The immunological responses produced by mice and amphibians to spargana

by

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CONTENTS

SUMMARY

1. The immunological responses stimulated in mice and marine toads(Bufo marinus) by the spargana of the Australian form of spirometrid tapeworm were investigated.

2. Mice experimentally infected with these spargana for 10 or more days developed an immediate(anaphylactic) form of hypersensitivity to their parasites.

3. This hypersensitivity was demonstrated by intravenously or intracutaneously injecting the infected mice with a saline extract of spargana. Uninfected mice failed to respond to injections of this extract but they could be passively sensitised with serum taken from infected mice.

4. Serum from infected mice which was treated with the extract of spargana became partially or completely incapable of passively sensitising uninfected mice

5. Immunodiffusion methods were used to demonstrate the presence of precipitating antibodies to spargana in the serum of mice infected with spargana for three or more weeks. Evidence which suggested that spargana may produce 10 substances that can stimulate the production of detectable antibodies (precipitins) by mice was obtained.

6 Although the humoral response of mice to spargana was variable, it seemed that infections of many spargana stimulated the production of more antibody than infections of few spargana and that serum taken from mice infected with spargana for five or more weeks could be considered to be strong anti-serum.

7. Proteins, carbohydrates and traces of lipids were detected amoungst the electrophoretically separated constituents of the extracts of spargana. However, the tests used to detect carbohydrates and lipids failed to show that these substances were present in the bands of precipitate which formed when the standard extract of spargana reacted with serum from infected mice. It therefore seemed likely that most or all of the antigens produced by spargana were proteinaceous.

8. Spargana induced local pathological changes in the tissues of mice. Most spargana that had been in mice for three or more weeks were encapsulated. Many eosinophilic leucocytes and macrophages accumulated in the walls of capsules formed by mice around spargana.

9. Spargana induced no marked general pathological changes in mice. Mice infected with five or 10 spargana each gained weight at an abnormally high rate and mice infected with 20-27 spargana each showed no clear signs of illness.

10. Mice were highly and equally susceptible to primary and superinfections of mature spargana given per os but they were resistant to primary infections of procercoids given per os.

11. Approximately 20 per cent of procercoids injected intra-peritoneally as primary infections into mice developed into spargana. Evidence which indicated that mice developed an acquired resistance to superinfections of intra-peritoneally injected procercoids was obtained.

12. Some evidence which suggested that antibodies produced by mice to spargana may have a direct effect on procercoids and that the cells present in the peritoneal cavities of infected mice were sensitive to mature spargana was obtained. Whether or not the direct action of antibodies or sensitised cells was responsible for the acquired resistance of mice to procercoids could not be determined.

13. Some hypotheses which may account for the apparent differences in the immunogenicity of the spargana of the Australian and the North American forms of spirometrid tapeworms have been proposed and discussed.

14. Evidence which indicated that marine toads(Bufo marinus) produced precipitating antibodies and marked local reactions to naturally acquired infections of spargana was obtained. Some of the substances present in spargana seemed to act as antigens to both mice and toads.

15. Attempts to investigate the immunological responses produced by laboratory-bred amphibians(Limnodynastes tasmaniensis) to experimentally introduced spargana failed because the amphibians could not be infected consistently.

Statement required by the University of Adelaide
from candidates for the Degree of Doctor of Philisophy

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge and belief it contains no material previously published or written by any other person except where due reference is made in the text of the thesis.

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SECTION 1 INTRODUCTION

Spargana are parasites which live in the tissues of vertebrate animals. They are the plerocercoid larvae of pseudophyllidean tapeworms that belong to the genus Spirometra (Wardle and McLeod, 1952, p.592). Spargana have been known to parasitologists for more than 100 years. During this period numerous workers have studied some of the more common spirometrid tapeworms. However, attention has previously been concentrated mainly on the life histories and some of the physiological and morphological characteristics of these tapeworms and their larvae. The immunological relationships between larval spirometrids and their hosts either have been ignored or have been studied only superficially. This lack of interest in the immunological aspects of the relationship between spargana and their hosts

may have resulted from the fact that spirometrid tapeworms and their larvae are not very important from the medical or economic viewpoint. Nevertheless, the immunological aspects of the relationships between spargana and their hosts may be considered to be important from the academic viewpoint. The evidence to justify this belief is given in the following pages.

