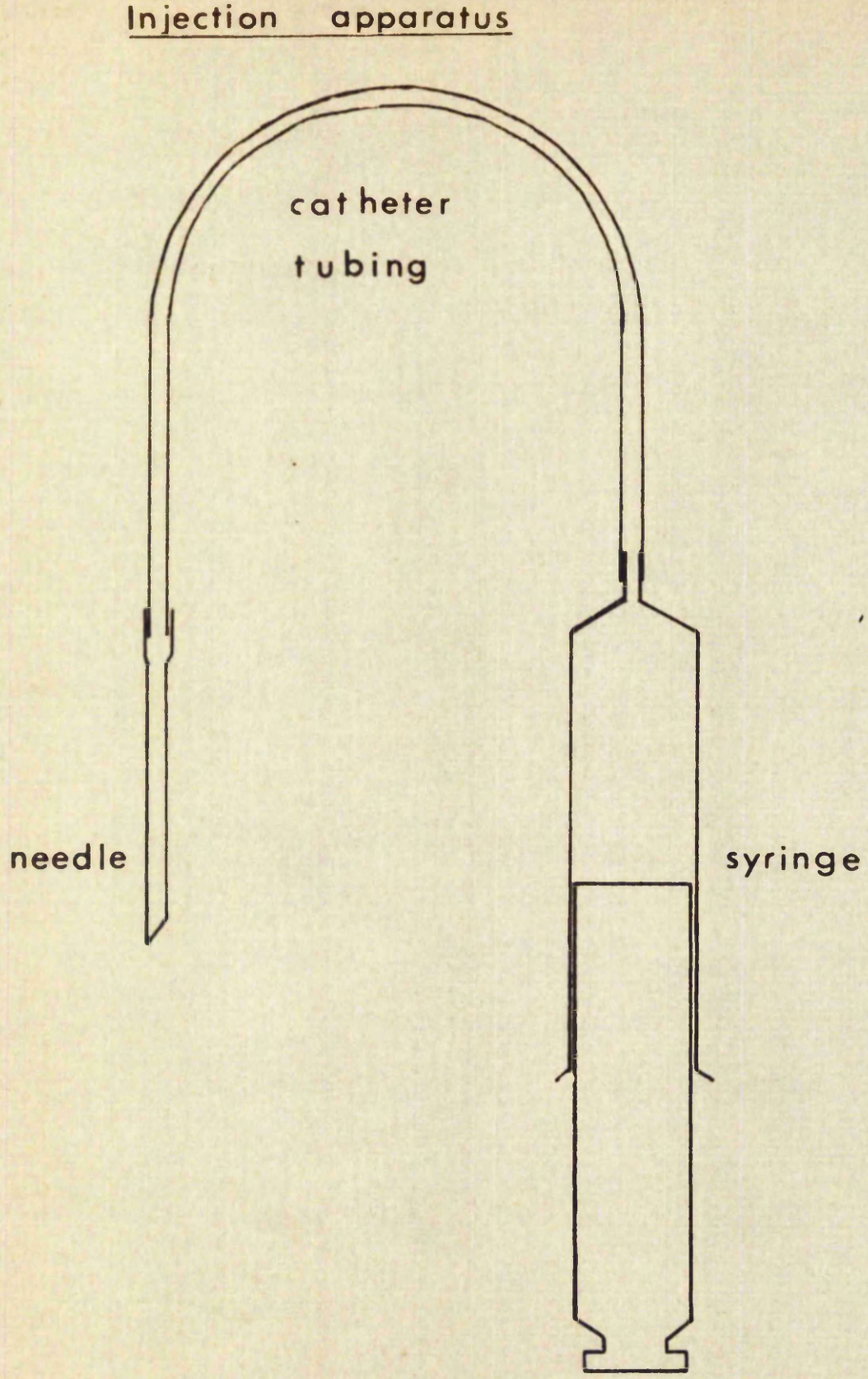


Figure 2. Apparatus used to inject adult N. brasiliensis directly into the duodenum of the rat.

adjusted with warm saline until 5 ml. contained the required number of worms to be transferred to a single rat. 5 ml. of this suspension, mixed carefully so that the worms were evenly distributed, was removed with a broad mouthed pipette and delivered into a tapered 15 ml. centrifuge tube placed in a 37°C. water bath. Throughout these operations care was taken to maintain the worms as near to 37°C. as possible, and to expose them to a minimum of mechanical damage.

Just before transfer to the recipient rat the worm sample along with 1 to 2 ml. suspending liquid was cautiously drawn up into a length of catheter tubing (2 mm. diameter) fixed to a 5 ml. syringe. (c/f fig. 2). A number 1 needle (B.S.W.G.) was then placed on the free end of the tubing. The worms tended to swim out along the catheter tubing forming a suspension, free from clumps, which was easily injectable.

The recipient rat was anaesthetised with "Trilene"* and the abdomen shaved and washed. An incision was made in the skin and then through the muscle layers on the right side just caudal to the ribs, exposing the duodenum. The worms were then slowly injected directly through the wall of the duodenum with the apparatus described. Suturing of the intestinal wall after this operation was not required. The muscle layers, then the skin were each closed with a single everting nylon suture. Care was

*"Trilene", (trichloroethylene B.P.), I.C.I., Pharmaceuticals Division, Wilmslow, Cheshire.

Table 1

Number of worms recovered from the small intestine of normal rats following an infection of adult worms surgically transferred to the duodenum

No. of rats per group	No. of worms transferred on day 0	Mean No. of worms recovered on Day 4	Percentage of introduced worms established
21	1,000	672 ± 231	67%
11	750	513 ± 74	68%
12	500	319 ± 116	64%

taken to ensure that the surgical procedure was moderately aseptic. Very few deaths occurred and the treated rats usually showed full recovery within a day or two.

In this experiment 21 rats were each given 1,000 adult worms, 11 rats were each given 750 adult worms and 12 rats were each given 500 adult worms. The three groups were killed on the 4th day following transfer and the mean worm burden for each group was found.

Results and discussion

It can be seen that a very uniform percentage of the administered dose of adult worms became established in the gut of the recipient rat. (table 1). Over the range used in this experiment, the number of introduced worms becoming established was an average between 60 to 70 per cent.

The uniformity of the size of the resulting adult worm burden brought about by this method of infection was better than that resulting from larval infections designed to give adult worm burdens similar to those created in this experiment. In the previous experiment where a large dose of infective larvae was given to each rat, only about 25% of these matured to adult worms. The variation in take between individual animals was also much greater. It was found by Haley (1962) with small initial larval

infections administered subcutaneously that about 60% of these larvae appeared as adults in the small intestine 10 days later. The fate of these larvae lost during parenteral migration is not known, though it is thought that they may not have migrated at all (Taliaferro and Sarles, 1939; Twohy, 1956) or they may have been lost during migration by being swept into some organ from which they were unable to escape (Weinstein, 1956) or they may have been overcome by innate resistance mechanisms.

The importance of parenteral migration and these "lost larvae" in the immunological response shown by the host to the adult worm population is difficult to assess. No such unknown factor results from initial infections created by the surgical transplant of viable adult worms from one rat to another. In this way the immune expulsion of the adult worm burden can be studied in the absence of complicating factors.

Summary

(1) A method is described for the successful transfer of adult worms from the gut of one rat to another.

(2) "Takes" of adult worms introduced to the recipient rat are regular and the variations involved sufficiently small to make the method of quantitative value.

CHAPTER VI

The fate of an adult worm population transferred to rats of varying immunological status

Introduction

It is known that a previously infected rat when challenged with a further dose of larvae will resist this infection. Resistance is manifested by a reduction in the number and size of the adult worms maturing in the small intestine, a reduction in egg production and a more rapid elimination of this worm population when compared with previously uninfected rats (Taliaferro and Sarles, 1939). It is also possible, using the serum of highly resistant rats, to transfer passively, to some extent, all the effects of a naturally acquired resistance (Chandler, 1937; Sarles, 1939).

The study of resistance to re-infection with *N. brasiliensis* has involved, in the past, the effect of the rat's immune response upon a challenge of infective larvae. It seemed that a less complicated appraisal of that part of the host's immune response, which brings about the expulsion of the adult worms parasitising the small intestine, might result from a study of the fate of a challenge, comprising of adult worms, surgically transferred to the duodenum of the challenged rat. That this method of infection is reliably quantitative has been shown in the previous Chapter.

In the following experiment, a study was made of the rapidity and severity of the expulsion of a population of adult worms, surgically introduced to the small intestines of rats immunised in different ways.

Methods and materials

(a) Preparation of hyper-immune rats and immune serum

A large group of rats were given four infections of N. brasiliensis larvae. Each rat received 3,000 larvae, then 5,000 larvae, then 7,500 larvae and finally 10,000 larvae, at fortnightly intervals.

Twenty of these rats made up Group 4 (the hyper-immunised group) of the present experiment. The challenge of 1,000 adult worms was given 20 days after the final larval infection.

The remainder of the hyper-infected rats were bled by cardiac puncture, 10 to 14 days following the final larval infection. The serum obtained in this way was administered to the rats constituting Group 2 (the passively immunised group).

(b) Experimental animals

Hooded rats of both sexes, each weighing about 175 gms. at the time of experiment, were divided into 4 groups and treated as follows:-

Group 1 consisted of 40 rats which had had no previous experience of the parasite *N. brasiliensis*. This was the 'control group' of animals.

Group 2 consisted of 48 rats. Each rat was weighed and passively immunised by the injection, intra-peritoneally, of 4.0 ml. of immune serum per 100 gm. live weight. The serum was injected in two doses, the first during the morning and the second in the evening of the day immediately before that of challenge. The method of preparation of the immune serum is described above. This was the 'passively immunised group' of animals.

Group 3 consisted of 48 rats. Each rat in this group had received a primary infection of 3,500 infective *N. brasiliensis* larvae, injected subcutaneously 30 days before the day of challenge. This was the 'single infection group' of animals.

Group 4 consisted of 20 rats. Each rat in this group had undergone a series of larval infections. Details of the size and frequency of the four larval infections given to each rat, are given above. This was the 'hyper-immunised group' of animals.

Each rat of each group was challenged at day 0 of the experiment, by surgically transferring 1,000 adult *N. brasiliensis* to the duodenum. The adult worms were isolated from the small intestines of the donor rats 10 days after a primary larval infection. The procedure used is described fully in Chapter V.

Table 2

Autopsy schedule of rats challenged on day 0 with 1,000 adult worms each

Day following challenge	Number of rats killed from each group			
	Group 1 (controls)	Group 2 (passively immunised)	Group 3 (single larval infection)	Group 4 (several larval infections)
1	5	6	6	4
2	5	6	6	4
3	5	6	6	4
4	5	6	6	4
5	5	6	6	4
6	5	6	6	-
7	5	6	6	-
8	5	6	6	-

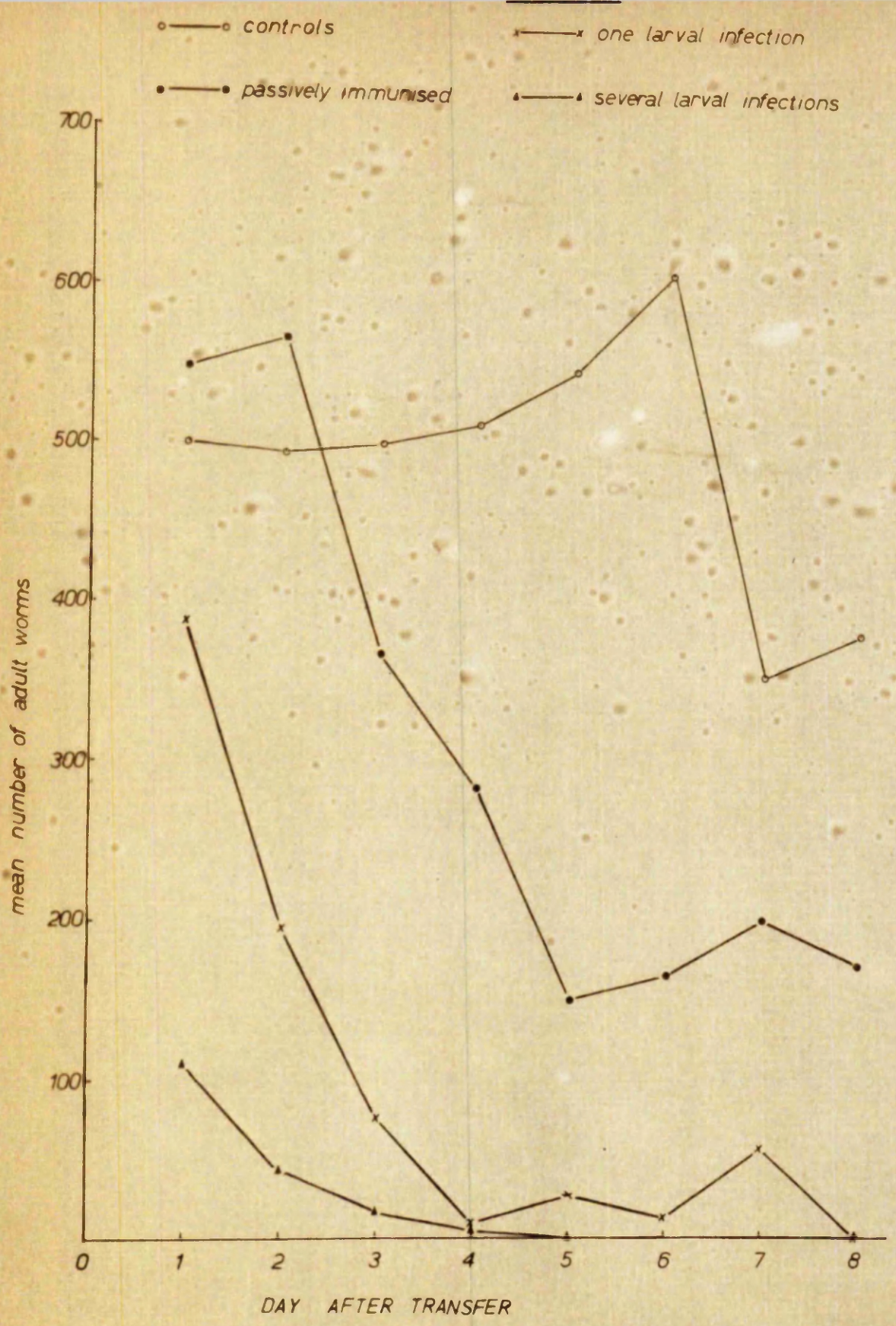


Figure 3. Number of adult N. brasiliensis isolated from the small intestines of rats of different immunological status following transfer of 1,000 adult worms to each rat on day 0.

Several rats from each group were sacrificed each day after challenge (details are indicated in table 2) and the number of adult worms present in the small intestine of each rat determined, in the way described in Chapter III.

Results

The mean number of adult worms, resulting from the transferred infection, recovered from the small intestines of the rats comprising each group is shown graphically in fig. 3. Over the experimental period i.e. 8 days following transfer, it can be seen that the control animals, Group 1, harboured more or less a constant number of adult worms. However, it would appear, on days 7 and 8 following transfer, that some reduction in the number of worms was taking place. This indicated that the host's immune response, which is manifest by expulsion of the worm burden, was becoming evident at this point.

Resistance, as shown by a reduction in the size of the adult worm burden, only became evident in the passively immunised group (Group 2) on day 3 following transfer. Over the first 2 days of the experiment no difference in the size of the introduced worm population was seen between this group and the control group. Also, with the passively immunised group, it can be seen that further expulsion of adult worms ceased from day 5 until the end

Table 3

Number of stunted worms recovered from the small intestines of rats comprising Group 4 (several larval infections)

Day after challenge	No. of rats	Mean no. of stunted worms	Mean no. of transferred worms
1	4	38 ± 50	110 ± 90
2	4	69 ± 71	43 ± 6
3	4	44 ± 41	17 ± 13
4	4	19 ± 25	5 ± 4
5	4	10 ± 13	0

of the experiment. As a result the size of the adult worm population remained almost constant over this period.

Both Group 3 (single previous larval infection) and Group 4 (several previous larval infections) displayed a reduction in the number of transferred adult worms becoming established, when compared with the control group. From day 1 onwards it is evident that the recipient host's immune response was immediate in both groups of animals and that it was stronger with Group 4. The worm burden found in this group was smaller on each corresponding day than Group 3 animals, and complete elimination of the introduced worms was effected earlier.

When the rats comprising Group 4 (several previous larval infections) were autopsied in this experiment, a number of small stunted adult N. brasiliensis along with adult worms derived from the transferred infection were found in the small intestines of some rats. These stunted worms were quite distinct from the introduced adult worms which showed no evidence of becoming stunted during the short experimental period. It is probable that these stunted worms had originated from a previous larval infection. The mean numbers found are shown in table 3. Although the number of rats killed each day was small, it appeared that those rats which harboured stunted worms also harboured a greater number of transferred adult worms. It can be seen in table 3 that the expulsion of the introduced adult

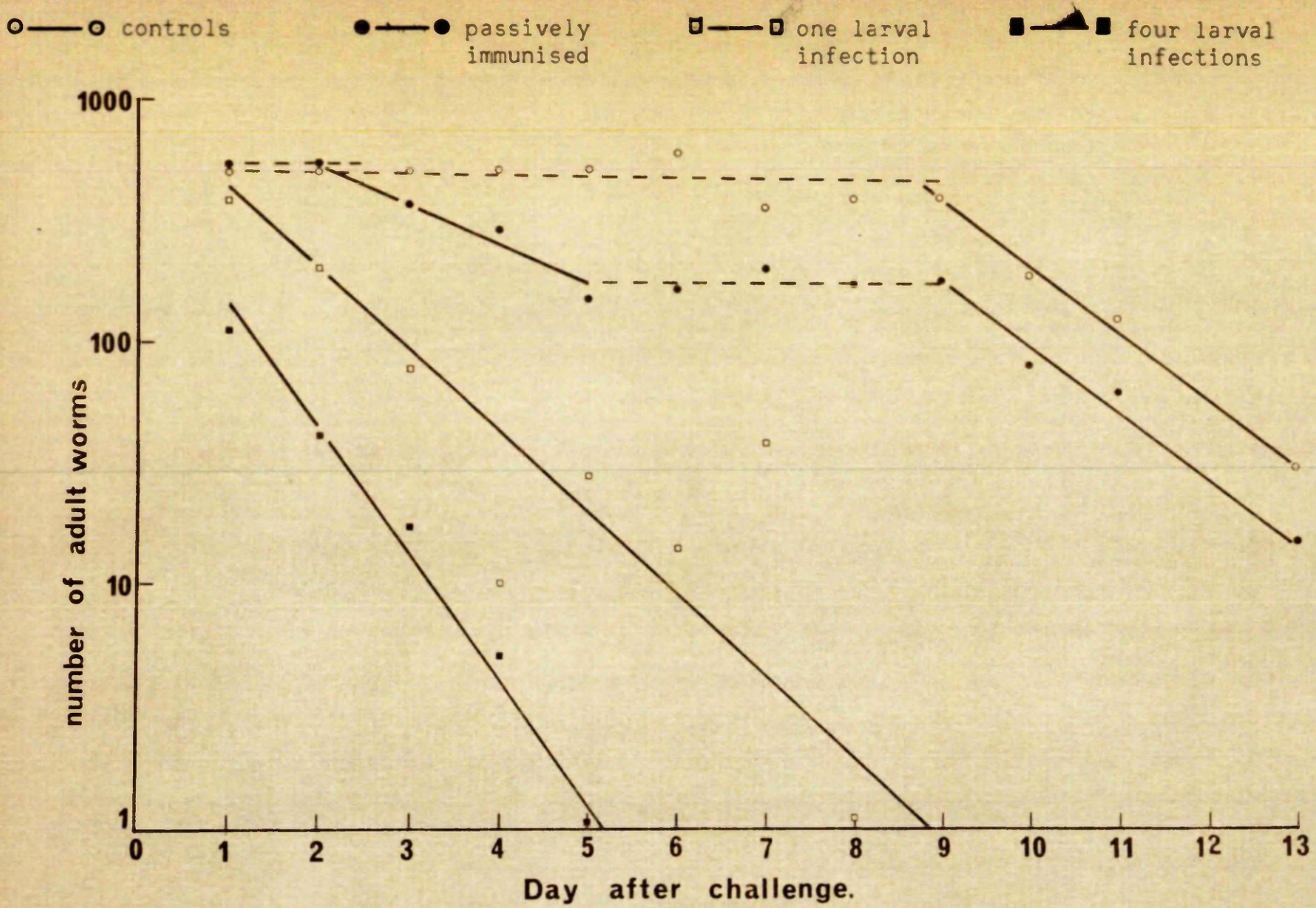


Figure 4

Figure 4. Number of adult N. brasiliensis isolated from the small intestines of rats of different immunological status following transfer of 1,000 adult worms to each rat on day 0.

Table 4

Biological half-lifes of adult worm populations in rats of different immunological status

Group		time after infection	biological half life	half-life for entire period of infection
1	normal rats	days 1 to 9	infinity	
		days 9 to 13	24 hours	104 hours
2	passively immunised rats	days 1 to 2	infinity	
		days 2 to 5	44 hours	
		days 5 to 9	infinity	
		days 9 to 13	24 hours	64 hours
3	actively immunised rats (single infection)	days 1 to 9	22 hours	22 hours
4	actively immunised rats (hyper-infected)	days 1 to 5	14 hours	14 hours

worms is more rapid than the expulsion of the stunted worms. No similar stunted adult worms were found in Group 3 which had experienced only one previous larval infection.

Wade et al (1960) studying the elimination of D. viviparus from the lungs of guinea pigs estimated the biological half-life of the worm population by plotting the logarithms of the numbers of worms recovered against time after infection. The data obtained in this experiment covering that period from day 0 to day 8 of an infection combined with data obtained in an earlier experiment covering the period day 9 to day 13 has been treated in this way. (fig. 4).

In this graph regression lines have been fitted, and from the slope of these lines the approximate half-life of the worm population can be determined. A comparison can be made of the rate of elimination of the worms during the expulsion phases by assessing the biological half-lifes over these periods. These results are shown in table 4, along with an estimated half-life value for the entire period of the infection for each group of rats.

Discussion

Rats which have had no previous experience of the parasite *N. brasiliensis* do not exhibit an immune response to the worm burden until 12 to 14 days post infection when infected with larvae, or until 8 to 10 days post infection when infected by introducing adult worms surgically into their small intestines. This is the case with intermediate or large infections. With small infections of larvae or adult worms, the onset of expulsion may be delayed (Taliaferro and Sarles, 1939; Haley and Parker, 1961; Spindler, 1936; Chandler, 1936). This has been confirmed with the control group of rats in this experiment so far as infection by adult worms is concerned. It can be seen with this group that the self cure reaction can be stimulated by infection with the adult worm. It would appear that the parenteral migration of larvae is not a prerequisite for this immune response to occur.

The effect of acquired immunity upon an adult worm infection is made evident from a study of the expulsion of that infection by rats comprising groups 3 and 4. Group 3 rats had experienced only one previous infection with larvae, whilst Group 4 rats had been exposed to several large infections with larvae. In both groups, the mechanism whereby the host rids itself of its worm burden is initiated immediately. The extent and severity of

this reaction, as judged by the reduction in numbers of introduced adult worms remaining, is greater in Group 4 than in Group 3. This suggests that more than one previous larval infection is required, in the case of N. brasiliensis in rats, before maximal immunity to re-infection is obtained. This is in agreement with the protection offered by passively immunising rats with serum from rats having suffered one infection and from rats having undergone a number of infections. The latter serum is more protective than the former (Mulligan et al, 1965).

Faliaferro and Sarles (1939) found that when previously infected rats were challenged with N. brasiliensis larvae, fewer worms matured in the small intestine and they were expelled sooner than in previously non-infected rats. These workers also reported that the size of the mature worms was decreased. Stunted adult worms have also been found in calves repeatedly infected with D. viviparus (Jarrett et al, 1959). In the present experiment stunted small brown adult N. brasiliensis were found at autopsy in the small intestines of some, but not all, of the rats comprising Group 4. As the rats of this group had recently received several large infections of N. brasiliensis larvae, there is no doubt that these stunted adult worms originated from these immunising infections. Faliaferro and and Sarles (1939) also found stunted adult worms in the small

intestines of rats which had been repeatedly infected with large doses of larvae. These workers also showed that the stunted worms, on being transferred to the duodenum of a 'clean' rat, developed into normal adult worms and began egg production. It was evident that an immune response on the part of the host was responsible for the stunted nature of the worms, and it was thought that the parasites were unable to feed properly on the immune host's tissues. This failure to feed may have reduced sufficiently the antigenic stimulation by these worms, so avoiding the onset of the expulsion mechanism.

Although the number of animals harbouring stunted worms in Group 4 in this experiment was small, and caution is required in interpreting these results, it did seem that the immune expulsion stimulated by the introduction of healthy adult worms also brought about some expulsion of these stunted worms. However, the expulsion of the stunted worms was not so effective as the expulsion of the normal worms. The immune expulsion may not only depend upon the creation of an unsuitable environment within the gut, but also a specific anti-parasite function of either humoral or cell-bound antibody. If this were so, then the stunted worms, which presumably did not feed as actively as the newly introduced adult worms, may not have been exposed to the same extent to these antibodies which may specifically

inhibit some metabolic or feeding processes vital to the worm. In this way the stunted worms may not have been exposed to the full anti-worm action of the host's defences.

Another feature, which more meticulous experimentation will clarify, was the finding that those hosts which harboured stunted worms also harboured a greater number of adult worms from the introduced infection. It may be that some rats are incapable of developing such a strong acquired resistance as shown by other rats.

Passive transfer of resistance to infection with N. brasiliensis has been shown by Sarles and Taliaferro (1936), Chandler (1937), Sarles (1939) and Mulligan et al (1965), and it is generally accepted that the serum from a rat which has had several larval infections confers greater resistance than serum from a rat which has experienced a single infection. The passive transfer of resistance to several other parasitic worm species has been established, among them, Trichinella spiralis (Culbertson, 1942), Ascaridia galli (Sadun, 1949) and D. viviparus (Jarrett et al, 1955). There can be no doubt that humoral antibody is playing a role in this phenomenon.

Group 2, in which each rat was passively immunised with serum obtained from rats which had experienced several larval infections, shows an interesting response to its challenge

infection. Over the first and second days following transfer there is no significant reduction in the number of worms harboured, when compared with the control group. However, it would appear that on day 3 a trend, whereby the worm burden is being reduced, is being initiated, and becomes clearly evident on days 4 and 5 following transfer. There is no further expulsion of the surviving worms over days 6, 7 or 8. It is difficult to explain the time lag of 3 days in the appearance of resistance in rats composing Group 2. In both the actively immunised groups, Groups 3 and 4, resistance as shown by expulsion of the introduced worms, was immediate. Urquhart et al (1965) have proposed that the possession of resistance by rats to adult N. brasiliensis may depend upon a state of hyper-sensitivity in the rat in which local anaphylactic reactions in the gut create an unsuitable environment for the parasite. This could lead either to the ultimate expulsion of a resident population, as seen in Group 1 in this experiment, or failure to allow the establishment of an introduced adult worm population, as seen in Groups 3 and 4. Soulsby (1961) proposed that the 'self cure' in H. contortus infection in sheep, depended upon a state of specific hypersensitivity in the abomasum. On the arrival of a dose of newly obtained larvae the sensitised abomasum would show an anaphylactic reaction in response to this antigenic stimulus.

In Groups 3 and 4 in this experiment it could be argued that the introduced worms provide the antigenic stimulus for an anaphylactic reaction to ensue. It is also possible that the guts of Group 4 rats are more highly sensitised causing a more violent reaction, resulting in a more rapid elimination of the worms in this group.

With regard to the time lag between the introduction of worms and the onset of expulsion in the passively immunised Group 2, it may be that a certain period of time is required for a sufficient amount of the introduced antibody to become fixed at the site of the antigenic stimulation. If this were so, then under the conditions prevailing in this experiment, 3 days are necessary for this to happen. It would be interesting to carry out an experiment, giving immune serum at different times before and after the time of transfer and noting, when the expulsion of the worms begins.

The necessity for an interval of time of about 2 days to elapse before expulsion of the worm burden of passively immunised rats is contrary to the usual speed of a reaction based on anaphylaxis. Ogilvie (personal communication) was unable to bring about the expulsion of an adult worm population by anaphylaxis resulting from sensitisation with ovalbumin with H. pertussis killed organisms as adjuvant. It may be that the

creation of an environment which is physically unsuitable for the worms in the gut through anaphylaxis, is not the entire story. It is possible that this is a mechanism whereby sufficient quantities of serum, containing antibody, can gain access to the worms and then cause some anti-parasite effect which then debilitates the worms in some way, making them prone to expulsion.

It has been pointed out by Clark (1952) that sometimes the equation, or a graph, representing the course of a process as if it were that of a reaction of the first order, describes very well the decline in the number of bacteria exposed to a toxic agent or a high temperature. It is recognised that sterilisation against bacteria by specific agents such as heat or disinfectants is a complicated matter. However, it has been shown that, when the logarithms of the numbers of surviving anthrax spores, after exposure to heat or phenol, are plotted against time, a straight line graph is obtained. Furthermore, the more severe the sterilisation procedure, the steeper is the slope of the graph. Hence the time course of this phenomenon or the rate of killing of the spores, satisfies the conditions of, and can be described as, a reaction of the first order.

It is possible to describe the expulsion of adult N. brasiliensis in a similar way. As a working hypothesis, it seems reasonable

that antibody, either free or in association with cells, may be toxic to the adult worm. The degree of toxicity or anti-parasite effect of antibody would be proportional to the concentration within the host and hence to the strength of the acquired resistance possessed by the host.

It was seen in fig. 4, where the logarithms of the numbers of worms isolated from the different groups of rats was plotted against time that a straight line graph was obtained for each expulsion phase. This suggested that the process of expulsion of the adult worms conformed to the kinetics of a reaction of the first order. This being so it was possible to calculate the half-life of each adult worm population during each expulsion period, from the slope of the corresponding line (c/f table 4). In this way it was demonstrated that the rate of expulsion of adult worms was greatest with hyper-infected hosts. Rats which had suffered one previous larval infection showed a rate of expulsion slightly greater than that of rats undergoing self cure to a primary infection. Expulsion brought about by passive immunisation gave the slowest expulsion rate. The actual cessation of egg production followed by expulsion of the worms may be the result of an anti-parasite effect possessed by host serum antibodies, which gain optimum access to the worm through an anaphylactic shock of the gut. If this were so then the

graded rate of expulsion manifest by Groups 2, 3 and 4 may be a reflection of the concentration of anti-worm substances (i.e. antibodies) in the serum of the rats comprising the different groups.

Another interesting feature is the cessation of the expulsion phase in the passively immunised group (Group 2) from day 5 to day 8 after transfer. In the actively immunised groups, especially Group 4, virtually the entire population of worms was eliminated by day 5. The half-life of homologous rat serum is around 5 days (Bangham and Terry, 1957) hence it is quite possible that in rats of Group 2, the concentration of introduced antibody could have been reduced to a level, by day 6 which was too low to confer an anti-parasitic effect. Another difficulty of comparing results for passively immunised and actively immunised rats is that, in the latter case a secondary antibody response may be provoked by this introduction of an adult worm burden. No such increase in antibody can be expected in the passively immunised rats.

It appeared to the author that the mechanism of the self cure reaction may depend primarily upon a specific anti-worm action and not upon gross cellular changes in the gut, though the latter may well assist in expulsion. Subsequent experiments were designed to find a mechanism whereby host antibody could

gain access to the adult worm in appreciable amounts, and to demonstrate that such antibody could interfere with the well being of the parasite.

Summary

Summary

(1) Adult *N. brasiliensis* were transferred to the small intestines of rats of different immunological status.

(a) previously non-infected rats (Group 1)

(b) passively immunised rats (Group 2)

(c) rats which had experienced one previous infection with larvae (Group 3)

(d) rats which had experienced 4 previous infections with larvae (Group 4).

(2) Rats were killed serially over 8 days following transfer and the number of the worms present in their small intestines determined.

(3) Group 1 rats displayed no significant reduction in worm burden over the period of the experiment.

(4) Group 2 rats showed some reduction over days 3, 4 and 5 post transfer, thereafter the worm burden showed no further reduction.

(5) Group 3 rats showed an immediate reduction in worm burden which approached zero by day 5.

(6) Group 4 rats showed an immediate reduction, even more drastic than that found with Group 3. Complete expulsion of the introduced worms was effected by day 5. Stunted worms derived from a previous larval infection were found in the small intestines of some rats in this group.

(7) The pattern of elimination was very different for passively immunised and actively immunised rats.

CHAPTER VII

(a) Blood loss into the gut of rats infected with N. brasiliensis (short term experiments)

Introduction

The ingestion of blood by parasitic worms is not only important for its possible deleterious effect upon the health of the host but also from the point of view of parasite physiology and host resistance.

With many host parasite systems all the effects of a naturally acquired resistance can be passively transferred hence implicating humoral antibodies in this phenomenon. That serum antibodies may play an important role in the 'self cure' reaction of rats infected with N. brasiliensis has been suggested by Sarles and Taliaferro (1936), Chandler (1937) and Mulligan et al (1965).

The presence of whole red blood cells and fragments of red cells has been noted in the oesophagus and intestine of adult N. brasiliensis isolated from the small intestines of infected rats. From this histological evidence it has been suggested that the host's blood may serve as a source of nutrition for this parasite (Taliaferro and Sarles, 1939; Taliaferro, 1942; Weinstein, 1956; Haley, 1962). Hence evidence has been

obtained to show that adult N. brasiliensis suck blood and that humoral antibody plays some part in the host's immune response to this helminth.

If during 'self cure' humoral antibody acts directly upon the adult worm, possibly debilitating it in some way, so making it prone to expulsion, then the blood sucking activities of this parasite may provide a vehicle whereby serum antibodies gain close access, in reasonable quantity to the adult worm. It is feasible that such feeding activities could also facilitate the acquisition of worm antigens by the host, especially if secretory enzymes, as suggested by Chandler (1937) are important antigens.

It was decided to try and obtain a quantitative measure of the amount of whole blood consumed by a population of adult N. brasiliensis. This problem is particularly suitable for study by isotopic methods because of the ease with which blood can be labelled. Of the 3 isotopes with which it is possible to label red cells, i.e.

(a) ^{32}P, which becomes incorporated in organic phosphorous compounds within the cell, which do not readily diffuse out,

(b) ^{59}Fe, which is incorporated into haemoglobin, and

(c) ^{51}Cr, which becomes firmly bound to proteins within the cell, the radio-chromium method was chosen as it has advantages over the other methods, e.g.:-

(a) The label is firmly attached and is only released on destruction of the cell.

(b) There is no significant reabsorption of ^{51}Cr. from the lumen of the gut.

Gray and Sterling (1950) showed that it was possible to label red cells by incubating them with sodium chromate ($Na_2{}^{51}CrO_4$).

It is suggested that red cells are permeable to anions, and so allow the inward diffusion of the chromate anion. Inside the cell the chromate anion is converted to chromic cation which is bound firmly by the haemoglobin moiety of the red cell. Once bound in this way, the label is only lost on haemolysis of the cell. ^{51}Cr. tagged red blood cells have been used both in blood volume and red cell survival studies (Sterling and Gray, 1950; Necheles et al, 1953; Weinstein and Le Roy, 1953; Mollison and Veall, 1955; Small and Verloop, 1956). The technique has also been applied to the detection of gastro-intestinal haemorrhages (Owen et al, 1954; Matsumoto and Crossman, 1959), and to estimate the amount of blood ingested by Fasciola hepatica in guinea pigs, rabbits and sheep (Jennings, 1962; Pearson, 1963) and in hookworm infection in man and dogs (Roche et al, 1957; Gilles et al, 1961).

In this initial experiment rats were injected with ^{51}Cr. labelled homologous red cells, killed 3 hours later and the radioactivity of the worms compared with that of a blood sample taken at autopsy.

Methods and materials

(a) Initial considerations

In this experiment labelled red cells are injected into the infected host and after a reasonable interval of time, the activity of the parasite is compared with that of the circulating blood at slaughter. In order to be able to detect the amount of blood which the parasite has ingested one must consider two points - (a) If the worm sucks blood, what volume would it contain when fully gorged? From consideration of the dimensions of the oesophagus of N. brasiliensis (Haley, 1961) and assuming this organ to be a cylinder, then the volume of liquid it could contain is approximately 0.7 µl. Hence a population of 1,000 worms should be able to contain about 700 µl. of blood. No details are given by Haley as to the potential capacity of the intestine of this parasite.

In any event, it was decided that the specific activity of the host's blood should be such that 1.0 µl. of worm ingested blood could be detected.

(b) How much isotope should be added to the red cell preparation in order to give a specific activity in the circulation of the host which will allow the detection of 1.0 µl. "blood" in the parasites?

An accurately measurable count in the scintillation counter used was considered to be 2 times the background count of the machine. With the counter used this value was 2 x 300 counts/minute or 600 counts/minute.

The efficiency of the counter for $^{51}Cr.$ = 4%.

Hence the true activity must be $\frac{600 \times 100}{4}$ = 15,000 counts/minute.

i.e. 0.001 ml. blood must give 1.5×10^4 counts/minute.

The approximate blood volume of a 150 gm. rat = 10 ml.

This dilution of the injected labelled red cells must be allowed for.

∴ 10 ml. of blood must have 1.5×10^8 counts/minute.

The efficiency of labelling of the red cells by chromate is approximately 80%.

∴ Total disintegrations required = $\frac{1.5 \times 10^{10}}{80}$

$= 0.19 \times 10^9$ counts/minute.

If 1 µc. of $^{51}Cr.$ yields 2.2×10^6 dis/min. what quantity of $^{51}Cr.$ yields 0.19×10^9 dis/min.?

i.e. 86 µc. $^{51}Cr.$ is the desired amount.

As the above calculation is approximate it was decided to use 100 µc. of $^{51}Cr.$ in each preparation.

Hence 100 μc. of $Na_2{}^{51}CrO_4$ was added to a suitable aliquot of blood from a 150 gm. rat in order to give a level of radioactivity which would allow the detection of about 0.001 ml. "blood" ingested by a parasite population.

(b) Labelling of red cells with ^{51}Cr.

Approximately 0.5 ml. of venous blood from the tail of each rat was collected separately into tubes containing the requisite quantity of heparin. The heparinised whole bloods were washed several times with cold isotonic saline and the washings discarded.

To about 0.5 ml. of red cells suspended in saline, 100 μc. of ^{51}Cr. as sodium chromate was added. The preparations were incubated at 37°C. with frequent mixing for one hour. The uptake of ^{51}Cr. by red cells is rapid (Mollison and Veall, 1955) and a one hour incubation period allows maximum uptake of label. The labelled red cells were then washed three times with approximately 5.0 ml. of saline each time, and were finally suspended in 1.0 ml. of saline for injection. The labelled red cells were injected intravenously, the tail being the site of injection, each rat receiving its own red cells.

After a period of 3 hours the rats were anaesthetised and exsanquinated by cardiac puncture. The samples of blood from each rat were delivered into heparinised tubes. 0.1 ml. aliquots of each sample of whole blood were measured carefully into separate counting tubes and laked with normal sodium hydroxide. The

radioactivity of each sample was assessed for a period of 100 seconds. Two such determinations were carried out on each sample and where the duplicate counts were not in good agreement, a third determination was made.

(c) Experimental animals

Three groups of rats were selected.

Group A This group had been infected with 5,000 N. brasiliensis larvae 5 days previously.

Group B This group had been infected with 5,000 N. brasiliensis larvae 12 days previously.

Group C This group consisted of rats which had had no experience of the parasite.

At autopsy the small intestine of each rat was excised, slit longitudinally, placed in a muslin bag, and suspended in warm saline in a 250 ml. beaker at 37°C. for one hour. During this time, virtually all the adult worms, where present in the gut, swam out and settled on the bottom of the beaker along with a certain amount of intestinal debris.

The worms, where present, and the intestinal debris were collected with a broad mouthed Pasteur pipette, washed once with saline and placed in a glass counting tube. The radioactivity of each sample of worms was measured in a well type scintillation counter.

Table 5

Radioactivity of blood and adult worms plus intestinal debris samples

	Rat No.	Count rate of 0.1 ml blood	Count rate of worms and debris	No. of worms	'blood' volume in µl.
Group A (5 days after infection)	1	73,240	0	1,000	0
	2	82,600	22	1,100	0.03
	3	112,700	70	1,200	0.06
Group B (12 days after infection)	1	93,800	9	1,310	0.01
	2	46,600	25	1,200	0.05
	3	78,640	48	1,300	0.06
	4	93,220	62	1,600	0.07
Group C (non-infected rats)	1	98,650	0	0	0
	2	61,700	31	0	0.05
	3	82,100	16	0	0.02

The number of worms present in each sample was counted with the aid of a binocular microscope.

Results

The volume of "blood" ingested by the parasitic worms over the 3 hour period of the experiment was estimated by using the formula -

$$\text{Vol. blood (V } \mu\text{l.)} = \frac{\text{Net worm sample count rate}}{\text{Net 1.0 } \mu\text{l. blood count rate}}$$

It can be seen from table 5 that the count rates of all three groups do not differ nor are they significantly greater than the background count given by the scaler.

Correspondingly the worms present in Group A and B possess no more radioactivity than the intestinal debris obtained in Group C.

Discussion

The results indicate that the adult worms infecting the two groups of rats (i.e. Group A and B) used here did not ingest any appreciable quantity of host's blood, under the conditions prevailing during the experiment.

This type of measure, however, has several unsatisfactory features. The most important of these is the arbitrary nature

of the time interval between injection of the labelled red cells and the autopsy of the experimental animal. It may well be that during this time interval none or only few of the parasites may have fed. In this experiment rats at day 5 and day 12 following larval infection were chosen. From a study of the life cycle of N. brasiliensis in rats (Haley, 1961), day 5 and day 12 should represent periods where the nutritional requirement of the worm population is high. For example at day 5 the immature adults should be growing into mature adults whilst day 12 represents the peak egg laying period. Little is known concerning the feeding habits of the parasite i.e. do they feed slowly and steadily or do they feed voraciously over short intervals? If they fed rapidly over a short period then it may be that the choice of time interval between injection of labelled red cells and slaughter is too long, in that the ingested label may have been excreted by the worm.

In order to over come these difficulties a long term experiment was planned in which homologous labelled red cells were injected into infected rats and the appearance of radioactivity in the faeces followed throughout the period of infection.

CHAPTER VII

(b) Blood loss into the gut of rats infected with N. brasiliensis (long term experiments)

Introduction

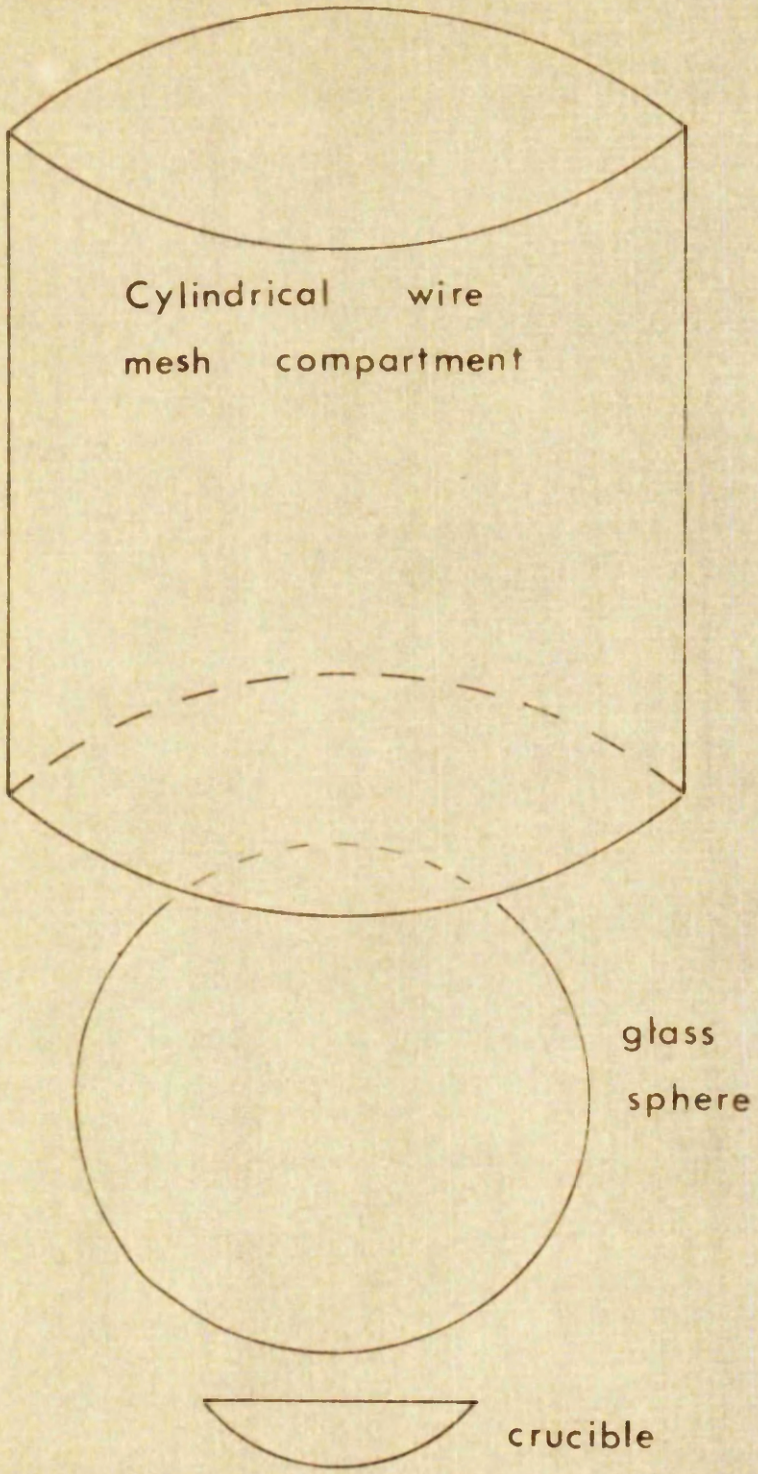
Red blood cells labelled with ^{51}Chromium will only lose their isotopic label substantially on destruction of the red cell. On intravascular haemolysis the isotope largely appears in the urine, though part of this released ^{51}Cr. may combine with plasma proteins. ^{51}Cr. is not reabsorbed from the lumen of the intestine.

By injecting ^{51}Cr. labelled homologous red cells and following, over a period of time, the appearance of radioactivity in the faeces, it is possible to detect and measure gastro-intestinal haemorrhages (Owen *et al*, 1954). This principle has been used to assess the blood loss in rabbits and sheep infected with *F. hepatica* (Jennings, 1962; Pearson, 1963).

By applying this approach to rats infected with *N. brasiliensis* it is possible to measure the host's blood loss over the entire period of infection. If the adult worm causes blood loss into the gut, then the ^{51}Cr. content of the faeces of infected rats will be greater than that of non-infected rats.