Because much of what has been written about spargana is either repetitive or is of no significance in the context of the present study, no reference has been made to a number of papers published by previous workers. Most of the omitted references may be found in publications by Iwata (1933), Mueller (1965a), Strauss and Manwaring (1954) or Wardle and McLeod (1952 pp.591-605).

It is also important to point out that apart from some observations related to the taxonomy of the genus Spirometra, all of the information presented in section 1 of this thesis was published at or before the time when the present study was started. The section was written in this way in order to show (1) why at first it seemed reasonable to assume that the topic of the study was worthy of investigation and (2) why it seemed the sparganа of the Australian form of spirometrid tapeworm were suitable parasites to use. Subsequently, it was found that at least the latter of these two assumptions was, perhaps, unjustified.

1.1 THE BIOLOGY OF SPARGANA AND SOME ASPECTS OF THE RELATIONSHIPS BETWEEN SPARGANA AND THEIR HOSTS

Before the biology of spargana is discussed, it is necessary to briefly comment on the taxonomy of the spirometrid tapeworms. According to Wardle and McLeod(1952, pp.591-605) the genus Spirometra contains seventeen species of tapeworms but there is considerable doubt about the validity of many, if not most, of these species. In response to a request for information on the taxonomy of the spirometrid tapeworms Mueller(1967) expressed the opinion that "the many 'species' described by Faust, Campbell and Kellogg and others have no validity whatever". Similar doubts about the validity of the species Diphyllobothrium reptans, D. okumuri, D. decipiens, D. ranarum and D. houghtoni were expressed by Iwata(1933) who proposed that all these 'species' represented varieties of the species D. erinacei. These comments were made by Iwata(1933) before the genus Spirometra was erected by Mueller (1937). At that time, spirometrid tapeworms were classified amoungst the diphyllobothriid tapeworms(see section 2.11)

How many species of spirometrid tapeworms exist is at present not known but even if the described species are valid, many of them resemble each other so closely that they can be identified only with difficulty. Furthermore, spargana of spirometrid tapeworms cannot be identified even tentatively on the basis of their

morphological characteristics (Wardle and McLeod, 1952, p.593). Thus many of the spirometrids used by previous workers may have been incorrectly identified or they could not be identified at all if they were in the form of spargana. If therefore seemed reasonable to omit specific names from the introductory discussion of the biology of spargana although in some instances the geographical origins of the spirometrids discussed have been mentioned. For example, the so called species Spirometra mansoni, S. erinacei, S. ranarum and S. decipiens are sometimes collectively referred to as Oriental spirometrid tapeworms whereas the apparently valid species Spirometra mansonoides is referred to as the North American spirometrid tapeworm. Spirometrid tapeworms were referred to in this way initially by Mueller (1965a). The taxonomy of the spirometrid tapeworms will again be discussed in section 2.11 and section 5.