Figure 5

Diagram of Metabolism Cage



Methods and materials

(a) Labelling of red blood cells

A blood sample from each rat was obtained and labelled with ^{51}Cr. as described in the previous section.

(b) Experimental animals

36 male rats, around 150-160 gms. weight, were selected, and placed in individual cages a few days before the experiment began.

(c) Collection of urine and faeces

In this type of experiment it is desirable to have efficient separation of faeces from urine. ^{51}Cr. activity will be found in both forms of excreta, that in the urine from intravascular breakdown of labelled red cells and that in faeces from bowel haemorrhage. The experimental animals must, therefore, be caged in such a way as to avoid contamination of urine by faeces and vice versa. A diagram of the metabolism cage used in this experiment is shown in fig. 5. The principle of the method is that the faecal pellet will, when passed, fall through the wire mesh floor of the cage, bounce off the glass sphere, and land on the bench. The urine, on the other hand will, when coming in contact with the glass sphere, adhere to its surface and run down to the lowest point, where it will form a drop which, when large enough, will drop off into the crucible. Male rats were

used as there is less chance of these urinating against the wall of the cage. Urine passed against the wall tends to run down the apron of the cage and drop onto the bench. The method worked satisfactorily, so long as the consistency of the faecal pellet was firm. In this experiment diarrhoea did not occur, hence the problem associated with soft pellets did not arise.

(d) Injection of labelled red cells

4 days after the rats had been placed in the individual cages, blood samples were taken, labelled with ^{51}Cr. and re-injected. Each rat was injected with its own labelled red cells. 3 days later 18 of the rats were each infected with 5,000 *N. brasiliensis* larvae. Faeces and urine samples from each rat were collected separately each morning and represented a 24 hour sample. Before determining the radioactivity of the faecal sample, the pellets were placed in 5.0 ml. of water and homogenised. The radioactivity of the total 24 hour sample of both urine and faeces was measured.

On day 2, 9, 15 and 19 following injection of the ^{51}Cr. labelled red cells, heparinised blood samples were taken from the tail of each rat. Care was taken during this operation to ensure that the small incision, made in the rat's tail, had stopped bleeding before the animal was placed in its cage so avoiding contamination of the urine and faeces with blood. The radioactivity of a carefully measured volume of whole blood was

Figure 6

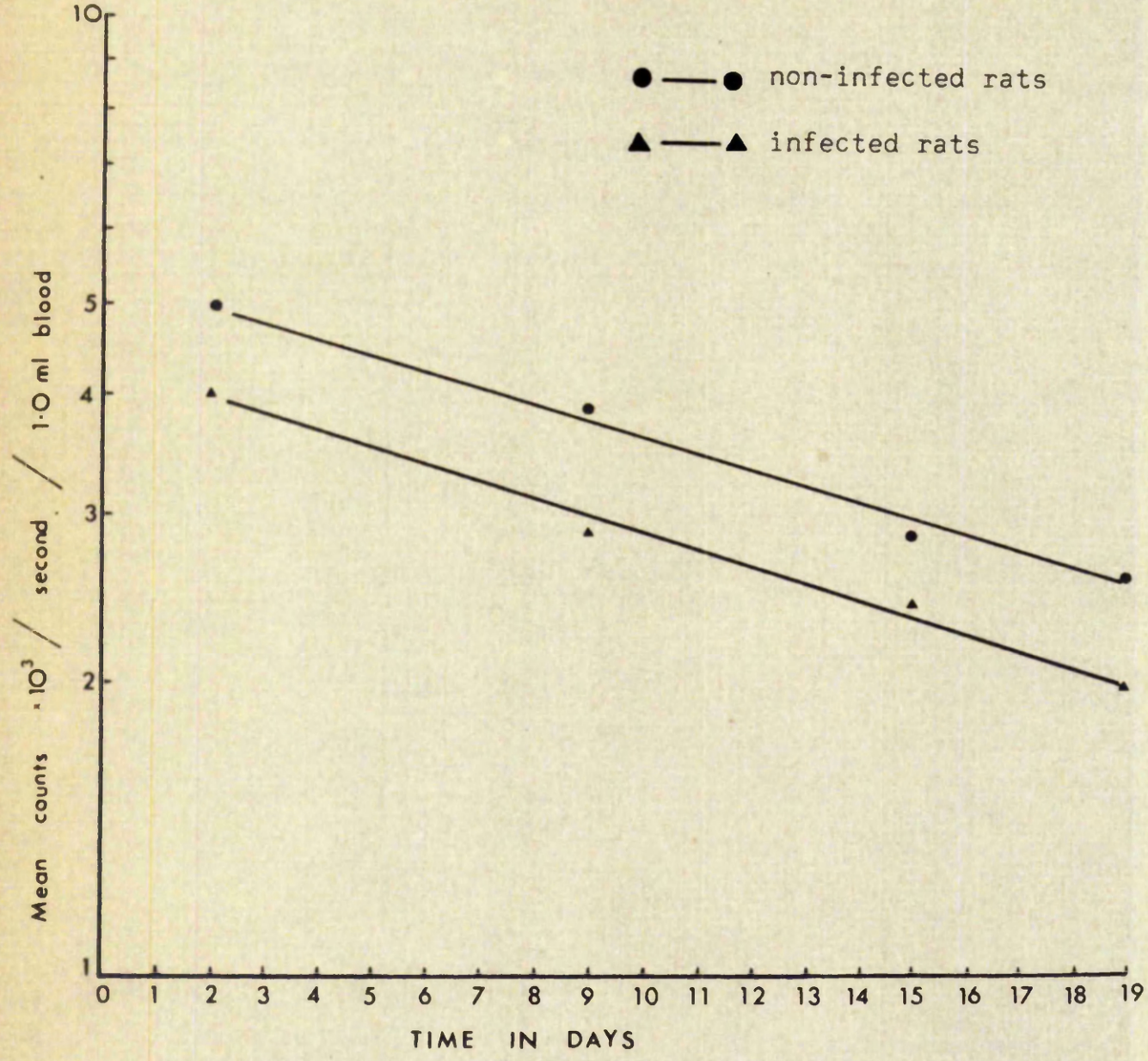


Figure 6. Mean activity of the blood of the infected and non-infected groups of rats following injection of labelled r.b.c. on day 0.

measured. This data for each rat was plotted against time. From this curve it was possible to compare the radioactivity of a known volume of whole blood with the activity of the faecal and urine sample for the same 24 hour period. In this way the radioactivity of 24 hour faecal and urine samples for each rat could be expressed in terms of volume of whole blood.

(e) Collection of adult worms

2 rats from the infected group and simultaneously 2 rats from the non-infected group were sacrificed on days 2, 4, 6, 8, 10 and 12 following the day of infection. The worms from the small intestine of the infected rats along with any intestinal debris, were collected as described in the previous section. In a similar manner the intestinal debris from the non-infected rats was collected. The number of parasites present and the radioactivity of each sample was obtained and expressed in terms of μl. of blood of the host animal.

All radioactivity measurements were carried out in a well type scintillation counter and corrected for background and isotopic decay.

Results

Fig. 6 shows the mean activity per ml. of blood plotted against time, for both infected and non-infected animals. The

Figure 7

Corresponding day after infection	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

Figure 7. Volume of "blood" appearing in the urine of infected and non-infected rats.

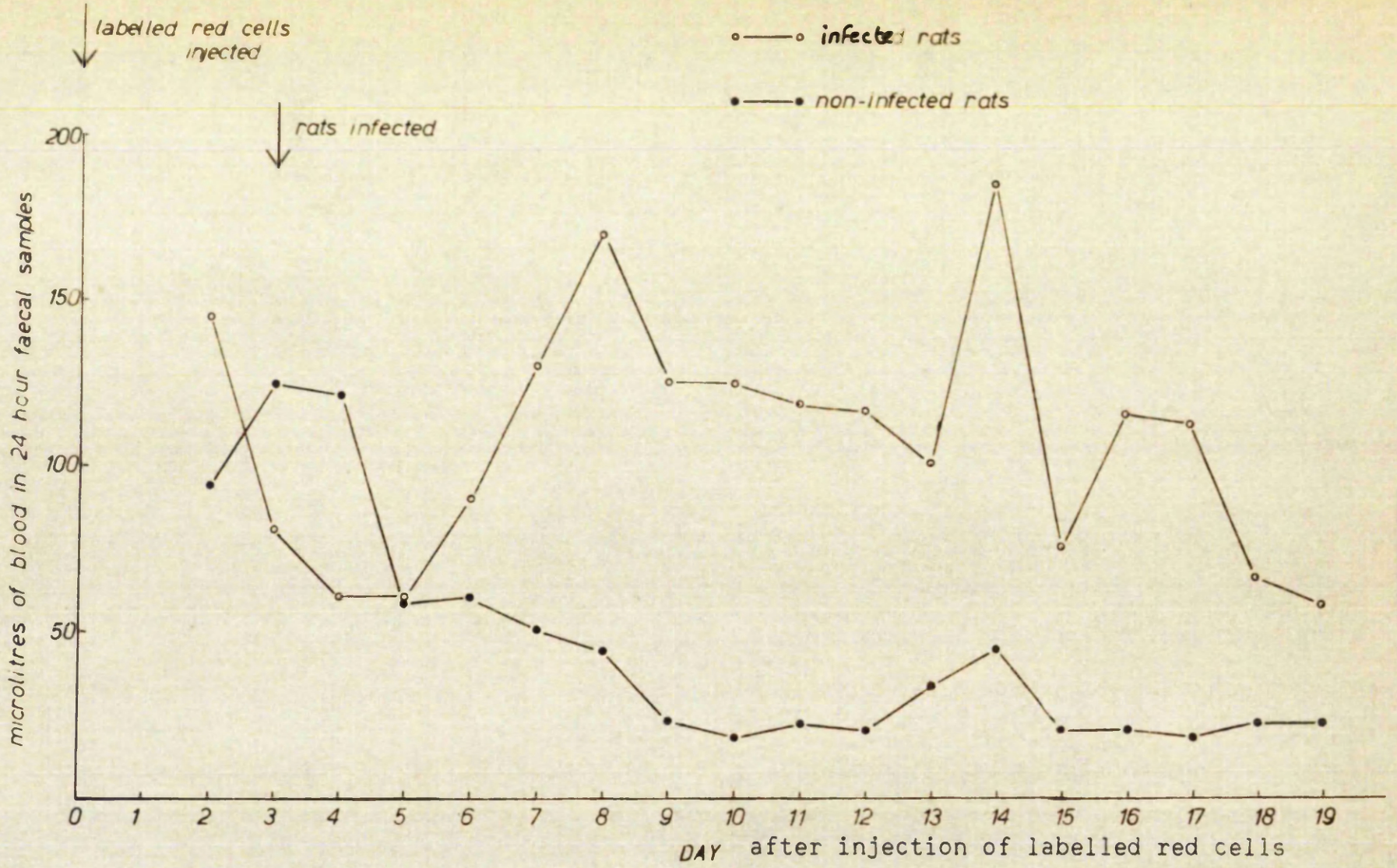


Figure 8

Corresponding day after infection	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

Figure 8. Volume of "blood" appearing in the faeces of infected and non-infected rats.

slope of both lines was similar indicating that the rate of loss of radioactivity from the blood of both groups of animals was approximately the same.

The loss of radioactivity from the blood, due to the normal turnover of red cells is roughly indicated by measuring the radioactivity appearing in the urine of each animal. Over the period of the experiment this was similar for both infected and non-infected animals (fig. 7).

The mean volume of blood appearing in 24 hour faecal samples, of both the infected and non-infected groups is shown in fig. 8. It can be seen that from day 4 of the infection a significantly greater volume of blood appeared in the faeces of infected animals when compared with non-infected controls. This trend continued until day 16 of the infection. By subtracting the value for the volume of blood appearing in the faeces of the non-infected animals from that of the infected animals, a figure indicating the volume of blood lost by the host, due to the presence of the parasite, was obtained (table 6). This blood loss was about 100 µl. per 24 hours in each rat.

From a knowledge of the number of adult worms found at autopsy of the infected rats, serially killed, and from the number of parasite eggs found in the faeces of infected animals, the extent of the infection can be assessed. 2 infected and 2

Table 6

Volume of 'blood' expressed in microlitres
present in 24 hr faecal samples from infected rats
less the volume present in similar samples from non-infected rats

Day after infection with 5,000 larvae	µl. of blood lost due to the parasitic worm
1	0
2	2
3	30
4	79
5	126
6	102
7	107
8	96
9	96
10	67
11	140
12	57
13	95
14	94
15	44
16	36

Table 7

Volume of 'blood' expressed in micro-litres,
present in the intestinal contents
(i.e. intestinal debris plus worms where present)
collected from infected and non-infected rats killed serially

Day after infection with 5,000 larvae	No. of rats per group	Non-infected group		Infected group	
		µl blood in contents	No. of worms	µl blood in contents	No. of worms
2	2	0	0	0.09	0
4	2	0.16	0	0.09	465
6	2	0.43	0	0.06	1055
8	2	0.08	0	0.09	1205
10	2	0	0	0	1110
12	2	0.36	0	0.12	1035
16	2	0.13	0	0	54

non-infected rats were sacrificed on days 2, 4, 6, 8, 10, 12 and 16 following infection. The contents of the small intestine from both groups were isolated, washed and the radioactivity of the sample assessed. The count rate obtained was not significantly greater than the background count rate, even when the infected group samples contained as many as 1,000 worms. When the count rate was expressed in terms of volume of blood of the host, it is evident that the parasitic worms contained no significant amount of blood (table 7).

Discussion

It has been shown, using red cells labelled with ^{51}Cr. that a rat harbouring an infection of around 1,000 adult *N. brasiliensis* loses about 100 µl. of blood every 24 hours into the lumen of its small intestine due to the presence of this parasite.

Hughes-Jones (1961) whilst studying the fate of ^{51}Cr. labelled red cells in the rat found that there was a small random loss of red cells from the circulation. It was proposed that this loss occurred at 2 sites; at the spleen where destruction of the red cells takes place and at the gastro-intestinal tract where some red cells are lost directly into the lumen of the gut. This mechanism could explain the radioactivity appearing in the faeces of non-infected rats.

It appears (table 7) that adult worms do not ingest a significant amount of this blood, or more accurately the worms do not ingest any red cells. This was shown by the fact that the adult worms collected, at intervals throughout the infection contained no measurable radioactivity.

Before a comparison can be made of the host blood loss due to the presence of different parasitic worms it is necessary to allow for the large variation in size of these organisms. A valid comparison of the blood sucking activities of different worms can be made by expressing blood loss in terms of worm tissue nitrogen. With F. hepatica it was found that flukes equivalent to 1.0 mg. of fluke nitrogen caused a blood loss of about 650 µl. each 24 hours, from the host, and that the parasite ingested this blood such that flukes equivalent to 1.0 mg. of worm nitrogen contained about 60 µl. of host blood (Jennings et al, 1955). A similar calculation with N. brasiliensis based on the figures obtained in the previous experiments show that 1.0 mg. of adult worm nitrogen cause a loss of about 100 µl. of host's blood over 24 hours and that at autopsy the volume of blood contained by worms equivalent to 1.0 mg. of worm nitrogen was zero. Hence it would appear that adult N. brasiliensis causes haemorrhage in the host's small intestine, probably as a result of trauma inflicted during feeding by the worm upon

the host's tissues. However, ingestion of red cells by the worms does not occur to any extent.

Whilst the parasite itself does not consume the host's blood, it is probable that the blood lost by the host into the gut will come into close external contact with the worms. A further point is that if intestinal haemorrhage is being caused by the traumatic feeding habits of the worm then it is probable that secretions produced by the worm will find their way into the host's vascular system. Taliaferro and Sarles (1939) studying the termination of a N. brasiliensis infection in rats noticed that a marked inflammatory response developed in the gut mucosa and precipitates were seen in and around the parasite. A similar situation has been found with Trichinella spiralis (Larsh and Race, 1954). These reactions are held to play a part in the immune elimination of the worms, since the inhibition of the inflammation, which is possible with cortisone, allows the parasite to remain longer in the host (Weinstein, 1955; Coker, 1956).

Although adult N. brasiliensis do not ingest red cells, it is possible that they may be quite capable of ingesting plasma proteins released in the vicinity of the feeding worm. It is felt that the host's blood loss of about 100 µl. per 24 hours may well be a means whereby antibody gains access to the worm.

When rats, which have had no previous experience of the parasite, are infected with 5,000 third stage larvae, immature adult worms begin to appear in the small intestine about 72 hours post infection. These early adults mature and egg laying commences around day 6 or 7, and maintains a high level until around day 11 or 12 when it begins to subside rapidly. Elimination of the main bulk of the worm burden by the host occurs around day 13 or 14. This scheme of events is probably reflected in the parasites efforts to gain nutrition.

The appearance of a significantly greater volume of blood in the faeces of the infected animals as compared to the non-infected animals occurred at day 4 following infection (c/f fig. 8). This trend increased reaching a peak on day 5 post infection. It seems reasonable to speculate that this peak was caused by the large numbers of immature adult worms appearing in the gut at this time, seeking a position on the intestinal mucosa at which to feed and in so doing causing haemorrhage. Following this over days 6, 7, 8, 9 and 10, the amount of blood in the faeces fell gradually until on day 10 it was barely significantly different from that contained in the faeces of the non-infected group. It could be, that over days 6, 7, 8, 9 and 10, the adult worm population, having become established and in the absence of any immunological

response from the host, was feeding on the host tissue causing only slight trauma. About day 10 it is possible that a local anaphylactic reaction at the site where each worm was feeding may have occurred (Urquhart et al, 1965) causing that position to become "unsuitable" for the worm. This could have forced the worms to seek a new position on the mucosa where they could successfully feed. This activity could explain the increase in the volume of blood appearing in the faeces of the infected rats on day 11. Due now to the hyper-sensitive state of the rat intestine an immediate response is elicited against the parasite which is again dislodged, hence the low faecal activity of day 12. One could speculate that on day 13 and 14 the worms were seeking a place on the mucosa on which to feed but without success, as the worm egg count dramatically declined from day 11 onwards, with a corresponding reduction in the numbers of worms as from day 13.

Summary

(1) Red blood cells of rats were labelled with ^{51}Cr. by in vitro incubation with labelled sodium chromate.

(2) After injection of the labelled r.b.c. to both non-infected and infected rats, the animals were placed in individual metabolism cages. 24 hour samples of faeces and urine were

collected throughout the entire course of the infection from both groups of animals. By comparing the radioactivity of these samples with the radioactivity of the blood it was possible to express the activity in the faecal sample as volume of blood.

(3) During the patent period of the infection about 100 µl. of blood were lost into the gut every 24 hours by infected rats. This could well be a means whereby host antibody could gain direct access to the worm.

(4) Rats were killed serially and the activity in the worms present was measured. This was always zero indicating that the worms did not ingest any significant amount of the blood lost by the host.

CHAPTER VIII

Studies on the loss of macromolecules into the gut using ^{131}I labelled P.V.P.

Introduction

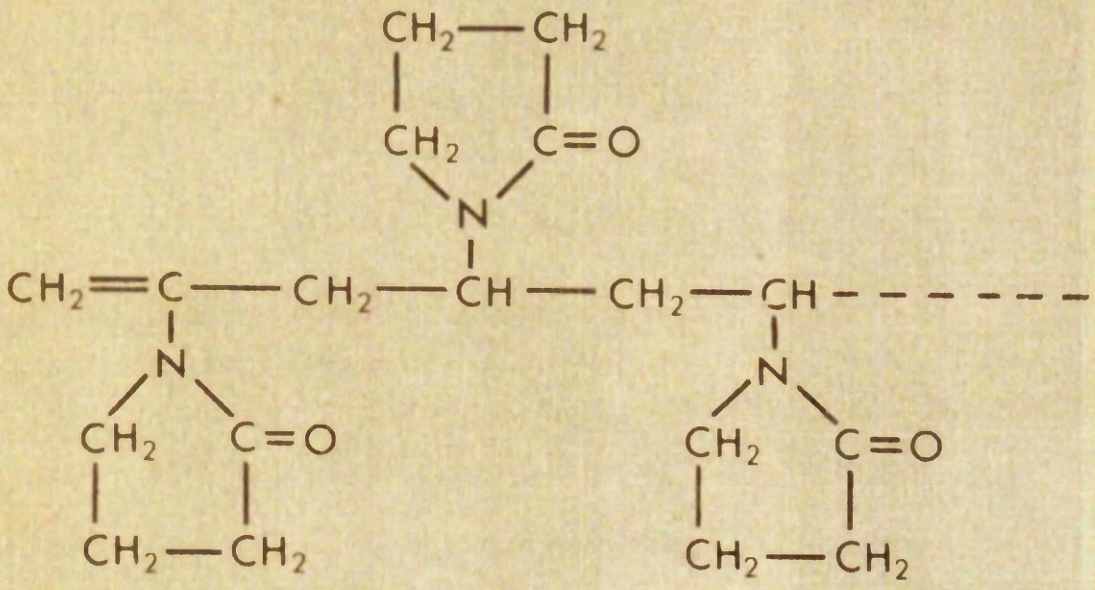
Circulating antibody may play an important part in the self cure reaction found in rats infected with *N. brasiliensis*, by being capable of directly debilitating the adult worm hence making it prone to expulsion. It has been shown in the previous Chapter that the trauma, caused by a population of around 1,000 adult worms, leads to a blood loss of about 100 micro-litres per 24 hours into the lumen of the gut. It is conceivable that haemorrhage caused in this way would take place at the site where the worms were present. In this way humoral antibody, should it play a part in the self cure reaction, could gain direct access to the worm.

It has been proposed by Urquhart *et al*, (1965) that the mechanism of the self cure reaction associated with a primary *N. brasiliensis* infection, may be comparable to the suggested mechanism for the self cure reaction found in sheep infected with *H. contortus*; i.e. it is suggested that the expulsion of adult *N. brasiliensis* may rely upon a local anaphylactic reaction taking place in the gut (Urquhart *et al*, 1965; Mulligan *et al*,

1965). The increased capillary permeability brought about by such a reaction might provide a further vehicle whereby humoral antibody in appreciable amounts might come in close contact with the adult worm. It was decided to investigate this aspect using ^{131}I labelled polyvinyl pyrrolidone, a substance which has been successfully used in the study of abnormal permeability of the gastro-intestinal tract of humans to macromolecules.

Polyvinyl pyrrolidone (P.V.P.) has been described as an effective isotonic plasma substitute by Ravin et al (1952). These workers made a close study of the excretion distribution and metabolism of this synthetic macromolecular polymer labelled with ^{131}I in man, dog, rabbit and rat. They found, with a P.V.P. preparation of about 40,000 molecular weight, that around 60% of the injected material was excreted in the urine within 3 days. The polymer is not metabolised to any significant degree and 90% of an orally administered dose appeared in the faeces. Gordon (1959) made use of the fact that P.V.P. labelled with ^{131}I, evades enzymic protein breakdown in the gastro-intestinal tract. In his studies on the abnormal permeability of the gastro-intestinal tract to plasma proteins, he injected into the blood stream ^{131}I labelled P.V.P. and related radioactivity in the faeces to leakages into the gut.

Figure 9



Polyvinyl pyrrolidone

Figure 9. Formula of polyvinyl pyrrolidone

In the following experiment an attempt was made to find if there was an abnormal loss of macromolecules into the gut at some point during an infection of N. brasiliensis in the rat.

Methods and materials

(a) Labelled polymer

Polyvinyl pyrrolidone, av. M.wt. 40,000 was obtained from May and Baker.

Carrier free sodium iodide ($Na\ I^{131}$) was obtained from Radiochemical Centre Amersham, England. The method used for labelling P.V.P. with ^{131}I was as described by Gordon (1958). Unbound activity was removed from the preparation by overnight dialysis against water.

(b) Experimental animals

A group of over 100 hooded rats, of both sexes, each around 175 gms. live weight, were each infected with 3,500 infective N. brasiliensis larvae, subcutaneously and faecal egg counts made daily.

On each day post infection a group of 6 rats was removed and 1.0 ml. of the labelled P.V.P. preparation was injected intravenously into each rat via a tail vein. As the injection had to be quantitative, great care was taken. The injection

was carried out each day at 11 a.m. All 6 rats were killed, by a blow to the head, 3 hours following injection, and the amount of P.V.P. in various parts of the gut measured as described below.

(c) Preparation of intestinal samples

The small intestine and the large intestine including the caecum were removed separately and the exterior of each washed with warm physiological saline to remove any traces of blood. The contents of the small intestine were washed out by forcing warm saline down through the lumen. This was facilitated by starving the rats the night before the experiment. The washings were collected and bulked. The contents of the large intestine were collected by slitting the bowel longitudinally and swirling it around in 15 ml. of warm saline in a small beaker. This action was sufficient to wash off all faecal material.

The mucosa of the small intestine was obtained by opening the gut longitudinally and scraping off as much of the mucosa as possible with a glass slide. After the radioactivity of each mucosa sample had been assessed they were examined for adult N. brasiliensis, and the number present in each sample noted.

The above experimental procedure was also carried out on 2 groups of control animals (rats which had had no experience of the parasite) 1 group near the beginning of the experiment and 1 group near the end.

Table 8

Amount of P.V.P. (As % of injected dose) in intestinal contents and mucosa 3 hours after injection in rats at different stages of infection with N. brasiliensis

Day after infection	No. of rats	Mean P.V.P. Content as % injected dose			
		Contents S.I.	Contents L.I.	Mucosa S.I.	Total
2	6	2.5	1.6	0.4	4.5
3	5	1.0	0.8	0.3	2.1
4	6	2.3	1.0	0.3	3.6
5	6	1.4	2.8	0.4	4.6
6	6	2.0	1.7	0.5	4.2
7	6	1.5	1.0	0.5	3.0
8	6	4.0	3.5	0.6	8.1
9	5	3.1	2.3	0.5	5.9
10	5	2.8	2.2	0.6	5.6
11	5	3.1	2.3	0.6	6.0
12	6	2.7	1.8	0.5	5.0
13	6	3.1	1.8	0.7	5.6
14	6	3.4	2.1	0.6	6.1
15	6	3.1	1.5	0.6	5.2
17	5	3.4	1.7	0.5	5.6
Non-infected rats	12	2.1	2.0	0.2	4.3

Figure 10

Figure 10. Mean amount of polyvinyl pyrrolidone found in the contents of the gut and the mucosa of the small intestine of rats infected with N. brasiliensis on day 0.

Figure 11

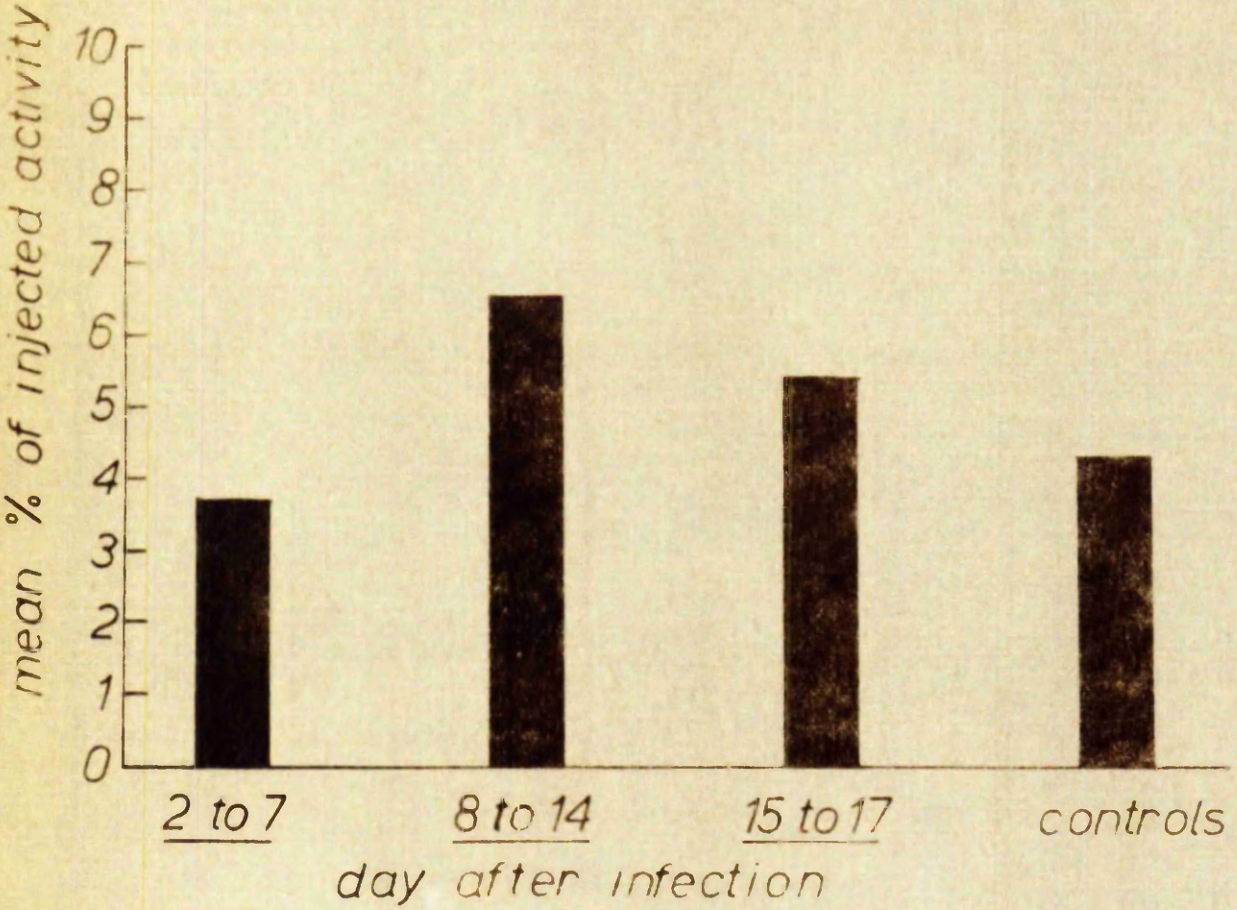


Figure 11. Mean amount of P.V.P. present in the contents and mucosa of the small intestine of infected and non-infected rats.

(d) Radioactivity measurements

The radioactivity of the various samples was found using a well type scintillation counter. The resulting counts were expressed as a percentage of the total counts injected to each animal.

Results

In table 8 the mean percentage of the injected P.V.P. present in the various samples from rats over the whole period of the infection, is shown. Comparable figures for non-infected rats is also shown. The total amount of P.V.P. expressed as a percentage of the injected dose is shown graphically in fig. 10. It is clear that from day 2 until day 7 of the infection there was no appreciably greater loss of the macromolecule into the gut of infected rats compared to non-infected rats. However, on day 8 of the infection there was a dramatic increase in the appearance of radioactivity in the gut contents and mucosa and this higher level was maintained until the end of the experiment. It appears that from day 8 of the infection onwards there was an increased leakage of labelled P.V.P. into the gut of infected rats.

In fig. 11 the mean amount of P.V.P. recovered in the samples for these periods of the infection is shown. The values obtained for the period, day 8 to day 14, show the extent of the leakage

Figure 12

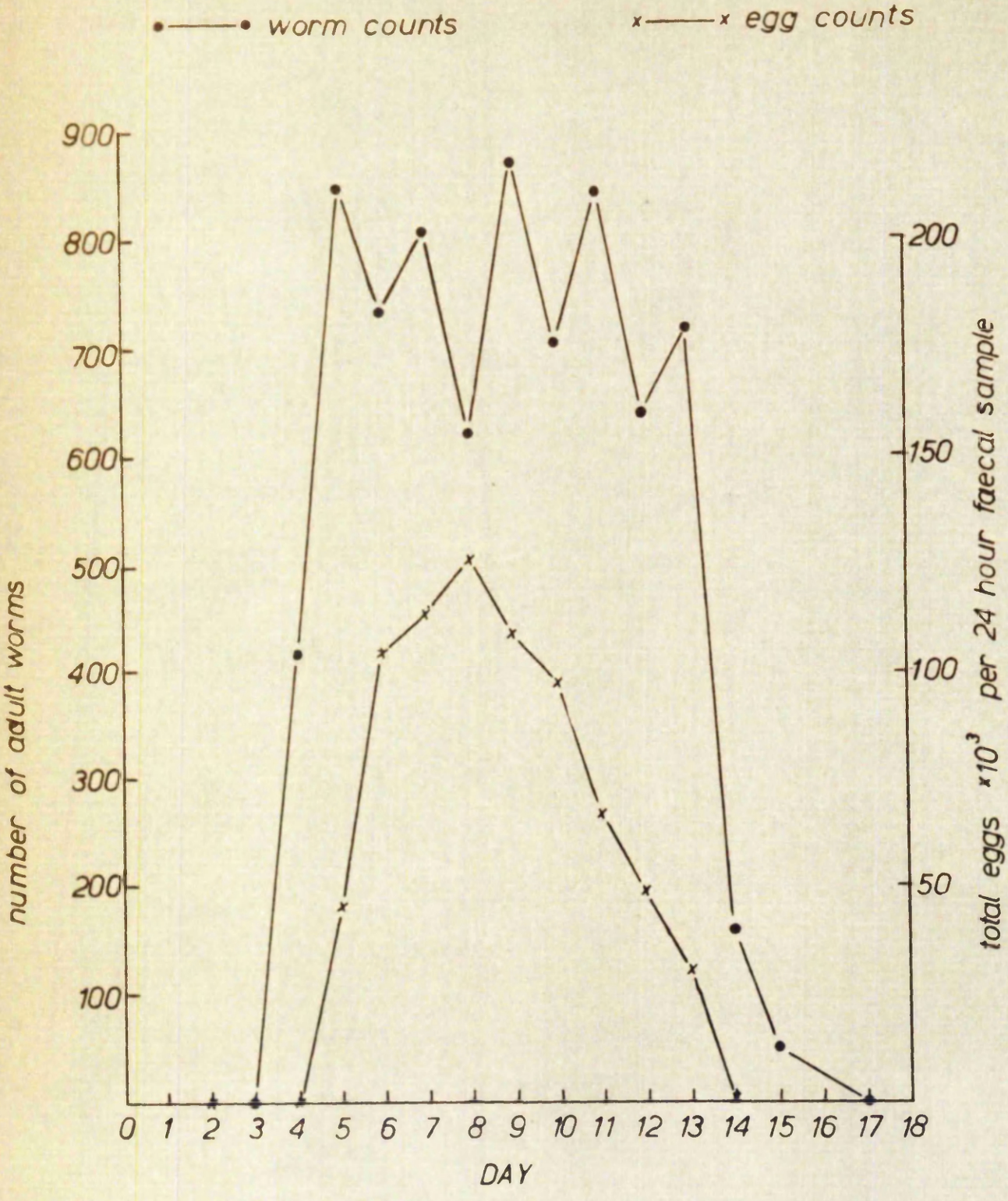


Figure 12. Faecal egg counts and numbers of adult worms found at autopsy of rats infected on day 0.

compared to the other periods of the infection and to the situation with non-infected rats. This period, day 8 to day 14, coincides with the onset and expression of the immune response leading to the expulsion of the adult worm population (fig. 12).

Discussion

Parallel work in this laboratory has shown that when Evans Blue was administered intravenously to rats with a patent *N. brasiliensis* infection, dark blue patches occurred where the adult worms were located. It was proposed that this was caused by increased capillary permeability at these areas (Urquhart *et al*, 1965). Further experiments where Evans Blue and adult worm antigen were injected intravenously into rats which had recently undergone self cure from a primary infection, showed marked changes in the integrity of the small intestine compared to previously uninfected rats. These changes included, intense blueing, marked hyperaemia and an excessive production of mucus, indicating an anaphylactic type reaction. It was postulated that a similar type of reaction may occur during self cure, the adult worm population providing the antigenic stimulus.

The increased leakage of P.V.P. into the gut of infected rats from about day 8 of the infection onwards offers support to

the suggestion that the self cure reaction is associated with local anaphylaxis in the gut. This may, however, be only one part of the immune expulsion mechanism. For example, Ogilvie (1964b) was able to induce a severe anaphylactic shock in rats sensitised with ovalbumin and Haemophilus pertussis killed organisms as adjuvant while currently infected with N. brasiliensis. The gross physical changes in the state of the gut due to the induced anaphylactic shock did not result in the expulsion of the adult worm population.

These findings tend to suggest that while there is a marked inflammatory response in the infected rat's gut at the time of the immune expulsion of adult N. brasiliensis, there probably is some specific anti-parasite action by either humoral or cell bound antibody, possibly interfering with the adult worms' metabolism or feeding ability.

The preceding experiment has shown that rats infected with N. brasiliensis display a greater permeability of the gut to macromolecules around the time of self cure. The polyvinyl pyrrolidone used in this experiment had an average molecular weight of 40,000, and it must be borne in mind that this is smaller than albumin and much smaller than globulin molecules of rat plasma. Therefore care must be taken in equating the amount of this P.V.P. preparation which leaks into the lumen of the gut with the possible amount of plasma proteins and especially globulin which may leak through, at or near the time of self cure.

A more critical examination of the situation may be possible by employing a sample of P.V.P. with an average molecular weight nearer that of the plasma proteins. In this way interpretation of the results obtained with P.V.P. of this size in so far as it reflects the efflux of plasma proteins into the gut, could be made with more conviction. This problem could also be investigated more directly by labelling the plasma proteins of the infected animal, with a label which was not reabsorbed by the small intestine. This approach may prove feasible by labelling plasma proteins with ^{51}Cr.

Summary

(1) Non-infected rats and rats infected with N. brasiliensis were given intravenous injections of P.V.P. labelled with ^{131}I. 3 hours later the rats were killed and the radioactivity of the S.I. and L.I. contents, and the S.I. mucosa was found. It appeared that from day 8 to day 17 of the infection a greater amount of the labelled macromolecule appeared in the samples taken from infected rats than those taken from non-infected rats.

(2) This increase in gut permeability took place just before and at the time of self cure of the infected rats.

(3) This increased gut permeability to macromolecules may well be the mechanism whereby appreciable amounts of antibody gain access to the adult worm.

CHAPTER IX

Studies on passive transfer of resistance

(a) Preparation of various immune sera and an investigation of their ability to confer passive resistance

Introduction

Several experiments presented in this thesis were carried out to investigate the effects of antibody upon the metabolism of adult N. brasiliensis. As a preliminary to these studies, it was necessary to produce an anti-serum containing the highest possible level of antibody against the adult worm. To obtain such a serum it is important to consider the degree and type of antigenic stimulation and the time interval between sensitisation and collection of the anti-serum from the donating rat.

Little success has been achieved, in the past, with attempts to stimulate resistance by injecting dead whole-worm material. This is true with N. brasiliensis as well as with other parasitic worms (Chandler, 1936; Oliver-Gonzalez, 1956; Urquhart et al, 1954; Soulsby, 1957; Jarrett et al, 1960). Vaccines prepared from dead worm material have proved almost ineffectual in stimulating functional immunity. Slightly better resistance has been achieved on administering to host animals, the excretory and secretory products of living worms. This has been shown

with N. brasiliensis and Trichinella spiralis (Thorson, 1953; Campbell, 1955; Chute, 1956).

With N. brasiliensis in rats the strongest acquired immunity has resulted from infection with living worms (Chandler, 1937). The complexity of the antibody response to an organism like a parasitic helminth, which is the source of a multitude of potential antigens, has been recognised. The antigenic stimulus may also vary in nature as the parasite completes each phase of its life cycle, usually in different tissues of the host. It is not surprising that this results in a complex antibody response.

It is difficult with conventional serological tests to associate the appearance of the various kinds of antibody, as detected by the various tests, with functional immunity. However, with the appropriate anti-serum, it is possible to passively transfer all the effects of a naturally acquired resistance (Sarles and Taliaferro, 1936).

It was decided in the following experiment to assess the protective capacity, and hence the anti-parasitic activity of the various sera prepared, by the ability of these sera to confer passive immunity upon rats, challenged with an infection of transferred adult N. brasiliensis.

With N. brasiliensis in rats different workers have carried out different infection regimes and bleeding schedules to prepare

immune serum. However, it is agreed that, to gain a potent anti-serum, it is necessary to give a rat several large doses of larvae (Sarles, 1939; Chandler, 1937).

It is not known with certainty, how long resistance, stimulated in this way is maintained. Chandler (1935) suggested that resistance stimulated by a single larval infection declined after 29 days but Porter (1935) suggested that it may be more long lived.

Methods and materials

(a) Preparation of various sera

Large groups of rats, which were to act as serum donors, were infected with *N. brasiliensis* in a variety of ways.

Some rats were infected with 3,500 larvae each. Half were sacrificed and bled on the 13th day following infection (single larval, 13 day serum) while the remainder were bled 20 days post infection (single larval, 20 day serum).

Another large group of rats were given a series of larval infections of increasing size at fortnightly intervals. A first infection of 3,500 larvae, a second infection of 5,000, a third infection of 7,500 larvae and a final infection of 10,000 larvae, were given to each rat subcutaneously. This group of rats was divided into 3 sub-groups. The first was

Table 9

Group	No. of rats in group	Description of serum injected intraperitoneally to appropriate group
1	20	saline
2	12	normal rat serum
3 a.	5	single larval 13 day serum
b.	11	single larval 20 day serum
4 a.	10	series of larval infections 7 day serum
b.	10	series of larval infections 14 day serum
c.	10	series of larval infections 20 day serum
5	5	single adult transfer 20 day serum
6	8	series of adult transfer 20 day serum

bled 7 days after the last infection (series of larval infections, 7 day serum). The second was bled 14 days after the final infection (series of larval infections, 14 day serum). The third group was bled 20 days after the final infection (series of larval infections, 20 day serum).

A third group of rats each received 1,000 adult N. brasiliensis injected into the small intestine. This group was bled 20 days after transfer (single adult transfer serum).

A fourth group of rats each received a series of adult infections injected into their small intestines at fortnightly intervals. 3 transfers, each of 1,000 adult worms, were given and the rats bled 20 days following the final transfer (series of adult transfer serum).

The rats were bled by cardiac puncture and the sera were stored at -20°C. until required.

(b) Passive immunisation and challenge of experimental animals

Groups of rats of both sexes, all around 175 gms. weight, were given the appropriate serum, intraperitoneally at a dose rate of 4.0 ml. serum per 100 gm. live weight, 2 days before challenge. Table 9 gives details of the serum given to the various groups of rats used in the experiment. The challenge infection consisted of 500 adult N. brasiliensis, freshly isolated from donor rats 10 days following an initial infection of 3,500 infective larvae. The worms were injected directly into the duodenum of each experimental rat.

Table 10

Number of adult worms recovered from the small intestine of rats passively immunised with various sera (c.f. Table 9 for descriptions of group)

Group	No. of worms transferred on day 0	No. of worms recovered on day 4	t-test of significance
1	500	335 ± 132	-
2	500	349 ± 93	not significant
3 a.	500	325 ± 76	not significant
b.	500	225 ± 121	not significant
4 a.	500	144 ± 77	significant
b.	500	182 ± 106	significant
c.	500	124 ± 76	significant
5	500	307 ± 57	not significant
6	500	336 ± 118	not significant

The degree of protection gained from the serum treatment was estimated from the number of adult worms found in the small intestine of each experimental rat 4 days following introduction of the challenge infection.

The technique employed for transferring the adult worms is given in Chapter V.

Results and discussion

It is evident from table 10 that resistance was passively transferred only with immune serum from rats which have received several large larval infections. Resistance in this experiment was measured by the extent to which an infection of adult worms failed to become established, in the passively immunised host.

Sarles and Taliaferro (1936) challenged passively immunised rats with an infection of *N. brasiliensis* larvae. Resistance to this challenge was only obtained with serum from rats given 3 or more infections of 3,000 or more larvae. It would appear that the antigenic stimulation resulting from administration of dead worm material, 1 or 3 infections of 1,000 adult worms direct to the duodenum, or a single infection of 3,500 larvae, is not sufficient to give a high level of circulating antibody.

An interesting result in this experiment is the inability of serum obtained from rats given 3 infections of adult worms, to

confer resistance passively. It was noted during the preparation of this serum that the rats showed a strong resistance to the second and third transferred adult worm infections, as judged by the host's faecal egg count. In fact the egg counts were minimal. A similar situation was found with a small group of rats by Chandler (1938), who failed to get any transfer of resistance using serum from rats given 4 infections of 1,000, 80-hour larvae transferred directly into the duodenum. He challenged the passively immunised rats with infective N. brasiliensis larvae. He also found that serum from rats immunised with dead larvae gave no protection on passive transfer. These findings led Chandler (1935, 1937) to suggest that a localised, isolated and distinctive response confined to the intestinal mucosa was responsible for the expulsion of a transplanted adult worm population and that this local response did not involve humoral antibody. To stimulate humoral antibody parenteral migration associated with several large infections of larvae was necessary.

It is quite possible that transferred adult worms sensitise the tissues of the small intestine giving local production of cell-bound antibody. Whether a combination of the local production and the local fixation of antibody in the tissues of the gut excludes appearance of antibody in the circulation is

not known. That the host's response to N. brasiliensis is a two part phenomenon, an anti-larval mechanism as distinct from an anti-adult mechanism, has been refuted by Taliaferro (1940), who maintained that the expulsion of the adult worm is a local manifestation of a general humoral and cellular phenomenon.

In later experiments, where the in vitro effect of humoral antibody upon adult N. brasiliensis was studied, the source of antibody was serum obtained from rats which had experienced several large larval infections, as in Group 4 in this experiment.

Summary

(1) Rats were infected with N. brasiliensis using different levels of infection of either larvae or adults. Sera were collected at different times after the final infection.

(2) The sera from the different groups were assessed in their ability to confer passive resistance to a challenge of adult worms.

(3) Only serum from rats which had suffered several large larval infections, was capable of conferring a reasonable degree of resistance.

(b) An investigation of the dilution of the reagin-like component of immune serum during passive immunisation

Introduction

It has recently been shown that reagin-like antibodies may be important in the immunity shown by animals to helminth parasites. Ogilvie (1964a) reported that antibodies produced by infection with *N. brasiliensis* in the rat resembled human reagins, in that they became rapidly and firmly fixed to the skin of a normal rat on intradermal injection. These skin sensitising antibodies can be detected by the Prausnitz-Kustner test, used in the detection and measurement of human reagins (Stanworth, 1963).

Such antibodies could be involved in some of the hypersensitive states which are a feature of many helminth infections. Sprent (1963) has suggested that the inflammatory reaction provoked by gastro-intestinal worms and the granulomatous reactions associated with migrating larvae may be allergic phenomena. It is not difficult to realise that such allergic reactions may be of protective significance in that the associated oedematous changes and inflammatory reaction could make penetration and feeding by the parasite difficult. It must be noted, however, that hypersensitivity reactions need not necessarily involve only reaginic antibodies.