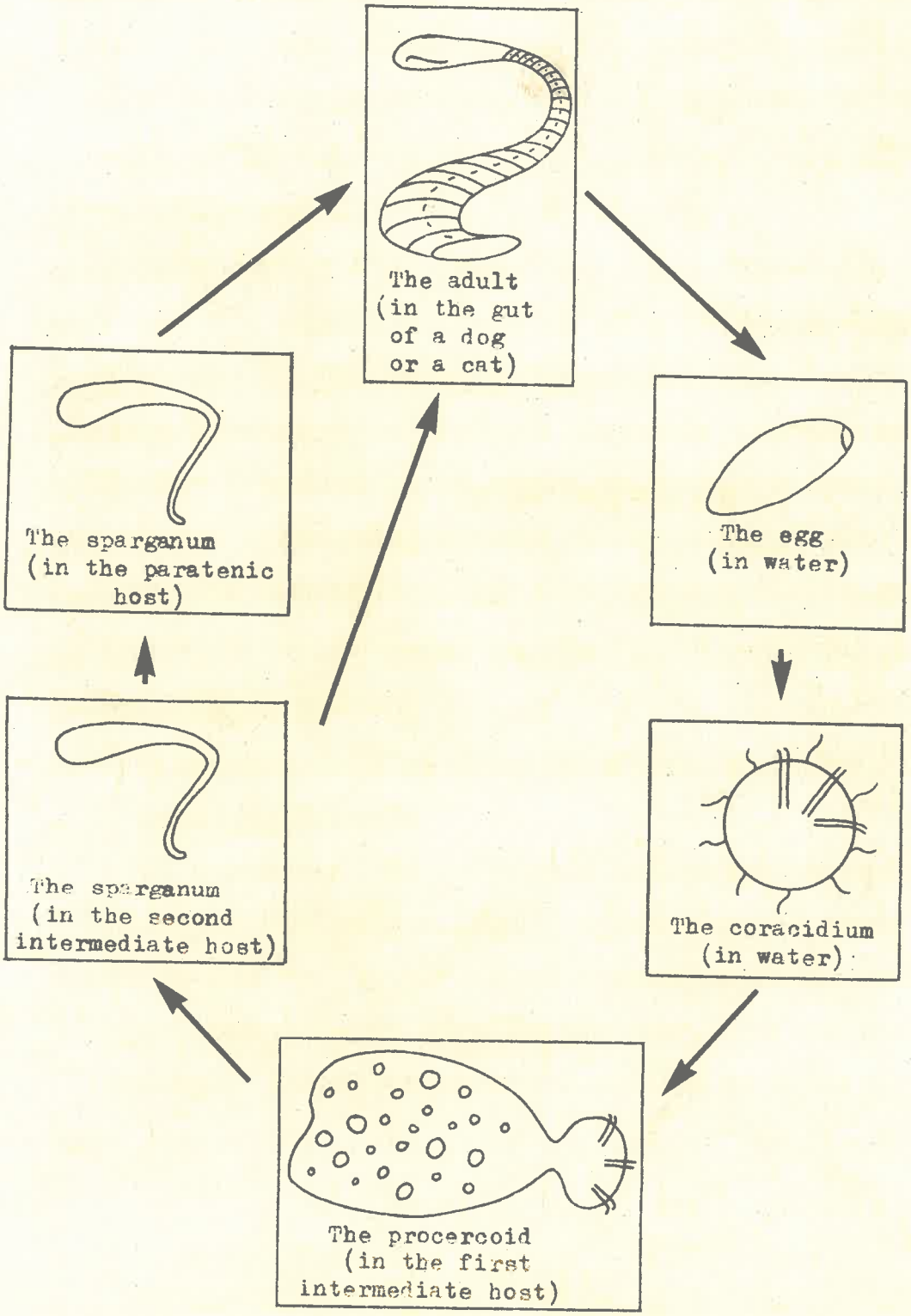
1.11 The position of the sparganum in the life cycle of a spirometrid tapeworm

The general form of the life cycle of a spirometrid tapeworm is shown by the diagram in Figure 1.1. This diagram is based on descriptions of life cycles published by Galliard and Ngu (1946), Li (1929), Okumura (1919) and Takahashi (1959a).

The adult tapeworms occur in the intestines of canine or feline carnivores. Fertilized eggs are released from the proglottids

Figure 1.1

The general form of the life cycles
of spirometrid tapeworms



of the adult parasite and pass to the exterior in the faeces of the host. Eggs which fall or are carried into water hatch after an incubation period of approximately 14 days to release ciliated larvae called coracidia. These coracidia actively swim through the water until they are eaten by a member of the family Cyclopidae or until they die.

Coracidia that are eaten by a cyclops lose their cilia and burrow through the wall of the intestine and into the haemocoel of the crustacean. In the haemocoel each coracidium grows into the next larval form, the procercoid. Cyclops therefore are the first intermediate hosts in the life cycles of spirometrid tapeworms.

If an infected cyclops is eaten by a suitable vertebrate animal, the procercoids leave the cyclops and burrow through the intestinal wall of the animal. Just before or during this stage of migration, each procercoid loses its cercomer and becomes a young sparganum. Each young sparganum passes through the wall of the intestine and enters the coelom of its host. The young sparganum then migrates to a site in the muscular or connective tissues of the host where it settles and grows into a mature sparganum. Animals that are infected with spargana in this manner are called second intermediate hosts.