With *N. brasiliensis* in the rat and indeed with most parasitic worms, passive immunisation has not resulted in complete protection against a subsequent challenge infection. Even when large volumes of serum from highly refractory rats have been administered this has not resulted in total protection against a challenge of *N. brasiliensis* larvae (Sarles, 1939) or adult worms (c/f Chapter IX(a) of this thesis). If reagin-like antibodies play a role in protection against infection with *N. brasiliensis* then this failure to confer a strong passive resistance may be due to the very low level of these antibodies in the circulation and it may be difficult to obtain sufficiently high circulating levels in animals being immunised because of the affinity of those particular antibodies for cells. For example the "mopping up" that may occur in the peritoneal cavity when serum containing reaginic antibodies is being introduced during passive immunisation may present special problems. With human reagins it has been shown from immunological data that sera from allergic individuals, capable of showing a positive P-K reaction, could contain as little as 0.1 mg. antibody N. per liter of serum (Stanworth, 1963). Hence reagins when compared to the major serum protein constituents are more or less a trace component.

Ogilvie (1964a) used the P-K test to follow the level of reagin-like antibody in the serum of rats infected with *N. brasiliensis*. In the following experiment an attempt was made to investigate the reagin-like antibody titre in rats passively immunised with serum from rats made highly resistant to further infection.

Methods and materials

(a) Preparation of immune serum and passive immunisation

Immune rat serum was obtained from rats given 4 infections (3,500, 5,000, 7,500 and 10,000 larvae) of third stage larvae at fortnightly intervals. The rats were bled, by cardiac puncture, 10 days following the final infection.

A group of 8 hooded rats, males and females, around 175 gms. live weight were passively immunised with the above immune serum, each receiving 4 ml. serum/100 gm. live weight intraperitoneally. 2 rats from the group were killed by cardiac puncture each day, over the 4 days following passive immunisation. The serum obtained from both rats on the day of killing, was pooled.

(b) P.C.A. test with serum from the passively immunised rats

The original immune serum and the serum samples subsequently obtained after passive immunisation were both serially diluted with normal rat serum.

Figure 13

Plan of injection sites and serum dilutions used

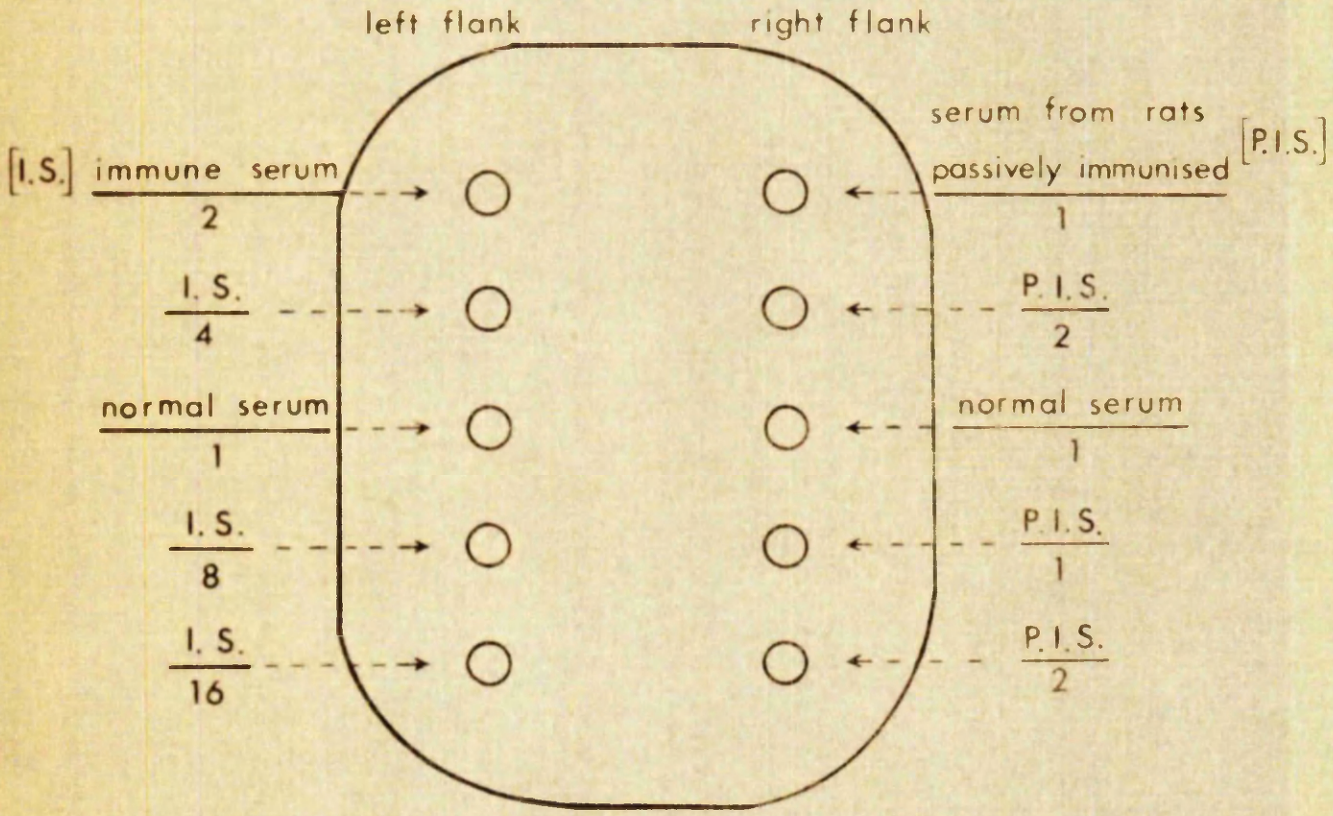


Figure 13. Plan showing the site of intradermal injection of the various sera.

Rat Pelts Showing P.C.A. Reaction

I.S. = Immune serum. N.S. = Normal Serum

P.I.S. = Serum from passively immunised rats.

0.02 ml. of each dilution of both sera was injected intradermally to normal rats. In order to facilitate injection the backs of the rats were carefully shaved. A plan showing the injection sites and the dilutions of the sera injected can be seen in fig. 13. It was found that the small volume of serum used in this experiment could be most accurately injected using a Burroughs-Welcome "Agla" micrometer syringe fitted with a No. 20 (S.W.G.) needle.

(c) Preparation and injection of antigen

Adult worm antigen was prepared as described in Chapter XI. The antigen solution was made 1% with respect to Evans Blue and 1.0 ml. of this mixture was injected via a tail vein to each rat three days following intradermal injection of serum.

The rats were killed 1 hour after intravenous injection of the antigen-Evans Blue mixture and the pelt removed for examination.

Results

A positive passive cutaneous anaphylactic (P.C.A.) response was indicated by a discrete blue patch, usually about 1 cm. in diameter. From the photographs of the pelts obtained it can be seen that positive reactions were obtained with the original immune serum diluted two fold and four hold with normal serum. The higher dilutions of this serum i.e. 1/8 and 1/16 gave no reaction.

No positive skin reactions resulted with the serum obtained from rats which had been passively immunised with the above immune serum.

Discussion

The same degree of resistance to a challenge infection of *N. brasiliensis*, as found in actively immunised rats, has not been achieved by passive transfer of hyperimmune serum, even when very large volumes of such serum have been administered (Sarles, 1938). The reasons for this failure are not fully understood.

Should antibodies which become firmly fixed to cells (e.g. reagins) be important in resistance to this parasitic worm then 2 factors may be of importance in passive immunisation. Firstly such antibodies may only be present in the plasma of the actively immune rat at very low concentrations and secondly when serum is administered intraperitoneally, during passive immunisation, then the reagin-like antibodies may be "mopped up" by cells in the peritoneal cavity.

In the experiment described, the titre of reaginic antibodies present in a given serum was found by a P.C.A. test. An attempt was made to compare the titre of reagin-like antibodies given by an immune serum before and after intraperitoneal injection into

a normal rat. Assuming no catabolism and no cell fixation of the introduced reagin within the recipient rat, then the injected serum would merely have equilibrated with the intravascular and extravascular systems so undergoing an approximate 3 to 4 times dilution. However, if the reagins present in the administered serum had been removed and fixed to cells then one could have expected a greater apparent dilution of this component within the recipient rat (this is called the in vivo dilution). The extent of this dilution was determined by a comparison of the skin reaction given by the serum obtained from the passively immunised rat with the skin reaction given by serially diluted original immune serum (this is called in vitro dilution).

It can be seen from the results that no positive skin reaction was obtained with the serum from passively immunised rats. The highest in vitro dilution of the original serum to give a positive skin reaction was 1 in 4. Hence the in vivo dilution must have been greater than 1 in 4.

This failure to obtain positiveskin reactions with in vitro dilutions greater than 1 in 4 was disappointing and limited the value of the experiment. Other workers using this technique (Ogilvie, 1964a) have reported positive P.C.A. reactions with immune rat serum diluted 1 in 200 and even with 1 in 1,600. Ogilvie (1964a) found that these high antibody titres occurred

in rats 3 days following a second or later larval infection. The titre remained high only for a short period and then fell to a low level until a further infection. The immune serum used in this experiment, which has proved effective in conferring some degree of passive resistance (c/f Chapter V) was collected 10 days following the final larval infection. It is possible that the reagin-like antibody level had fallen to a low level during this period of time. It may be that a stronger passive immunity would have resulted had this serum been collected 3 days after the final larval infection.

Differences between Ogilvie's (1964a) results and those obtained here may also have resulted from differences in the antigenicity of the crude worm extract administered as antigen.

Summary

(1) The level of reagin-like antibodies in a serum sample was determined by a passive cutaneous anaphyaxis (P.C.A.) test.

(2) Immune serum, which is capable of conferring resistance passively, was diluted with normal serum and tested (*in vitro* dilution). Immune serum was injected intraperitoneally at a dosage which gives passive immunity, and the recipient rat's serum tested (*in vivo* dilution).

(3) The greatest *in vitro* dilution to give a positive P.C.A. test was 1 in 4. No positive skin reactions were obtained with the serum after *in vivo* dilution.

(4) Due to the lack of sensitivity of the P.C.A. test under the conditions of this experiment, no estimate could be made of the *in vivo* dilution of the reaginic component of immune serum other than this was greater than 1 in 4. This indicated, however, that some "mopping up" of reaginic antibodies by cells in the recipient rat had occurred.

CHAPTER X

The in vitro effect of immune serum upon adult N. brasiliensis

(a) The fate of a transferred adult worm population after previous in vitro incubation in immune serum

Introduction

When infective N. brasiliensis larvae were incubated in homologous immune serum for a period of time, precipitates formed at the body openings of the larvae (Sarles, 1938). As no precipitate formation occurs when the larvae were incubated in normal serum it appeared that this phenomenon was related to acquired resistance. Jackson (1960) using fluorescent antibody, demonstrated that this precipitate formation resulted from an antibody-antigen reaction. He also showed that precipitates formed within the parasite's alimentary canal as well as on its body surface.

Thorson (1954b) incubated infective larvae, in immune serum and found that this treatment reduced, on some occasions, the infectivity of the larvae. This has also been demonstrated with the infective larvae of Ancylostoma caninum (Otto, 1948); and Trichinella spiralis (Mauss, 1940).

Hence it would appear, in the case of infective larvae, that pre-incubation in serum obtained from rats highly refractory to further infection with N. brasiliensis, debilitates the larvae to some extent resulting in a reduced infectivity.

Taliaferro and Sarles (1939) noted on occasion the presence of precipitates around the adult parasite in vivo and stated that they were similar to those found during their in vitro tests. When adult N. brasiliensis were incubated in immune rat serum precipitates sometimes formed at their body openings.

In the following experiment an attempt was made to find if pre-incubation in immune rat serum lowered the ability of an adult worm population, to become established when transferred to the gut of a rat which had had no previous experience of the parasite.

Methods and material

(a) Preparation of immune serum

This was prepared by infecting rats with N. brasiliensis larvae on 4 occasions as follows:-

Initial infection of	3,500 larvae,
followed 2 weeks later by	5,000 larvae,
followed 2 weeks later by	7,500 larvae,
followed 2 weeks later by	10,000 larvae.

The rats were bled 10 days following the final larval infection. In other experiments it was found that this serum conferred a good degree of passive resistance.

(b) Normal serum

This was obtained from rats which had had no experience of the parasite, N. brasiliensis.

Both sera had been stored at -20°C. until required.

(c) Incubation procedure

15,000 adult N. brasiliensis were obtained from the small intestines of rats infected with 3,500 infective larvae each, 10 days previously.

1,000 worms were placed in each of 15 Warburg flasks and suspended in the following media:-

(1) 5 flasks each containing 3.0 ml. of Krebs-Ringer phosphate buffer at pH 7.4.

(2) 5 flasks each containing 3.0 ml. of normal rat serum adjusted to pH 7.4.

(3) 5 flasks each containing 3.0 ml. of immune rat serum adjusted to pH 7.4.

Antibiotics were added to each suspension such that the final concentration was 100 units Penicillin and 10 µgm. Streptomycin per ml.

Each flask was fitted to a manometer and placed on the Warburg apparatus and allowed to shake at the lowest speed setting of the machine. The temperature of the bath was set at 37°C. and the flasks allowed to shake overnight under an atmosphere of air.

After 20 hours incubation the worms were removed from the flasks, divided into 2 portions of 500 worms each, and placed in tapered 10 ml. centrifuge tubes. The worms were washed 3 times with warm physiological saline, and finally suspended in saline.

(d) Transfer to experimental animals

40 rats of both sexes and of similar weight were chosen and divided at random into 4 groups of 10.

Rats forming Group 1 each received 500 adult worms which had been incubated in Krebs-Ringer phosphate buffer. Rats forming Group 2 each received 500 adult worms, incubated in normal serum. Rats forming Group 3 each received 500 adult worms which had been incubated in immune serum. Group 4 rats each received 500 adult worms which had been isolated from donor rats just before transfer.

In all cases the adult worms were transferred to the duodenum of the recipient rats by the method already described (Chapter V).

Table 11

Mean number of adult worms recovered from the small intestine four days after transfer of 500 adult worms to each rat

Group	Incubation medium	No. of rats	Mean No. of worms recovered on day 4	As % of transferred infection
1	Krebs-Ringer phosphate buffer	8	255 ± 42	51%
2	Normal rat serum	9	174 ± 59	35%
3	Immune rat serum	10	142 ± 73	28%
4	No pre-incubation fresh worms transferred	10	359 ± 75	72%

The number of parasite eggs contained in the faeces passed by each group per day was found. On the fourth day following transfer all groups were autopsied and the number of worms surviving in the small intestine counted.

Results and discussion

It can be seen, from table 11, that overnight *in vitro* incubation reduced the number of adult worms becoming established on transfer to the small intestine of a 'clean' rat. 72% of a transferred infection of freshly isolated worms succeeded in becoming established. The corresponding percentages for worms incubated for 20 hours before transfer in Krebs-Ringer phosphate buffer, normal serum and immune serum were 51%, 35% and 28% respectively. It is clear that a number of adult worms had been either killed or seriously weakened during this period of *in vitro* incubation.

Worms incubated in both normal and immune serum showed a reduced ability to become established when compared to worms incubated in phosphate buffer. These results tend to suggest that a factor or factors possessed by both sera in some way interfered with the well being of the worms such that their infectivity was reduced. There was a suggestion that preincubation in immune serum caused slightly greater debilitation

Table 12

Mean number of eggs per rat per 24 hours

Day after transfer	Group 1 phosphate buffer	Group 2 normal serum	Group 3 immune serum	Group 4 No. pre incubation
1	10,000	5,000	3,500	65,000
2	17,000	4,500	6,000	80,000
3	37,000	20,500	17,500	45,000
4	46,000	24,000	19,000	60,000

of the adult worms than similar treatment in normal serum. However, it must be pointed out that there is no statistically significant difference between the number of worms becoming established in these 2 groups.

The adverse effect of *in vitro* conditions upon the adult worm is also seen in its effect upon egg production (table 12). Of particular interest in this connection is a comparison of the egg laying potential of the 3 preincubated groups of worms as compared to the fresh worms over the initial period of the infection. The actual manipulation of transferring adult worms from the donor rat to the recipient rat does not cause a reduction in the egg production of the worms. This is seen with Group 4 in this experiment. A 20 hour preincubation period, on the other hand, no matter what the medium, causes a relative reduction in egg laying over the first 2 days in the new host. By day 3 post-transfer each worm appears to have regained full egg laying potential.

Towards the end of the incubation period the worms of each group were closely examined for the appearance of precipitates. As this test is far from being a quantitative one, it is difficult to interpret. No real significant difference as regards precipitate formation between normal and immune serum could be detected with any conviction, however, it appeared that there was probably less precipitate formation in the phosphate buffer preparations.

It has been suggested that the formation of Sarle's precipitates are the result of an antigen-antibody interaction and could be related to *in vivo* resistance, (Faliaferro and Sarles, 1939). Studies with *N. brasiliensis* infections in both normal and abnormal hosts were carried out by Lindquist (1950). In the abnormal hosts, cotton rat, guinea pig and hamster, an initial larval infection gave an extended prepatent period, stunted worms and a cellular response. All these features were similar to the result obtained with a larval challenge to a hyperimmunised normal host. However, it was noted, in the case of the abnormal hosts, that no granular precipitates were present around larvae trapped in the cellular nodules. However, the serum from previously uninfected abnormal hosts, like that of uninfected normal hosts, did not give precipitate formation around larvae on *in vitro* incubation, whereas serum from both hyperinfected abnormal and normal hosts did give Sarles precipitates. These studies suggested that natural resistance did not depend upon humoral precipitating antibodies but that acquired resistance to some extent might.

In order to clarify the position as regards the formation of precipitates by adult *N. brasiliensis* when incubated in rat serum, and their relation to antibody-antigen interaction it would be wise to use a fluorescent antibody technique similar

to that employed by Jackson (1959 and 1960) who investigated the nature of precipitate formation with larvae of N. brasiliensis and T. spiralis.

Summary

(1) N. brasiliensis adult worms were incubated for 20 hours in 1 of 3 media -

normal rat serum, immune rat serum and Krebs-Ringer phosphate buffer.

Precipitates were present around worms incubated in both sera but no such precipitate formation was found with worms in phosphate buffer.

(2) After incubation the adult worms were transferred to the small intestines of normal rats.

(3) The number of worms which had become established after 4 days, was assessed.

(4) It appeared that preincubation in immune rat serum did not reduce the infectivity of adult worms, when compared with similar treatment in normal serum.

(5) Preincubation in both normal and immune serum reduced the ability of adult worms to become established in a normal rat when compared with worms preincubated in Krebs-Ringer phosphate buffer.

(b) The effect of immune serum upon the respiration and metabolism of adult worms

Introduction

The respiratory metabolism of nematode parasites has been reviewed by several authors (Hobson, 1948; Mendes, 1949; von Brand, 1952; Bueding and Most, 1953; Rogers, 1962). It has been found that all metazoan parasites examined so far, respire aerobically when a supply of oxygen is available. However, respiratory rates vary considerably from parasite to parasite. This could result from a difference of the importance of the various metabolic routes involving oxygen.

The intermediary metabolism of parasitic worms has not been extensively investigated, however, there is sufficient evidence to suggest that the Embden-Meyerhof system of phosphorylating glycolysis is present in the tissues of parasitic adult worms and larvae (Rogers and Lazarus, 1949a; Bueding and Yale, 1951; Rathbone and Rees, 1955; Agosin and Aravena, 1959a). Part of the pentose phosphate pathway of glucose metabolism was found to be present in Ascaris lumbricoides and Echinococcus granulosis (Agosin and Aravena, 1959b; Entner, 1957; Entner and Gonzalez, 1959) however it is though that as with mammalian tissues this route is probably unimportant as a source of energy.

A common characteristic of the intermediary metabolism in the tissues of nematodes, is a high rate of carbohydrate metabolism associated with incomplete substrate oxidation. Although helminths take up oxygen when it is available fermentation rates remain high. Indeed, it has been suggested that these organisms depend upon anaerobic metabolism for survival and appear to be unable to utilise energy derived from oxidative reactions (von Brand, 1952). Only rarely is lactic acid the major end product of glycolysis, a variety of volatile fatty acids being produced in many cases. No definite information is available about the mechanisms involved in the formation of volatile acids by helminths but it is conceivable that pyruvate produced by glycolytic reactions, is the common precursor of these acids (Rogers, 1962). The importance and route of metabolism of pyruvate varies with different worms. For instance, with Nematodirus spp., Neoaplectana glaseri and Ascaridia galli, pyruvate metabolism seems to be similar to the Krebs tricarboxylic acid cycle of mammilian tissues (Massey and Rogers, 1951). Evidence for the participation of a cytochrome system in the oxidative metabolism has been demonstrated in the tissues of T. spiralis (Goldberg, 1957).

The wide variation in respiratory rates among parasitic worms could also result from the fact that some possess pigments capable of transporting oxygen whilst others do not (Lazarus,

1950). Nematodes in general possess no specialised respiratory surfaces and it is thought that oxygen enters via the general body surface and in the case of A. caninum via the intestine.

There is also an absence of a specialised circulatory system in these organisms. This lack may be compensated in part by the pseudocoel, in which are suspended the alimentary canal and reproductive system. This internal fluid is also in contact with the body wall and may be of importance in transporting oxygen to the internal organs. This function would be greatly facilitated if the pseudocoel contained a respiratory pigment which was capable of transporting oxygen. Haemoglobin is present in the pseudocoel of N. brasiliensis (Davenport, 1949) and transport of oxygen by this pigment has been shown to be of importance especially at oxygen tensions normally found close to the gut mucosa of the rat (Rogers, 1949a).

A survey of the literature suggests that N. brasiliensis utilises oxygen and that intermediary metabolism may depend upon a phosphorylative oxidation process. Earlier studies reported in this thesis have shown that the passive transfer of serum confers resistance manifest by expulsion of the adult worm. Conditions are also present whereby plasma proteins can gain ready access to the adult worm (c/f Chapter VIII). It has been proposed that the immune expulsion of adult N. brasiliensis may

rely upon some interference in the worm's feeding or metabolism. A worm debilitated in such a way could be easily prone to expulsion from the gut. An attempt to demonstrate the presence of an anti-metabolic factor in immune rat serum is described in this Chapter. The effect of immune serum, and hence humoral antibody, upon both the oxygen uptake and the phosphate metabolism was investigated.

(1) The effect of immune serum upon the oxygen uptake of adult N. brasiliensis

Introduction

Schwabe (1957) studied the respiratory rates of free-living and parasitic N. brasiliensis larvae whilst suspended in normal and immune rat serum. It was found that immune serum inhibited immediately the endogenous oxygen uptake of free-living but not that of parasitic larvae. However, on adding glucose to the sera the results indicated a trend whereby the respiratory rate of the parasitic larvae was now being depressed by immune serum, as well as that of the free-living larvae. These results were interpreted as giving some support to the enzyme-antienzyme theory of immunity to parasitic worms first proposed by Chandler (1936).

The respiratory rate of adult N. brasiliensis suspended in a balanced salt solution has been investigated (Rogers, 1949b; Roberts and Fairbairn, 1963) but no attempt has been made to find if immune serum interferes with normal respiration. The following experiments were carried out to find if immune serum had any effect upon the oxygen uptake of adult worms and to relate these findings to the immune expulsion of the parasite.

Preliminary experiments

It is feasible that if humoral antibody interferes with oxygen metabolism it is not necessary for its effect to act immediately. In the event of this being the case it was decided to find out how long it was possible to follow the oxygen uptake of adult N. brasiliensis before bacterial contamination of the preparation made this impossible.