The fate of a sparganum after it becomes established in a second intermediate host depends on what happens to that host. If the host is eaten by a suitable canine or feline carnivore, the sparganum matures into the adult tapeworm in the lumen of the carnivore's gut. If, on the other hand, the predator is not canine or feline, the sparganum usually penetrates the wall of its intestine and migrates to the muscular or connective tissues of the new host. Any animal which becomes infected with spargana by eating a second intermediate host is called a paratenic host.

The life cycle is completed when animals infected with spargana are preyed upon by canine or feline carnivores and the spargana mature to adult tapeworms in the intestines of these predators.

In the remaining part of the introduction, I will describe some observations made by workers who studied spargana and the relationships between spargana and their hosts before the present study was started.

1.12 The characteristics of spargana and some aspects of the relationships between spargana and their hosts

1.121 The morphology and behaviour of spargana

Descriptions, drawings and photographs published by Li (1929), Iwata (1934) and Mueller (1938a,b,1961) show that spargana are white, thread-like parasites and that each sparganum consists of a holdfast region (which is the precursor of the

holdfast of the adult tapeworm) and a tail region.

It has been shown that if the tail region is cut off a sparganum, the holdfast is able to regenerate a new tail (Mueller, 1963). The holdfasts of spargana apparently are able to produce tissues of the tail almost indefinitely with the result that spargana which have been in mice for approximately one year may be up to 400mm long (Mueller, 1961). The decapitated tail regions apparently are unable to regenerate new holdfasts when they are implanted in mice or amphibians (Takahashi, 1959a).

Spargana sometimes naturally lose pieces of their tails both in vitro in saline solution (Mueller, 1938a) and in vivo in amphibians (Joyeux, Baer and Martin, 1933) and mammals (Mueller, 1938b; Mueller and Coulston, 1941). Moreover, it has been shown that the whole tail region is lost when a sparganum burrows through the intestinal wall of a paratenic host (Kobayashi, 1931; Mueller, 1938b).

Spargana are able to survive for considerable periods in vitro in various culture media (Berntzen and Mueller, 1964; Mueller, 1959; Takahashi, Okamoto and Sonoe, 1959) although, during this period, the quantity of glycogen which is present in their tissues (Takahashi, 1959b) decreases with time (Takahashi, Okamoto and Sonoe, 1959).

1.122 The hosts of spargana

The results obtained by previous workers show that spargana are able to live in a wide variety of amphibians, reptiles, birds and mammals (see, for example, Bearup, 1948; Faust, Campbell and Kellogg, 1929; Houdemer, Dodero and Cornet, 1933; Johnson, 1911; Joyeux and Houdemer, 1928; Mueller, 1938a, b; Sanders, 1953; Yutuc, 1951). There have been a few reports of the failure of spargana to become established in some amphibians (Li, 1929; Mueller, 1938a) and birds (Joyeux, Houdemer and Baer, 1934; Mueller, 1938a) but it seems that only fish are consistantly resistant to infection (Faust, Campbell and Kellogg, 1929; Mueller, 1960). But even though most animals used in previous studies were found to be susceptible to infection with spargana some species of amphibians, reptiles and mammals seemed to be partially or completely resistant to infection with procercoids (Galliard and Ngu, 1946; Li, 1929; Joyeux, Houdemer and Baer, 1934; Mueller, 1938a, b; Takahashi, 1959a). Thus it is clear that a wide variety of animals can act as paratenic hosts and even second intermediate hosts to spargana and it seems that no species of vertebrate animals can be considered to be the normal host of spargana. However, Mueller (1963) pointed out that the usual natural hosts of spargana are water snakes and frogs and Galliard and Ngu (1946) proposed that the passage of spargana through amphibians is a necessary part of the life cycle

of a spirometrid tapeworm. Whether or not this means that amphibians or reptiles are the 'normal' hosts of spargana is uncertain.