Methods and materials

(a) Isolation and preparation of adult worms

Adult N. brasiliensis were isolated from the small intestine of rats each infected 10 days previously with 3,500 larvae. The adult worms were given 3 washes with warm sterile saline and finally suspended in Krebs-Ringer phosphate buffer at pH 7.4 (Umbreit et al, 1957). Dead adult worms, where used as controls, were killed by storing at -20°C. overnight.

Table 13

Description of flask contents	Micro-litres of oxygen consumed per hour per milligram worm tissue nitrogen			
	1st period 0-3 hrs.	2nd period 20-23 hrs.	3rd period 64-67 hrs.	4th period 90-93 hrs.
1) live worms, no antibiotic, no glucose	59.9	38.3	60.7	97.4
2) live worms, antibiotic (1) no glucose	53.5	26.8	21.2	11.8
3) live worms, antibiotic (2), no glucose	46.9	21.5	15.6	7.2
4) live worms, no antibiotic, M/20 glucose	44.9	22.1	71.4	53.5
5) live worms, antibiotic (1), M/20 glucose	49.7	18.9	77.3	74.2
6) live worms, antibiotic (2), M/20 glucose	40.8	17.5	13.0	32.4
7) dead worms, no antibiotics, no glucose	4.1	5.9	23.9	29.2
8) dead worms, antibiotics (1), no glucose	9.6	1.0	3.5	4.5
9) dead worms, antibiotics (1), M/20 glucose	10.4	0.8	74.1	86.2
10) washings from adult worms, antibiotics (1), M/20 glucose	0.8	0	0	0

Antibiotics were added to the Krebs-Ringer phosphate buffer where indicated at one of two concentrations.

(1) giving a final concentration of 250 units of penicillin G per ml. and 25 micrograms of streptomycin sulphate per ml.

(2) giving a final concentration of 2,500 units of penicillin G per ml. and 250 micrograms of streptomycin sulphate per ml.

Where indicated, glucose was added giving a final concentration of M/20.

(b) Manometric methods

These are described fully in Experiment 1. In the present experiment the gas phase of flasks was air. Flasks and manometers were equlibrated for 15 minutes in the water bath, set at 37°C, before readings were taken. The oxygen uptake was measured over a 3 hour period on each of 4 days. During the periods between measurements, the flasks and manometers were allowed to shake in the Warburg apparatus, the stopcocks of the manometers open to the atmosphere.

Results and discussion

The mean Qo_2^N values for each preparation are shown in table 13. These figures were obtained from duplicate measurements. Several points are worthy of note.

(a) The presence of antibiotics in the concentrations used had no adverse effect upon the worms.

(b) Both concentrations of antibiotics used were capable of suppressing bacterial contamination over the first 24 hours, however this ability was lost with increasing time, especially when glucose was added to the preparation.

(c) A main source of bacterial contamination seemed to come from dead and decaying worms.

From these findings it was decided, in future experiments, to add antibiotics such that the final concentrations were 250 units penicillin and 25 micrograms streptomycin per ml. of suspending fluid, and to limit the duration to 24 hours.

Experiment 1

Effects of normal and immune rat serum upon the endogenous respiration of adult N. brasiliensis

Methods and materials

(a) Isolation and preparation of adult worms

Adult N. brasiliensis were isolated from the small intestines of rats following a primary 3,500 larval infection. The worms were washed 3 times with warm sterile physiological saline. An aliquot was counted, and on the basis of this count, the volume of the original saline suspension of worms was adjusted so that 5.0 ml. contained 1,000 worms. As each Warburg flask was to contain 1,000 worms, 5.0 ml. aliquots of the worm

suspension were pipetted into individual 10 ml. calibrated centrifuge tubes. When the worms had settled at the bottom of the tube, as much as possible of the saline was removed. The worms were then suspended in either normal serum, immune serum or Kerbs-Ringer phosphate buffer, plus antibiotics, as the case might be, giving an exact final volume of 3.0 ml. This was then entirely removed with a broad mouthed Pasteur pipette and placed in the appropriate Warburg flask. During this operation care was taken to maintain the temperature of the worms at 37°C, and to expose the worms to as little mechanical damage as possible.

(b) Normal serum

Normal serum was obtained from rats which had had no previous experience of the parasite. The donor rats were starved overnight before being bled.

(c) Immune serum

Immune serum was obtained from rats which had been given a series of larval infections, as follows:- 3,500 larvae, 5,000 larvae, 7,500 larvae and 10,000 larvae, all at fortnightly intervals. The rats were bled, after being starved overnight, 10-12 days following the final infection. Serum prepared in a similar manner has been shown to confer a strong passive immunity against challenge. Both sera were stored at -20°C. until required.

(d) Krebs-Ringer phosphate buffer

Krebs-Ringer phosphate solution was prepared after the method of Umbriet et al (1957) and was made up fresh on the day required. The pH of the 3 media was adjusted to 7.4 just before use.

(e) Manometric methods

0.5 ml. of M/5 K.OH. was placed in the outer trough of the Warburg flasks followed by the 3.0 ml. of worm suspension placed in the central compartment. The flasks were then rapidly fitted to the manometer and placed in the Warburg bath set at 37°C. The apparatus was adjusted to give a shaking frequency of 80 strokes per minute, each stroke being 2 cm. The gas phase in this experiment was air. After 15 minutes equilibration, readings were taken over the initial 3 hour period. The flasks were allowed to shake, in the bath, overnight, with the manometer stopcocks open, and readings were resumed for a second and final 3 hour period next day. Hence the "initial period" was 0-3 hours and the "final period" 20-23 hours.

At the end of the experiment, worms plus media from each flask were removed separately and the worms washed thoroughly with saline. The total nitrogen content of each sample of worms was determined by the micro-Kjeldhal method. The volume of oxygen in microlitres consumed per hour per milligram of worm tissue nitrogen (Qo_2^N) was calculated for each flask.

Table 14

Suspending medium	No. of determinations	micro-litres of oxygen consumed per hour per mgm. worm tissue N. ($Q^{N}_{O_2}$ values)	
		initial period 0 - 3 hours	final period 20 - 23 hours
Krebs-Ringer phosphate buffer	24	44.6 ± 14.7	27.4 ± 8.7
Normal serum	16	53.1 ± 7.6	16.1 ± 5.2
Immune serum	13	54.3 ± 10.2	5.5 ± 3.1

Results

The endogenous oxygen consumption rates of intact adult N. brasiliensis was measured in normal sera, immune serum, and Krebs-Ringer phosphate buffer solution.

The resulting Qo_2^N values are shown in table 14. Over the initial 3 hour period it can be seen that there was no difference in the respiratory rate of the adult worms, despite the different suspending media. This was not the case during the final period i.e. 20-23 hours later. It is clear that the oxygen consumption rate was lower in immune serum than in both normal serum and Krebs-Ringer phosphate buffer. There was also an indication that there was some depression of oxygen uptake with worms suspended in normal serum when comparedwith Krebs-Ringer phosphate buffer. As the adult worms aged, their respiratory rate decreased. After 20 hours incubation in Krebs-Ringer phosphate buffer the respiratory rate had decreased by about 40%.

Experiment 2

Effects of normal and immune rat serum upon the respiration of adult N. brasiliensis in the presence of an exogenous substrate

Methods and materials

Adult N. brasiliensis were isolated and treated as before. To each of the 3 media, glucose was added to a final concentration

Table 15

Suspending medium	No. of determinations	micro-litres of oxygen consumed per hour per mgm. worm tissue nitrogen ($Q^{N}_{O_2}$ values) initial period 0 - 3 hours
Krebs-Ringer phosphate buffer	24	44.6 ± 14.7
Krebs-Ringer phosphate buffer plus glucose	11	49.6 ± 13.8
Normal serum	16	53.1 ± 7.6
Normal serum plus glucose	6	52.8 ± 9.1
Immune serum	13	54.3 ± 10.2
Immune serum plus glucose	6	46.4 ± 8.7

of M/20. The respiratory rates of the adult worms in Krebs-Ringer, normal and immune rat serum with added glucose were determined by the direct method of Warburg with a gas phase of air at 37°C. Due to unforeseen circumstances it was only possible to measure oxygen uptake over an initial 3 hour period.

Results

No significant increase in oxygen consumption rate over the endogenous rate resulted from the addition of glucose to the 3 media used. The mean Qo_2^N values (plus or minus one standard deviation) are shown in table 15. These results indicated that adult N. brasiliensis were unable to utilise this metabolite under the conditions of this experiment.

Experiment 3

Effects of environmental carbon dioxide on endogenous oxygen consumption of adult N. brasiliensis

Introduction

In the previous experiments the direct method of Warburg was used to estimate the oxygen consumption rates of adult N. brasiliensis in various media. This method demands that oxygen uptake be measured in an atmosphere free from carbon dioxide. Generally carbon dioxide has little effect upon the

rate of respiration of mammilian tissues (Umbriet et al, 1957 p.28) however, Schwabe (1957), investigating the endogenous oxygen uptake of free living and parasitic N. brasiliensis larvae found that while the presence of CO_2 in the gas phase did not affect the oxygen uptake of the former it did increase the oxygen uptake of the latter.

Methods and materials

Adult N. brasiliensis were obtained as before. The two rat sera, normal and immune, used in the previous experiments were gassed for 15 minutes using a sintered aerator, with the gas phase to be used in the experiment and the final pH adjusted to 7.4.

An atmosphere of air containing 1% CO_2 was used in this experiment. This was made possible by using the method of Pardee (1949) as described by Krebs (1951). The method is based upon the use of a "CO_2-buffer" in place of alkali in the trough of the Warburg flask. The 'buffer' used to maintain a constant pressure of CO_2 in the gas phase was an aqueous solution of diethanolamine. This compound binds CO_2 reversibly and removes CO_2 formed by metabolism so maintaining the desired pressure of CO_2 in the gas phase.

Table 16

Suspending medium	No. of determinations	% of CO_2 in gas phase	$Q_{O_2}^N$ values initial period 0 to 3 hours
Normal serum	16	0%	53.1 ± 7.6
Normal serum	6	1%	41.5 ± 9.4
Immune serum	13	0%	54.3 ± 10.2
Immune serum	6	1%	43.0 ± 8.3

Results

Table 16 shows the Qo_2^N values obtained for adult N. brasiliensis respiring in the absence of CO_2 from the gas phase and in the presence of a gas phase containing 1% CO_2. No appreciable difference in respiratory rate was obtained over the initial 3 hour period during which readings were taken. Hence the removal of CO_2 from the gas phase, as is the case in the direct method of Warburg, did not affect the endogenous respiratory rate of adult N. brasiliensis.

Discussion

The manometric methods, used in these experiments described so far, indicated that neither the presence of CO_2 in the gas phase nor the presence of glucose as a substrate influenced the respiratory rate of adult worms. Roberts and Fairbairn (1963) found that respiration was unaffected by the addition of glucose or other substrates to the medium and that these materials were not ingested in appreciable amounts by adult N. brasiliensis.

The endogenous respiratory rate of adult worms was significantly depressed following exposure to immune serum for 20 hours. Whether this inhibition is due to an anti-enzyme contained in the immune serum or to some other effect is difficult to decide.

In the above experiments air was used as the gas phase. The average pO_2 in air is 174 mm. Hg. It has been pointed out by Hobson (1948) and Rogers (1949a) that the applicability of results obtained when parasites, taken from an environment low in oxygen such as the host's small intestine, and examined in solutions in equilibrium with air, are open to question. Indeed it appears, to some extent, that the more oxygen available to parasitic worms, the more oxygen they will consume. This is the case with adult N. brasiliensis (Rogers, 1962).

It was decided to repeat the above experiments on the respiratory rates of adult worms in various media, with a gas phase containing a pO_2 similar to that present in the parasite's natural environment. Rogers (1949a) has demonstrated that the pO_2 close to the mucosa of the small intestine of the rat varies from 7.9 to 30.2 mm. Hg. The gas phase used in the following experiments contained 3% oxygen which is the equivalent to a pO_2 of 23 mm. Hg.

Experiment 4

Effects of normal and immune rat serum upon the endogenous respiration of adult N. brasiliensis in a gas phase containing 3% oxygen

Methods and materials

Each Warburg flask contained 1,000 adult N. brasiliensis obtained as already described, suspended in one of the following media:-

Normal rat serum, immune rat serum or Krebs-Ringer phosphate buffer.

These media were prepared as in Experiment 1, but in addition, in this experiment, they were gassed for 15 minutes using a sintered aerator, with the gas mixture to be used i.e. 3% O_2 + 97% N_2, before adjusting to pH 7.4. After placing the manometers with the flasks plus contents on the Warburg apparatus, the atmospheres in the flasks were flushed out with pure nitrogen for 15 minutes. This was done with the aid of a suitable manifold (Umbriet et al, 1957 p.70). This action is followed by flushing out the apparatus with the prepared 3% O_2 + 97% N_2 gas mixture for a further 15 minutes. Before initial readings were taken the flasks were allowed to equilibrate under this gas phase for 15 minutes. Readings were then taken over a 3 hour period, any CO_2 produced being absorbed by 0.5 ml. of M/5 K.OH. placed in the outer trough of each flask.

Table 17

Suspending medium	No. of determinations	$Q_{O_2}^N$ values	
		initial period 0 to 3 hours	final period 20 to 23 hours
Krebs-Ringer phosphate buffer	23	40.5 ± 8.1	26.7 ± 4.4
Normal serum	26	82.2 ± 13.9	17.3 ± 11.8
Immune serum	25	49.3 ± 10.4	15.8 ± 3.1

The flasks were left shaking overnight in the Warburg apparatus with the manometer stopcocks open to an atmosphere of 3% O_2 + 97% N_2. After equilibration readings were resumed for a further 3 hour period next day. The results were expressed in the terms of worm tissue nitrogen.

Results

As with the measurements taken with an atmosphere of air in the Warburg flask (table 14), the Qo_2^N values obtained in this experiment are similar despite the medium, over the initial 3 hour period (table 17). However, it is note worthy that the Qo_2^N values are all slightly lower with the gas phase containing less oxygen.

After 20 hours incubation in immune serum adult N. brasiliensis again show a reduced Qo_2^N value when compared to worms suspended in phosphate buffer. The reduction, however, is not so great in this experiment as it was with air as the gas phase. There was no significant difference in the respiratory rates of worms suspended in either serum.

Table 18

Suspending medium	No. of determinations	$Q_{O_2}^N$ values	
		initial period 0 to 3 hours	Final period 20 to 23 hours
Krebs-Ringer phosphate buffer	23	40.5 ± 8.1	26.7 ± 4.4
Krebs-Ringer phosphate buffer plus glucose	6	50.4 ± 9.7	23.9 ± 2.6
Normal serum	26	42.2 ± 13.9	17.3 ± 11.8
Normal serum plus glucose	6	29.7 ± 4.7	20.4 ± 6.7
Immune serum	25	49.3 ± 10.4	15.8 ± 3.1
Immune serum plus glucose	6	59.8 ± 10.3	23.6 ± 3.3

Experiment 5

The effect of glucose as an exogenous substrate upon the respiration of adult N. brasiliensis in a gas phase containing 3% oxygen

Methods and materials

Adult worms and the 3 media were prepared, placed in Warburg flasks and gassed with 3% O_2 + 97% N_2, as in Experiment 4. However, in this experiment each of the media contained a final concentration of M/20 glucose. Measurements were again made by the direct method of Warburg over an initial 3 hour period and a final 3 hour period, 20 hours later.

Results

Table 18 shows results obtained for the respiratory rates of adult worms in the 3 media in the presence or absence of glucose. Over the initial period the presence of glucose appeared to slightly increase the respiratory rates of worms suspended in phosphate buffer and immune serum, while failing to do so with worms suspended in normal serum. It is not clear why this should be so. Over the final 3 hour period there was no difference in the Q values of the worms in either of the 3 media where glucose is added. The presence of added glucose to immune serum appears to have abolished the decrease in Q value found over the final period, with the endogenous Q

Table 19

Suspending medium	No. of determinations	% CO_2 in gas phase	$Q_{O_2}^N$ values Final period 20 to 33 hours
Normal serum	26	0%	17.3 ± 11.8
Normal serum	6	1%	19.1 ± 5.4
Immune serum	25	0%	15.8 ± 3.1
Immune serum	6	1%	14.8 ± 2.4

values of worms suspended in phosphate buffer and immune serum. It may be that over the final period glucose was being utilised by the worms suspended in immune serum but not by worms suspended in phosphate buffer. A slight increase due to the presence of glucose was also found with worms in normal serum.

Experiment 6

The effect of environmental carbon dioxide on the endogenous oxygen consumption of adult worms in a gas phase containing 3% oxygen

Each Warburg flask contained 1,000 adult N. brasiliensis suspended in 1 of 2 sera:- normal rat serum or immune rat serum. In place of alkali in the outer trough each flask contained its appropriate volume of Pardee's solution to maintain a gas phase containing 1% CO_2 in each flask. Hence the gas mixture used in this experiment contained 3% O_2 + 96% N_2 + 1% CO_2.

The adult worms were stored in their respective media, in the Warburg apparatus, overnight under this gas mixture, and readings were only taken over the final period.

Results

Table 19 indicates that the presence of CO_2 in the gas phase has no effect upon the endogenous oxygen consumption of adult N. brasiliensis.

Discussion

During the patent stage of an infection with N. brasiliensis it has been shown earlier in this thesis that mechanisms may exist whereby host plasma proteins can gain access to the worms in appreciable quantities. Should specific antibody present in this serum fraction debilitate the worm then expulsion by the host would be facilitated.

It has been shown in the above experiments that after 20 hours exposure of adult worms to immune serum under an atmosphere of air, these worms assimilated less oxygen than did similar worms suspended in normal serum. The reduction in oxygen uptake of worms in immune serum was even greater when compared with worms in phosphate buffer solution. When the oxygen tension is reduced to that most likely encountered by the worms in their natural environment, then no such difference between the sera was found. In both sets of experiments, worms suspended in phosphate buffer showed a higher endogenous oxygen uptake after a 20 hour incubation, than worms in either sera, and especially so with worms in immune serum. That adult worms survived better in a balanced salt solution than in either immune or normal rat serum was shown in an earlier experiment (c/f Chapter X(a)). Those worms suspended in BSS. showed a better ability to become established in the new host than did worms suspended overnight in either serum.

The addition of glucose as an exogenous substrate to all 3 media in Experiment 5 (where the oxygen tension was small) resulted in the elimination of this lower endogenous respiratory rate, shown by worms suspended in immune serum. As our knowledge of the intermediary metabolism of carbohydrate by this parasite is scanty, it is difficult to interpret this result. More information upon the metabolism of glucose may be gained from use of the various ^{14}C. labelled glucose molecules as exogenous substrates. However, it may well be that antibodies present in immune serum were inhibiting to some extent, metabolic pathways which utilise oxygen, and that in some way it may be that the adverse effects of inhibition upon the worm's energy supply could have been made good by metabolising this substrate.

The relative contributions of anaerobic and aerobic metabolism towards the energy requirements of the parasitic worm are not known. However, it has been shown that under anaerobic conditions adult worms show complete loss of motility within 6 hours and death within 6 to 24 hours (Roberts and Fairbairn, 1963). Adult worms are also immobilised on adding cyanide, so inhibiting aerobic pathways (Rogers, 1949b). Hence it would appear that adult N. brasiliensis do rely upon energy gained from aerobic pathways for survival. Should these pathways be inhibited then the worm could be seriously weakened or even killed.

Adult worms are found burrowing deep into the mucosa of the small intestine. From measurements made by Rogers (1949a) the partial pressure of oxygen at this position varies in the non-infected rat from 7 mm. to 30 mm. mercury. It has been pointed out (Campbell, 1931) that the growth of fibrous tissue, inflammation and other pathological changes which lead to a decreased supply of blood also cause a fall in oxygen tension. In an infected rat it is conceivable that the oxygen tension may drop to a very low level. Under comparatively anaerobic conditions, the worms would soon die. A closer study of the oxygen tensions in the gut of an infected rat at the time of self cure is required. However, Ogilvie (1964b) has reported that the adult worms are not expelled by the gross changes in the integrity of the gut brought about by anaphylactic shock of rats sensitised to ovalbumin-H. pertussis antigen.

It is felt, however, that the above experiments do not afford sufficient information to state catagorically that immune serum inhibits the aerobic respiratory mechanism of the adult worm. Where oxygen consumption was reduced, this may have had nothing to do with a specific inhibition of oxidative metabolic pathways, but merely indicated that a higher proportion of the worms were dead or moribund. More will be said concerning this matter in the general discussion of this Chapter.

Summary

(1) The *in vitro* oxygen consumption of adult *N. brasiliensis* while suspended in 1 of 3 media (normal rat serum, immune rat serum or phosphate buffer solution) was determined in a Warburg respirometer.

(2) Measurements were taken over an initial 3 hour period and after overnight (20 hours) exposure in the suspending medium, for a final 3 hour period.

(3) A significant reduction in the endogenous oxygen consumption during the final period was found with worms suspended in immune serum, when compared to worms in phosphate buffer solution and normal serum.

(4) The presence or absence of CO_2 in the atmosphere or glucose as substrate did not affect oxygen uptake.

(5) The experiments were repeated with a gas phase containing only 3% oxygen. This represented the pO_2 that the adult worms are most likely to encounter in the rat's small intestine.

(6) Again a reduction in the endogenous oxygen consumption was found during the final period with worms suspended in immune serum when compared with worms suspended in phosphate buffer solution. However, under these conditions of reduced oxygen availability there was little difference in the respiratory rates between worms in normal or immune serum.

(7) The addition of glucose gave a slight increase in respiratory rate during the initial period. This increase was more marked over the final period in the case of worms in normal and immune serum. With the latter the increase was of such an extent that it abolished the difference between the endogenous oxygen uptake of worms in immune serum and phosphate buffer.

(2) The effect of immune serum upon the phosphate incorporation by adult N. brasiliensis

Introduction

Radiophosphorus has been extensively used in turnover studies of mammalian tissues and organisms such as yeast and virus (Hevesy, 1947). von Brand (1952) has mentioned the possibility of applying this technique to problems associated with the physiology of parasitic worms. Adult N. brasiliensis rely for survival to some extent upon energy derived from glycolysis of carbohydrates. Should humoral antibody interfere with this metabolic activity then this could be detected by abnormal utilisation and incorporation of labelled inorganic phosphate.

Methods and materials

(a) Isolation and preparation of adult worms

Adult *N. brasiliensis* were obtained from the gut of rats 10 days after a primary infection of 3,000 larvae. The worms were washed, counted and prepared as previously described.

The worms were incubated in 1 of 3 media, normal rat serum, immune rat serum and Krebs-Ringer phosphate buffer. The 3 media were similar to those used in the experiments investigating the *in vitro* oxygen consumption of adult *N. brasiliensis*.

(b) Isotopic methods

Radiophosphorous was obtained from the Radiochemical Centre, Amersham, as carrier free labelled potassium dihydrogen phosphate ($K.H_2{}^{32}P\ O_4$). 20 micro-curies of the isotope were added per ml. of medium. After addition of radiophosphate an aliquot was removed and the specific activity was calculated for each medium (counts per 100 second per milli-micromole of orthophosphate).

Radioactivity measurements were carried out in an M.6 liquid counter connected to a Panax scaler.

(c) Inorganic phosphate determinations

Orthophosphate concentrations in the samples were determined by the method of Martin and Doty (1949) which is a modification of the Berenblum and Chain (1938) procedure.

(d) Incubation procedure

1,000 freshly isolated washed adult worms were placed in 3.0 ml. of the appropriate medium in a 15 ml. test tube and placed in a roller drum for 20 hours at 37°C. The pH of each incubation medium was 7.4 and the atmosphere in each tube was air. Antibiotics were added giving a final concentration of 250 units Penicillin and 25 mgm. Streptomycin per ml. As a control, dead worms, killed by freezing overnight, underwent the same experimental procedure. These controls gave an estimate of any non-specific uptake of phosphate and this was subsequently deducted from the values gained with living worms.

(e) Determination of the acid soluble organic phosphate fraction

At the end of the incubation period the worms were washed thoroughly with ice cold saline, until no residual radioactivity was detected in the washings. Each sample of worms was finally suspended in 3.0 ml. of cold distilled water, and homogenised using a Potter-Elvehjem pestle homogeniser followed by several short bursts in a M.S.E. Ultra-sonic disintegrator. During these operations the samples were kept cold. The acid soluble phosphate fraction was isolated by the method of Nielsen and Lehninger (1955) summarised briefly as follows:-

An aliquot of 1.0 ml. of the worm homogenate was removed from each sample and the total nitrogen content measured. Another

1.0 ml. was removed from each worm homogenate and 0.4 ml. of a 25% Trichloroacetic acid solution made 0.05 M with respect to KH_2PO_4, added. The T.C.A. extracts the acid soluble phosphate fraction. 0.6 of this T.C.A. soluble fraction was extracted twice with water saturated benzene--isobutanol (v/v) solution, following addition of 0.8 ml. of 10% ammonium molybdate, to remove inorganic phosphate. After this treatment the aqueous phase, containing the acid soluble organic phosphate fraction, was free of inorganic phosphate. A volume giving a suitable count rate was quantitatively removed from the aqueous layer.

It was possible to calculate the amount of orthophosphate in milli-micromoles taken up by 1.0 ml. of the original worm homogenate from the following equation:-

$$\text{Orthophosphate uptake per 1.0 ml. worm homogenate} = \frac{B}{A} \times \text{total vol. of acqueous phase} \times \frac{b + a}{c} \text{ milli-micromole}$$

where B = counts per 100 seconds of 1.0 of the acid soluble organic phosphate acqueous phase

A = specific activity of the medium (i.e. counts per 100 second per milli-micromole orthophosphate contained in the respective medium)

b = 1.0 ml. (volume of original aliquot)

a = 0.4 (volume of T.C.A. added)

c = 0.6 (volume of acid soluble phosphate fraction used).

Figure 14

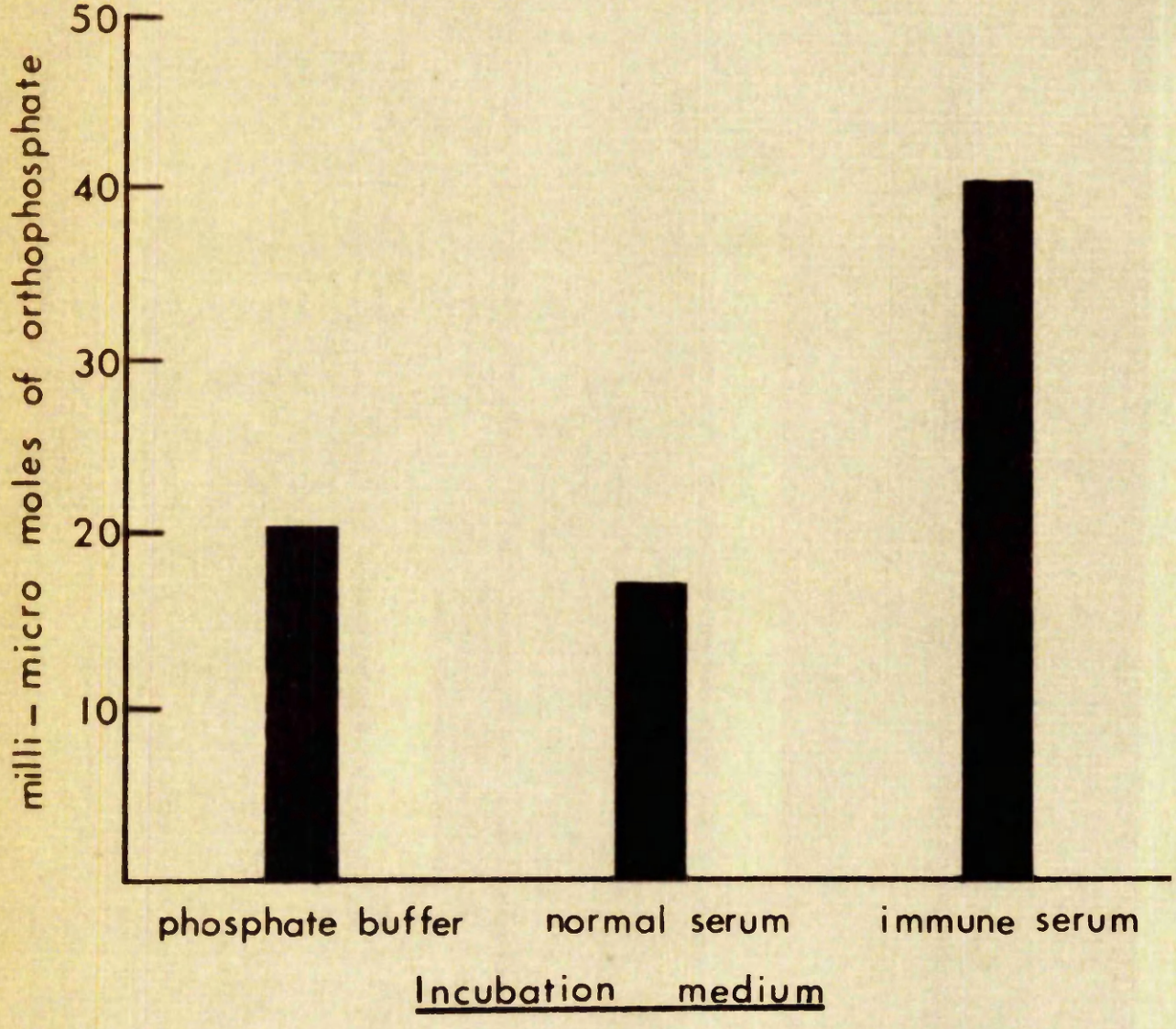


Figure 14. Amount of inorganic phosphate incorporated into the "acid soluble organic phosphate fraction" of adult worms after 20 hour incubation in various media.

From a knowledge of the total nitrogen content of 1.0 ml. of the original worm homogenate, it was possible to calculate the number of milli-micromoles of orthophosphate taken up per milli-gram worm tissue nitrogen over the 20 hour incubation period.

Results and discussion

In this experiment an estimation has been made of the amount of inorganic phosphate incorporated into the 'acid soluble organic phosphate' fraction of worms suspended in different media. The 'acid soluble organic phosphate' fraction isolated by cold T.C.A. extraction contains almost all the phosphorylated esters involved in the Embden-Meyerhof pathway of glycolysis (Umbriet et al, 1957). Over the incubation period it appeared that a greater amount of labelled inorganic phosphate was incorporated into the 'acid soluble organic phosphate' fraction of adult N. brasiliensis incubated in immune serum than in either of the other 2 media (fig. 14). A possible explanation for this phenomenon is that some factor present in immune serum and not present in normal serum or Krebs-Ringer phosphate buffer caused inhibition of glycolysis leading to a subsequent increase in phosphorylated intermediates. It is probable that antibody may be the factor involved.

Bueding (1949) reported that under aerobic conditions the filarial nematode Litomosoides when incubated in a medium containing ^{32}P labelled inorganic phosphate, incorporated at least 3.5 times more ^{32}Phosphate into the 'acid soluble organic phosphate' fraction in the presence of glucose than in its absence. All 3 media in the experiment described here were standardised with respect to glucose content, glucose being estimated by the glucose oxidase method (Huggett and Nixon, 1957). Hence the greater uptake of phosphate by worms incubated in immune serum was not due to a greater glucose concentration in that medium.

Mansour and Bueding (1954) studied the effect of trivalent antimony compounds on glycolytic enzymes of Schistosoma and showed that these drugs inhibit phosphofructokinase activity. Later Bueding and Mansour (1957) demonstrated that the addition of purified mammalian phosphofructokinase to homogenates, poisoned with trivalent antimonials, reversed the inhibition. From this it was taken that the chemotherapeutic effect of trivalent antimonials in schistosomiasis can be attributed to the inhibition of parasite phosphofructokinase, mammalian phosphofructokinase being relatively unaffected by the drug. Massey and Rogers (1950) stated that phenothiazine is an inhibitor of phosphatases. Hence it would appear that some anthelmintics have an inhibiting effect upon certain metabolic pathways of the parasitic worm.

While the mechanism remains in doubt, it is clear that the phosphate incorporation of N. brasiliensis incubated in immune serum is different from that of worms incubated in normal serum and Krebs-Ringer phosphate buffer.

Summary

(1) Adult worms were incubated for a 20 hour period in either immune serum, normal serum or phosphate buffer solution, to which had been added ^{32}P labelled inorganic phosphate.

(2) The amount of phosphate incorporated into the worm's acid soluble organic phosphate fraction was determined for each medium. It was found that those worms suspended in immune serum incorporate a significantly greater amount of inorganic phosphate than did worms suspended in normal serum or phosphate buffer solution.

General discussion

The basis of the immune mechanism to parasitic worms has been postulated to be "anti-enzyme" in character (Chandler, 1953). This has been substantiated by Thorson (1956) who noted that oesophageal gland extracts of hookworms possessed proteolytic activities which could be inhibited by immune serum. Soulsby (1957) has considered this to be "external" enzyme inhibition by immune serum. He has also pointed out that the inhibition of

"internal" enzymes, those responsible for the life processes of nematodes, may also be involved in immune mechanisms.

In the above experiments it was found that after 20 hours exposure to immune serum under a gas phase of air adult worms showed a significantly greater reduction in respiratory rate than did worms suspended in normal serum or Krebs-Ringer phosphate (table 14). After the same period of time under a gas phase containing only 3% oxygen worms suspended in immune serum showed a reduced respiratory rate when compared with worms in Krebs-Ringer phosphate buffer. However, under these conditions there was no significant difference between worms in normal or immune serum (table 17). On adding glucose to each of the 3 media it was found that there was now no difference in respiratory rate (table 18).

After incubating adult worms for 20 hours in the 3 media, containing ^{32}P labelled inorganic phosphate, it was found that those worms suspended in immune serum incorporated into their "acid soluble organic phosphate" fraction, about double the amount of inorganic phosphate than did worms suspended in normal serum and Krebs-Ringer phosphate buffer.

A possible explanation for these results could be that antibody contained in immune serum, caused partial inhibition of the metabolism of glucose. As a result of such inhibition the oxygen requirement would be reduced, and an accumulation of

phosphorylated intermediate compounds could occur. The greater availability of glucose, made possible by adding this substrate to the media, may have helped to overcome the inhibitory effect of antibody, hence restoring the normal rate of oxygen consumption.

Great care must be taken with the interpretation of results obtained from the type of experiment described in this Chapter. It must be recognised that, during these *in vitro* studies, the adult worm has been removed to an unnatural environment. Under these conditions it is inevitable that from the moment of removal from the gut the worms are in a process of dying. This alone could have far reaching effects upon the normal physiological processes of the worm, and attempts to measure a specific interference in the worm's metabolism by immune serum are made difficult. It may be possible to overcome this difficulty in 2 ways.

Firstly, it may be better to utilise worm homogenates when investigating anti-metabolic effects possessed by immune serum. However, such an approach brings with it the difficulty of relating findings obtained with homogenates to the situation in the intact viable worm. Despite this such studies should be carried out in parallel with experiments on the living worm.

Secondly, successful *in vitro* culture of the parasitic worm would eliminate the complicating factors caused by the worms

dying. Studies on the worm's physiology and effects thereupon by various substances could be carried out. It is unlikely that the metabolic activity of adult worms maintained in this way should differ markedly from that of worms in their natural environment.

The appearance of precipitates around adult N. brasiliensis on in vitro incubation in immune serum have been reported (Taliaferro and Sarles, 1939). Similar precipitates forming around the body orifices of larvae have been shown to be the result of an antibody-antigen interaction (Jackson, 1960). Whether the materials which arise from the body openings of worms are "external" enzymes is not known. It is conceivable that they may be waste end products from the "internal" enzyme moderated metabolic systems and whether an antibody against such products will inhibit the precursors is a matter of conjecture.

However, it is possible that the presence of a cap of precipitate at the body orifices of the worm may seriously debilitate it. This adverse effect may in turn seriously interfere with the worm's physiological processes. Hence it is quite possible that the differences in oxygen consumption and phosphate incorporation obtained with worms suspended in the 2 media used in the experiments described above may have resulted from a non-specific effect of this nature. It has

been suggested, for example, that precipitates occurring on the vulva of worms mechanically obstruct the passage of eggs, and similarly precipitates on the excretory pore block the excretory system (Culbertson, 1941).

The experiments described in this Chapter have been concerned with the effect of a serum factor upon the metabolism of the worm. Cell or cell extracts from the normal or resistant host were not employed. It is quite possible that cell bound antibodies are operative in the immune expulsion. Experiments investigating the effect of cellular components as well as serum factors, upon the worm's metabolism are worthy of attention. In the following Chapters experiments, designed to investigate the presence of cell-fixed antibody specific to the parasite, are described.

CHAPTER XI

Studies on the presence of cell fixed antibody using ^{131}I trace labelled adult worm antigen

Introduction

It has been suggested by Urquhart et al, (1965) that the expulsion of a primary infection of adult N. brasiliensis, from the small intestine of the rat may result from a specific immediate hypersensitivity in the tissues of the gut to worm antigen.

It is thought that before antibodies can participate in an immediate hypersensitivity reaction they must not only react with specific antigen but also be attached to tissues (Ovary and Karush, 1961). It also appears that, in some animal species, the participation of complement seems to be important to the occurrence of hypersensitivity reactions of the immediate kind (Osler et al, 1959). A specific affinity of antigens for certain cells from lymphoid tissue and spleens of immunised animals has been demonstrated using the Coons fluorescent antibody technique (Coons et al, 1955) and by the specific adsorption of ^{131}I trace labelled antigen by these cells (Boyden and Sorkin, 1960).

The following experiments were carried out to find if antigen prepared from adult worm tissue, would be specifically adsorbed by cells obtained from rats highly refractory to further infection

with N. brasiliensis, and by cells from normal rats which had been pretreated with serum obtained from highly immunised rats. The trace labelling technique with ^{131}I was used to measure antigen adsorption.

Methods and materials

(a) Antigen preparation

Adult N. brasiliensis worms were obtained from rats on the 10th day following larval infection. The adult worms were washed with sterile saline and finally suspended in saline, giving 1,000 worms per ml. The worms were then homogenised using a Potter-Elvehjem pestle homogeniser followed by several short bursts in an M.S.E. utra-sonic disintigrator. Particulate matter was removed by centrifugation. During these operations the worm sample was kept cold. This material is referred to as "antigen" although it is realised that it will contain a multitude of antigenic materials.

(b) Isotopic labelling of the antigen preparation

The protein nitrogen concentration of the antigen preparation was determined by the micro-Kjeldhal method. Labelling of the antigen preparation with ^{131}I was carried out by the iodine monochloride method of McFarlane (1958).

Labelled Antigen (preparation 1)

The total volume of this adult worm antigen preparation was 17 ml., containing a calculated 30-40 mgs. protein per ml.

8.5 ml. of glycine buffer (pH 9.0)* was added to the 17 ml. of antigen preparation. 0.5 ml. of I Cl solution (containing 0.42 mg I/ml. as I Cl) with 12 uc. ^{131}I as NaI (thiosulphate-free) was added to 8.5 ml. of glycine buffer (pH 8.5)** and immediately added to the buffered antigen solution, in a conical flask. The mixture was swirled for a minute then transferred to a dialysis sack and dialysed overnight against a large volume of cold isotonic saline. The saline was changed and dialysis continued for a further 24 hour period. The labelled antigen preparation was then removed from the dialysis sack centrifruged at 2,500 r.p.m. for 20 minutes and stored at -20°C. until required.

The nitrogen content of a measured aliquot was determined by the micro-Kjeldhal procedure.

(c) Preparation of cell suspensions

Spleens from normal rats and from rats which had been given 4 larval infections of 3,500, 5,000, 7,500, 10,000 at fortnightly intervals were obtained and each treated as follows:-

The spleen was cut with scissors into fragments of about 2.5 mm., and these were immediately immersed in Medium I (10% normal rat serum in Eagle's medium). The tissue fragments were pressed through nylon with a pestle, taken up and down into a syringe several times, and were then washed twice in Medium II

* pH 9.0 buffer: 8 ml. M-glycine in M/4 Na.Cl. + 2 ml. N - Na.OH.
** pH 8.5 buffer: 9 ml. M-glycine in M/4 Na.Cl. + 1 ml. N - Na.OH.

(5% normal rat serum in Eagle's medium). The cells were finally suspended to 20% in Medium I (1 volume of packed cells + 4 volumes of Medium).

Small intestines were obtained from normal and immune rats and Peyer's patches removed using a sharp scalpel blade. A cell suspension was prepared as described for spleen tissue.

Following removal of the Peyer's patches the small intestines were slit longitudinally and the mucosa lightly scraped off in each case with a glass slide. A cell suspension was obtained as already described.

In this way cell suspensions of spleen, Peyer's patches and small intestinal mucosa were obtained from both normal and immune rats.

(d) Pretreatment of cell suspension with immune rat serum

In one experiment a cell suspension of small intestinal mucosa, from normal rats, was pretreated, with immune serum before incubation with labelled antigen.

1.0 ml. of immune serum was added to 1.0 ml. of the 20% cell suspension and the mixture kept in iced water for 1 hour, the tubes being shaken regularly every 10 minutes. The cells were finally washed 4 times with Medium II and resuspended in Medium I as before.

As a control this procedure was also carried out with normal serum.

(e) Incubation procedure

The following standard procedure was adopted for each cell suspension.

A volume of labelled antigen preparation containing 10 ugm. of antigen N was added to 1.0 ml. of each 20% cell suspension. The mixtures were then incubated at 37°C. for 1 hour, the tubes being shaken every 10 minutes. The contents of each tube were then washed 4 times with Medium II and finally suspended in 5 ml. saline.

(f) Isotope assay

Radioactivity determinations of samples of washings, antigen preparation and cell suspensions were carried out in a well type scintillation counter. Before counting each sample was made to a standard volume of 5 ml. in a counting tube. The efficiency of the counter was about 15% and all samples gave counts of at least twice and usually many times background.

Results

Experiment 1

Viable cell suspensions prepared from various tissues of both normal rats and rats highly immune to *N. brasiliensis* were incubated with antigen 1. prepared from adult worms. As the antigen preparation used was labelled with ^{131}I it was possible

Table 20

Adsorption of ^{131}Iodine trace labelled adult worm antigen by rat cell suspensions

Source of cell suspension	No. of determinations	Mean counts/second adsorbed
Normal rat spleen	4	349
Immune rat spleen	4	348
Normal rat Peyer's patches	2	78
Immune rat Peyer's patches	2	59
Normal rat intestinal mucosa	6	554
Immune rat intestinal mucosa	6	832

10 µgm of antigen nitrogen giving 4504 counts/second added to each cell suspension.

Table 21

Adsorption of ^{131}Iodine trace labelled adult worm antigen by rat cell suspensions pre-treated with either normal or immune rat serum

Description of cell suspension	No. of determinations	Mean counts/sec. adsorbed
Cells from the intestinal mucosa of normal rats pretreated with normal serum.	5	596
Cells from the intestinal mucosa of normal rats pretreated with immune serum	5	621
Cells from the intestinal mucosa of normal rats, no pretreatment	6	554
Cells from the intestinal mucosa of immune rats, no pretreatment	6	832

10 µgm of antigen nitrogen giving 4504 counts/second added to each cell suspension.

to measure the relative amounts adsorbed by the various cell suspensions. After incubation the cells were washed 4 times with Medium II. By following the amount of radioactivity present in the washings it was ascertained that all radioactivity, not bound to the cells, had been removed.

The results of this experiment are given in table 20. They show that cells obtained from the mucosa of the small intestine of rats immune to the N. brasiliensis adsorbed a significantly greater amount of antigen preparation than did similar cells obtained from normal rats. Neither spleen cells nor cells obtained from Peyer's patches showed a difference in antigen adsorption.

In a further experiment with the same antigen preparation cells obtained from the small intestinal mucosa of normal rats were pretreated with serum from highly immune rats. These cell preparations showed a similar adsorption capacity to that of normal cells both treated with normal serum and untreated, and did not exhibit the enhanced adsorption gained with cells from an immune rat (table 21).

Experiment 2

This was essentially a repeat of experiment 1, however, a different antigen preparation was used. Despite the fact that this antigen preparation was made in an exactly similar manner

Table 22

Adsorption of ^{131}Iodine trace labelled adult worm antigen by rat cell suspensions

Source of cell suspension	No. of determinations	Mean counts/second adsorbed
Normal rat spleen	2	1674
Immune rat spleen	2	2247
Normal rat Peyer's patches	2	1309
Immune rat Peyer's patches	2	1069
Normal rat intestinal mucosa	6	2424
Immune rat intestinal mucosa	6	2280

10 µgm of antigen nitrogen giving 7837 counts/second added to each cell suspension.

to the earlier one, there was a distinct difference both in the nitrogen concentration and in the specific activity.

The results contained in table 22 indicated that there was no difference in adsorption of the labelled antigen by similar cell preparations from normal and immune animals.

Discussion

In one set of experiments it was shown that a cell suspension prepared from the intestinal mucosa of rats with a strong acquired immunity to re-infection with N. brasiliensis took up and held quite firmly a greater amount of ^{131}I labelled adult worm material (called adult worm antigen) than did similar cells obtained from normal rats.

The main problem in interpreting results of this kind is the extreme heterogenity of the antigen preparation. The exact chemical composition of the adult worm material was not known. It is probable that a great variety of molecules present in this crude aqueous worm extract would have become labelled with ^{131}I and probably with widely different specific activities. The way in which the labelled worm material was taken up and held by the cell preparations cannot be stated definitely. It is not known to what extent small labelled molecules were adsorbed to the cell surface. Non-specific uptake, by the cells, of labelled material

was high and, indeed, may have masked any specific antibody-antigen adsorption in the final experiments where no greater uptake by mucosa cells from immune animals compared to similar cells from normal rats was found. It should be noted that the worm antigen material in these final experiments had a much higher specific activity than the worm antigen material in the earlier experiments. It was also impossible to assess the relative concentrations of antigen and antibody in these experiments.

Crude aqueous adult worm extracts when injected intravenously to highly immune rats resulted in a severe anaphylactic shock (Urquhart et al, 1965). Hence this crude adult worm aqueous extract must contain antigens to which the host becomes sensitised during infection. It is generally accepted that immediate hypersensitivity, including anaphylaxis and spontaneously occurring atopies are a consequence of the interaction of antigen with antibody when the latter is suitably adsorbed on to tissues and, in some instances able to fix complement (Raffel, 1963).

Despite the failings already mentioned inherent in the experimental procedure, it was shown in the earlier experiments that intestinal mucosa cells from immune rats took up more radioactivity than similar cells from normal rats. It may well be that this greater uptake of radioactivity by mucosa cells from immune animals was a result of the union of cell fixed

antibody with worm antigen. Boyden and Sorkin, (1960) reported that spleen cells obtained from animals immunised with human serum albumin (H.S.A.), adsorbed ^{131}I labelled H.S.A. when incubated together in vitro. It was found that adsorption was highest with cells taken from spleens of animals killed 5 to 6 days after a booster injection of the antigen. Humphrey and Mota (1959) immunised guinea pigs with rabbit or rat anti-H.S.A. antibody and with horse anti-diptheria toxoid. After 2 to 3 days the animals were killed, their mesenteries removed, washed and incubated with the appropriate ^{131}I labelled antigen. It was found that the rabbit and rat anti-H.S.A. gamma-glubulin antigens were specifically taken up by the corresponding mesentery when compared to controls. The evidence for specific uptake of labelled diptheria toxoid by mesentery sensitised with horse antibody was much less definite. These workers reduced non-specific uptake of ^{131}I H.S.A. by firstly "screening" the antigen by passage through normal guinea pigs. This procedure was not possible with diptheria toxoid which coincidentally gave a less clear cut result. Turk (1960) has reported slightly greater uptake of radioactive tuberculin by lymphoid cells of tuberculin-sensitive guinea pigs. As with the adult worm antigen used in the present experiments, Turk found that the mycobacterial antigens used in his system were adsorbed in considerable amount by normal cells, hence tending to mask any specific uptake.

More clear cut differences are found with antigens which display relatively little adsorption on to normal cells. Turk (1960) reported that lymphoid cells from animals with delayed-type hypersensitivity to bovine serum albumin (B.S.A.) induced by immunisation with picrylated B.S.A. took up considerably more labelled B.S.A. than do cells from normal animals.

Non-specific uptake of labelled antigen was a serious interfering factor in all experiments employing heterogenous antigen preparations with cell suspensions containing different cell types. How may non-specific uptake and retention be reduced in our parasite system?

It has been pointed out by Thorson (1963) and others that metazoan parasites which are composed of so many tissues possess a large number of potential antigens. However, it is quite likely that only a small proportion of these actually exert an immunogenic effect on the host during infection. Recent evidence has underlined the possible importance of the secretions and excretions (called metabolic antigens) of the living worm in this connection. Bearing this in mind, specific uptake and retention by cells from immune animals of labelled metabolic antigens may be more clear cut and definite. In future work it may be wise to attempt to fractionate the antigen preparation and perhaps isolate and label the immunologically important

fractions. These could be identified by skin testing in an immune animal. Humphrey and Mota (1959) studying antigen uptake by sensitised mesentery reduced non-specific uptake by reducing exposure time of the 2 components to 1 minute.

Mucosa cells from normal rats pretreated with immune serum showed no increase in radioactivity uptake when compared to non-treated normal mucosa cells. However, it has been shown elsewhere in this thesis that similar immune serum when administered in fairly large doses conferred some degree of resistance passively.

Evidence has recently been put forward implicating reagin-like antibodies in immunity acquired by rats to infection with N. brasiliensis (Ogilvie, 1964). If this is true, then it may be that the level of such antibodies in the circulation of a highly immune rat may be small at any one time. This has been recognised with human reagins associated with the spontaneously occurring atopies such as asthma and hay fever (Stanworth, 1963).

Hence it may be, in the above experiment, that the volume of immune serum used to pretreat the normal mucosa cell suspension did not contain sufficient antibody. As a result any specific uptake and retention of labelled antigen which may have resulted from "fixed" antibody could have been so small that it was easily masked by the large non-specific uptake.

Summary

(1) Viable cell suspensions were preprepared from the spleen, Peyer's patches and intestinal mucosa of both normal and resistant rats.

(2) An antigen preparation made from freshly isolated adult *N. brasiliensis* was trace labelled with ^{131}I.

(3) The various cell suspensions were incubated with a standard quantity of labelled antigen preparation for 1 hour, after which they were thoroughly washed and the remaining activity determined.

(4) It was found that the mucosal cells from immune rats took up more radioactivity than did similar cells from normal rats. The other cell suspensions showed no difference.

(5) Mucosal cells from normal rats were pretreated with immune serum before incubation with labelled antigen. No greater uptake of radioactivity resulted when compared to non-treated normal cells.

(6) The cell suspensions contained many different cell types and the trace labelled antigen preparation was a crude aqueous extract of adult worms. The non-specific adsorption was high nevertheless it is felt that these experiments may indicate the presence of a specific cell-bound antibody associated with the small intestinal mucosa of rats resistant to *N. brasiliensis*. Such antibodies may play a role in the immune expulsion of the adult worm from the small intestine of resistant animals.

CHAPTER XII

General discussion and summary

The development of immunological studies in helminth infections has been reviewed on a number of occasions during the last 30 years (Taliaferro, 1929, 1940; Culbertson, 1941; Chandler, 1953; Urquhart *et al*, 1962; Soulsby, 1963).

In general the acquired immunity shown by a host, following previous parasitic infections, can be manifest in a number of ways, namely complete refractoriness to reinfection, complete or partial inhibition of both development and reproduction and complete or partial elimination of an existing infection. This last phenomenon has been the main subject of this thesis.

That acquired resistance could lead to the expulsion of all or part of an existing infection was first recognised by Stoll (1929) who recorded the phenomenon of "self cure and protection" in sheep exposed to *H. contortus*. This and the related phenomenon in Scottish hill sheep investigated by Morgan *et al* (1952) are effective and valuable mechanisms whereby the host can rid itself of part of its worm burden. These immune responses elicited by sheep to their gastro-intestinal parasites help to diminish clinical helminthiasis in a flock.

The immune expulsion of an existing infection has also been described for other helminth infections, among them T. retortaeformis in rabbits (Michel, 1952), T. spiralis in both rats and mice (Larsh and Race, 1954; Larsh, 1963) and N. brasiliensis in rats (Chandler, 1937).

N. brasiliensis, a small trichostrongyloid parasite of rats, has proved a very useful model system for the study of the mechanisms operating during the expulsion of adult worms from the gut of the host (Mulligan et al, 1965). There is no doubt that the self cure reaction is brought about by an immune response on the part of the host, and is not the result of a physiological change associated with ageing of the worm (Taliaferro and Sarles, 1937).

In the experiments described in this thesis it was shown conclusively that between days 10 and 20 following a primary larval infection, almost the entire adult worm population was expelled from the host's small intestine. Previously it was not known to what extent this immune state was due to the parenteral migration of larvae on the one hand and the presence of adult worms in the gut on the other, although it had been suggested that the adult stage alone is sufficient to stimulate the immune expulsion (Chandler, 1937). Chandler's hypothesis

was confirmed here by studying infections created by surgically transferring viable adult worms directly into the duodenum of previously uninfected rats.

In order to do these experiments it was necessary to develop a technique whereby reliably quantitative infections with adult worms could be carried out. Using this method it was possible to examine the immune elimination of adult worms in the absence of parenteral larval migration.

It was found that the pattern and kinetics of expulsion of adult worms between actively and passively immunised rats varied considerably. Distinct variation existed in the rate of expulsion of adult worms by rats possessing differing degrees of acquired immunity; i.e. it was more rapid with hyperinfected rats than with rats which had had only 1 previous infection. However, in both cases the onset of worm elimination was immediate and did not cease until all, or nearly all, the introduced infection had been expelled. There was little difference in the rate of the expulsion of adult worms in the case of rats which had had 1 previous infection, and rats which were undergoing self cure to a primary adult infection, however, expulsion was immediate with the former whereas, with the latter, expulsion did not begin until 9 to 10 days post infection.

With rats, passively immunised with large amounts of hyper-immune serum, no expulsion of the challenge adult worm infection took place over the first 2 days post infection (or the first 3 days after serum injection). However, adult worms were expelled over days 3, 4 and 5 post infection. The rate of expulsion from these rats was approximately half that of rats undergoing self cure to a primary infection. Furthermore, expulsion ceased over days 6, 7, 8 and 9 until the active immune response, brought about by the actual challenge infection itself, came into operation.

These different patterns in the time of onset and rate of expulsion of adult worms from rats of differing immunological status could be explained on the hypothesis that a hypersensitivity reaction in the gut is involved in the expulsion (Urquhart et al, 1965). For instance, the small intestine of a hyperinfected rat would be more highly sensitised than that of a rat which had experienced only 1 previous infection. Consequently, on challenge, the gut tissue reaction to the parasite in the former would be more violent than that of the latter.

However, the local anaphylactic reactions in the gut to worm antigens may not be the sole factor in expulsion. Recent work by Ogilvie (1964b) has shown that the gross changes in the integrity of the gut mucosa, brought about by inducing an

anaphylactic shock in rats with ovalbumin and H. pertussis, did not lead to any reduction in size of a current adult Nippostrongylus infection. Hence it appears that an acute inflammatory reaction in the gut per se does not lead to expulsion of the adult worm. The immune expulsion of adult worms may well be mediated by a specific effect or series of effects, directly upon the worm, by host antibody, either free or in association with cells. The differences obtained in the rate of expulsion of adult worms from rats of different immunological status may be a reflection of the availability of protective antibodies at the site of infection. Hence it was thought that the self cure reaction to N. brasiliensis may involve some debilitating effect of antibody directly upon the worm.

Chandler (1953) has maintained that immunity to parasites may well depend upon an anti-enzyme function possessed by antibody. In this connection great stress has been laid on the importance of the "metabolic products" produced by helminths and it is generally accepted that they might play an important role in stimulating immunity.

Soulsby (1957) has proposed that the anti-enzyme action of antibody could operate against "external" and "internal" enzymes. External enzymes are those involved in the feeding and migratory activities of the worm, while internal enzymes

are those concerned with the metabolic processes of the organism. Thorson (1956a) found that the oesophageal gland extracts of hookworms possessed proteolytic activities which could be inhibited by immune serum. This might represent external enzyme inhibition. Schwabe (1957) reported reduced endogenous oxygen utilisation in N. brasiliensis larvae exposed to immune serum and postulated that it might be due to the inhibition of enzyme systems regulating oxygen utilisation. This might represent internal enzyme inhibition.

Thus the expulsion of adult N. brasiliensis, at the time of self cure, might be mediated by inhibitory effects upon worm enzyme mechanisms brought about by antibody. Before the effect of immune serum upon the worm was investigated it was felt necessary to show that in an infected animal, serum in appreciable quantities could gain access to the adult worm at or near the time of self cure.

Experiments utilising red cells labelled with ^{51}Cr. demonstrated that infected rats suffered a loss into the gut of about 100 µl. blood per 24 hours due to the presence of a population of about 1,000 adult worms. Other studies using a macromolecule, polyvinyl pyrrolidone labelled with ^{131}I, indicated an increased gut permeability to large molecules in infected rats just before and during the time of self cure. This work showed

that, in an infected rat, conditions exist whereby greater than normal amounts of plasma protein and hence antibody can move from the vascular system into the gut.

The blood loss into the gut most probably resulted from haemorrhage associated with trauma inflicted by the feeding adult worm upon the host's gut mucosa. Not only is blood lost due to this action but is also presents a possible route for worm antigens to enter and sensitise the host. Acquired resistance to re-infection can be stimulated by infection with the adult worm in the absence of parenteral migration of larval stages. Hence it is possible that the metabolic products of the adult worm in the form of secretions and excretions are the important antigens.

It was recognised that the immune expulsion of adult worms might rely upon some debilitating effect of antibody acting directly upon the worm. In order to assess the importance a possible anti-parasite action of immune serum, it was necessary to study the effects of such serum upon the worm under *in vitro* conditions. Two approaches to this problem were followed. Firstly the effects of *in vitro* incubation in immune serum upon the adult worm's ability to become established on subsequent transfer to the gut of a 'clean' rat was investigated. Prior incubation in immune serum decreased only very slightly, the

number of worms becoming established, when compared with worms pre-incubated in normal serum. This result was disappointing, however it is feasible that the worms, on transfer to their natural environment, were able to recover from any possible adverse effects caused by the immune serum. For instance, precipitates formed around the worms during *in vitro* incubation could have been dislodged when the worms were transferred. Alternatively any interference in the worm's metabolic processes by immune serum during the 20 hour incubation which possibly weakened but did not kill the worm, may have been rectified on the arrival of the worms in the ideal environment provided by the small intestine of a non-resistant rat. In this connection it is interesting that Sarles and Taliaferro (1936) showed that if the "small brown" worms found in the intestine of immune rats 6½ days after infection were transferred to the duodenum of a normal rat, they completed development as shown by high egg production and the presence of normal adults at autopsy 8 days after transfer. Hence the effects of resistance upon the worm are not permanent, at least initially.

Secondly, attempts were made to demonstrate interference in the metabolic activity of adult worms suspended in immune serum compared with worms suspended in normal serum. Metabolism of the worms, and effects there upon by the 2 sera were judged by

2 types of measurements; the oxygen consumption and the inorganic phosphate incorporation of adult worms. Broadly speaking it appeared that worms, after 20 hours exposure to immune serum, consumed less oxygen and incorporated more inorganic phosphate than did worms exposed in a similar fashion to normal serum. There was also a suggestion that the addition of glucose to immune serum abolished the lower oxygen uptake found with worms in that medium.

It is attractive to think that these effects are a result of specific inhibition by antibody of energy producing metabolic pathways present in the worm's tissues. This would infer that the antagonistic mechanism of antibody upon parasite might be mediated through interference with "internal" enzymic pathways. However, the facts themselves do not provide sufficient evidence to confirm this hypothesis about the mode of action of antibody.

These effects obtained with worms suspended in immune serum may merely reflect the debilitating action of some other factor. For instance a plug of precipitate blocking the excretory system or some other body opening, could seriously damage the worm and interfere with its normal metabolism. In other words the apparent differences in oxygen uptake and phosphate incorporation might be secondary manifestations of a relatively simple effect. However, suffice to say that worms on in vitro incubation in

immune serum and hence exposed to antibody, were in some way affected by this treatment, when compared with worms similarly exposed to normal serum.

No study was made of the effect of serum plus cells from an immune rat, upon the in vitro metabolism of the adult worm. Evidence implicating the presence of specific cell bound antibody in the intestinal mucosa of hyper-infected rats was obtained. The presence of such antibodies strengthens the concept that hypersensitivity reactions may be important in the immune expulsion of this parasite. Resistance may depend upon the presence and action of antibodies firmly bound to cells at the site of infection. If this is so then failure to confer a strong resistance to re-infection by passive transfer of serum may be due to firstly, a low level of such antibodies in the serum of the donating rat and secondly to the "mopping up" of these antibodies present in the injected serum, at sites other than that involved in the immune reaction. Some evidence lending support to this concept was obtained. A closer study of the involvment of cell bound and reagin-like antibodies in acquired resistance is necessary.

A possible mechanism for the immune elimination of adult N. brasiliensis from the small intestine of the rat is described below.

Adult worms have been shown to be capable of providing adequate antigenic stimulation. The most important antigens are probably the secretory and excretory products of the organism, which gain access into the host through the feeding habits of the worm. As a result of this repeated sensitisation the tissues of the small intestine probably become hypersensitive. The hypersensitivity of the gut wall may at first, be local to the site at which the worm is feeding. However, as the worm changes its feeding position and the hypersensitivity of the gut wall becomes more general, then local anaphylactic reactions may well occur at any point at which the worm tries to feed. The increased gut permeability associated with this response would probably allow plasma proteins, and hence antibody, optimum access to the micro-environment of the worm. Such antibody, either free or in association with cells, or both, may seriously weaken, or even kill, the worm. Whether such effects are mediated by an external mechanism (e.g. the formation of precipitates at the body openings) or by an internal mechanism (e.g. the inhibition of metabolic pathways) has not yet been resolved. It is reasonable to assume that worms debilitated in such a way could be easily swept out of the small intestine.

A resistant rat could show this immune response rapidly, on re-infection, and immediately prevent adult worms from becoming established.

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APPENDIX

Figure 1

Days after infection	Number of adult worms	Eggs per gram faeces
6	-	4,000
7	-	22,000
9	-	27,000
10	1,225	24,000
11	-	29,000
12	-	16,000
13	-	2,000
14	600	20
16	-	0
17	75	0
18	-	0
19	-	0
20	0	-

Figure 3 and 4

1,000 adult worms transferred to each rat on day 0

Day after challenge	Number of worms recovered at autopsy			
	Group 1 (controls)	Group 2 (passively immunised)	Group 3 (single larval infection)	Group 4 (several larval infections)
1	500 ± 46	548 ± 90	388 ± 52	110 ± 90
2	495 ± 58	566 ± 69	196 ± 125	43 ± 6
3	500 ± 88	368 ± 103	77 ± 42	17 ± 13
4	512 ± 59	284 ± 66	10 ± 9	5 ± 4
5	526 ± 60	151 ± 97	28 ± 26	0
6	608 ± 88	166 ± 82	14 ± 11	-
7	355 ± 122	201 ± 216	38 ± 42	-
8	381 ± 64	173 ± 81	0	-

Figure 6

Day after injection of labelled r.b.c.	Mean counts/ml of blood/sec.	
	Infected rats	Non-infected rats
2	4,026	4,974
9	2,884	3,899
15	2,410	2,862
19	1,996	2,560

Figure 7

Volume of 'blood' in micro-litres present in 24 hour urine samples

Day after injection of ^{51}Cr red cells	Non-infected rats	Rats infected with 5,000 larvae in day 3
1	1297 ± 787	879 ± 331
2	256 ± 106	154 ± 55
3	115 ± 47	85 ± 32
4	219 ± 73	130 ± 25
5	169 ± 58	158 ± 45
6	173 ± 52	169 ± 49
7	181 ± 21	110 ± 57
8	174 ± 28	93 ± 49
9	100 ± 54	73 ± 27
10	125 ± 26	112 ± 16
11	134 ± 30	111 ± 27
12	115 ± 33	84 ± 33
13	123 ± 34	106 ± 43
14	114 ± 36	96 ± 22
15	113 ± 27	91 ± 34
16	98 ± 40	99 ± 18
17	114 ± 23	89 ± 26
18	131 ± 43	81 ± 16
19	97 ± 6	88 ± 32

Figure 8

Volume of 'blood' in micro-litres present in 24 hour faeces samples

Day after injection of ^{51}Cr red cells	Non-infected rats	Rats infected with 5,000 larvae on day 3
1	458 ± 440	523 ± 258
2	94 ± 51	145 ± 89
3	124 ± 83	81 ± 30
4	121 ± 52	61 ± 34
5	58 ± 30	60 ± 27
6	60 ± 21	90 ± 42
7	51 ± 29	130 ± 43
8	44 ± 30	170 ± 61
9	23 ± 20	125 ± 79
10	18 ± 10	125 ± 54
11	22 ± 12	118 ± 44
12	20 ± 13	116 ± 46
13	33 ± 22	100 ± 54
14	44 ± 16	184 ± 63
15	19 ± 8	76 ± 33
16	20 ± 14	115 ± 38
17	18 ± 6	112 ± 40
18	22 ± 14	66 ± 27
19	22 ± 9	58 ± 30

Figure 10

Day after infection	Mean % of injected P.V.P. present in intestinal contents and mucosa
2	4.5
3	2.1
4	3.6
5	4.6
6	4.2
7	3.0
8	8.1
9	5.9
10	5.6
11	6.0
12	5.0
13	5.6
14	6.1
15	5.2
17	5.6

Figure 11

Period after infection	Mean amount of P.V.P. (as % of injected dose) present in intestinal contents and S.I. Mucosa
day 2 to 7	3.7
day 8 to 14	6.5
day 15 to 17	5.4
Non-infected rats	4.3

Figure 12

Faecal egg counts and numbers of adult worms recovered from the S.I.

Day after infection	No. of rats	Faecal egg count total/244	Mean No. of adult worms/rat
2	6	0	0
3	5	0	0
4	6	0	417
5	6	45×10^3	848
6	6	104×10^3	736
7	6	114×10^3	810
8	6	126×10^3	620
9	5	109×10^3	870
10	5	97×10^3	710
11	5	58×10^3	850
12	6	49×10^3	640
13	6	31×10^3	720
14	6	0	160
15	6	0	50
17	5	0	0

Figure 14

Amount of inorganic phosphate incorporated into the 'acid soluble organic phosphate' fraction of adult worms whilst suspended for 20 hours in various media

Suspending medium	m.μ. moles phosphate incorporated per m.g.m. worm tissue nitrogen	No. of determinations
Krebs-Ringer phosphate buffer	21.1 ± 4.5	8
Normal serum	17.1 ± 3.9	8
Immune serum	44.9 ± 25.4	7

Immunological Studies on *Nippostrongylus brasiliensis* Infection in the Rat

A summary of a Thesis submitted for the degree of Doctor of Philosophy in the Faculty of Medicine of the University of Glasgow, by John Taylor McLaren Neilson.

The object of the work described in this thesis was to study certain aspects of the "self cure" of N. brasiliensis infections in the rat, with a view to throwing some light on the mechanism of immunity to gastro-intestinal parasites generally.

The starting point for the investigation was the immune expulsion of the adult parasites at the terminal phase of a primary infection. This was investigated in a more quantitative fashion than formerly and its rapidity and extent clearly demonstrated. It was shown that between days 10 and 20 following a primary larval infection almost the entire adult worm population was expelled from the host's small intestine. In order to study this reaction between host and adult parasite in an uncomplicated way, a reliable quantitative method had to be developed for the introduction of adult worms into the test animal.

A surgical technique was evolved which was safe and reliable and gave "takes" which were no less uniform than those resulting from infections with larvae.

Using the adult transfer method, the fate of parasites transferred to rats of different immunological status was studied and the "half-lifes" of these introduced populations measured. It was found that the pattern and kinetics of the expulsion of adult worms between actively and passively immunised rats varied considerably./

considerably. There was also a distinct difference in the rate of expulsion of adult worms by rats possessing differing degrees of acquired immunity, i.e. it was more rapid with hyperinfected rats than with rats which had had only one previous infection.

The most important question to be answered for this system is the mechanism of the immune expulsion. Parallel work by other colleagues in the department had indicated that local anaphylactic reactions in the gut might give rise to conditions which were "unsuitable" for the worms and thus lead to their elimination. It seemed to the author that the local anaphylaxis might only be one component in the expulsion mechanism and that the associated increased capillary permeability might allow plasma and, therefore, antibody, in quantity to come in contact with the parasite, and that the main effect might be due to "antibody v. parasite".

In order to try and assess the importance of any direct effect of antibody upon the adult worm in this system two parallel studies were conducted. The first was aimed at examining some of the ways in which antibody might come in contact with the worm in vivo. Experiments with red cells labelled with ^{51}Cr showed the extent to which this might occur due to blood sucking by the parasite or haemorrhage caused by it. Infected rats sufferred a loss into the gut of about 100 µl blood per 24 hours due to the presence of/

of a population of about 1,000 adult worms. The worms, however, did not ingest any significant amount of this blood. Other studies using a macromolecule, polyvinylpyrrolidone labelled with ^{131}I, indicated an increased gut permeability to large molecules in infected rats just before and during the time of self cure. This work showed that in an infected rat, conditions exist whereby greater than normal amounts of plasma protein and hence antibody can move from the vascular system into the gut.

The second part of the "antibody v. parasite" approach depends upon demonstrating that, if antibody does come in contact with the worm in quantity, it can do some harm. Again this was investigated in two ways. Firstly by incubating adult worms in immune serum before introducing them to the host, and secondly by studying the effects of immune serum on the in vitro metabolism of the worms. Broadly speaking it appeared that adult worms, after 20 hours exposure to immune serum, consumed less oxygen and incorporated more inorganic phosphate than did worms exposed in a similar fashion to normal serum. A variety of different ways for producing immune serum was included in this study.

The possible significance of cell-fixed or reagin-like antibodies in immunity to N. brasiliensis and the participation and importance of such antibodies in the passive transfer of resistance was studied. Attempts were made to demonstrate the presence of cell/

cell bound antibody in the mucosa of the small intestine and other tissues of resistant rats by *in vitro* incubation of viable cell suspensions of these tissues with a crude aqueous adult worm extract labelled with ^{131}I.