1.123 The distribution amd migration of spargana within the bodies of their hosts

Spargana have been found in various sites in muscular or connective tissues of their hosts. In adult amphibians they appear to congregate mainly in the muscles of the thighs (Galliard and Ngu, 1946; Sanders, 1953; Yutuc, 1951; Kobayashi, 1930,1931) whereas in tadpoles they have been found mainly in the tissues of the orbit or the base of the tail (Galliard and Ngu, 1946). In rats and mice they have been found in the subcutaneous tissues of the nape of the neck (Mueller, 1938a; Sanders, 1953; Yutuc, 1951; Kobayashi, 1930, 1931; Takahashi, 1959a) or in the muscles or subcutaneous tissues of the thighs (Mueller, 1938a; Sanders, 1953) and in man they have been found in the muscles and subcutaneous tissues of the thighs (Tashiro, 1924; Sanders, 1954; Swartzwelder, Beaver and Hood, 1964) and the trunk (Mueller, Hart and Walsh, 1963; Read, 1952; Swartzwelder, Beaver and Hood, 1946; Brooks et al., 1960).

Some work has been done to find out what path migrating spargana take in various species of hosts. Kobayashi (1930) found that young spargana of the Oriental spirometrid tapeworm burrow through the intestinal wall and enter the coelomic cavity

of the frog. They then pass through the body wall in the peri-rectal region and enter the femoral muscles where they settle. Most of the spargana Kobayashi (1930) examined reached the femoral muscles within 12-27 days after the frogs that he used were first fed with procercoids. He also showed that, in the mouse, young spargana migrate from the coelomic cavity to the sub-cutaneous tissues of the thorax and from there to the sub-cutaneous tissues of the neck (Kobayashi, 1930).

Similar observations were made by Takahashi (1959a) who found that the holdfasts of spargana migrated through the walls of the stomach and duodenum when fed to mice and appeared in the peritoneal cavities of mice infected for only forty minutes. The spargana then migrated mainly to the subcutaneous regions of the shoulder,neck and head although some remained in the subcutaneous regions of the abdomen for example (Takahashi,1959a). These results suggested that spargana follow a more or less direct route to the sub-cutaneous tissues of the neck or perhaps the abdomen and thighs. Intermittent migration of spargana in man also has been reported (Mueller, Hart and Walsh, 1963; Swartzwelder, Beaver and Hood, 1964).

1.124 The longevity of spargana

It has been shown that spargana are able to live for long periods in at least some species of hosts. There is

circumstantial evidence that spargana can live in man for as long as 20 years (Strauss and Manwaring, 1964) and that they can survive in mice for as long as the mice live (Mueller, 1963). Both uninfected mice and mice infected with up to 10 spargana live for approximately the same length of time (Mueller, 1963). Mice infected with more than 10 spargana also may live for the normal length of time provided that the spargana remain in the sub-cutaneous tissues and do not invade vital organs or muscles.

1.13 The immunological responses stimulated by spargana

Up until the time when the present study was started relatively little information on the immunological aspects of the relationships between spargana and their hosts had been published. Furthermore, the immunological responses of only a few different kinds of hosts to spargana had been examined. Even so, it seemed that spargana stimulated at least some of their hosts to respond in a manner that could not be explained easily. The responses to spargana observed by previous workers are described in sections 1.131 and 1.132 and the peculiarities of these responses are discussed in section 1.2.

1.131 The humoral responses stimulated by spargana

Relatively few previous workers attempted to examine the humoral responses stimulated by spargana. Mueller and Chapman (1937) reported positive results for complement fixation tests

in which serum taken from Rhesus monkeys infected with spargana was used. Poor results however were obtained when similar tests were done with serum taken from experimentally infected human beings (Mueller and Coulston, 1941).

Mueller (1961) showed that precipitates formed on the surfaces and around the excretory pores at the ends of the tails of spargana which were placed in serum taken from mice described as being chronically infected. The length of time for which these mice were infected was not stated precisely. No such precipitates formed on the surfaces of spargana that were put into serum from uninfected mice. Mueller (1961) therefore tentatively suggested that these precipitates may represent complexes formed by the union of antigens released by spargana and the antibodies formed by mice to these antigens. Kajiwara (1960) observed that rabbits produced precipitins to spargana and he noted that the increase in the titre of precipitins produced was paralleled by an increase in the concentrations of the γ-globulin fraction of the serum of the infected rabbits. The responses of rabbits to spargana also were examined by Nakabayashi (1961) who showed that an Arthus response was stimulated when the tissues of sensitized rabbits were injected with the protein fraction of an extract of spargana. She (Nakabayashi, 1961) therefore presumed that the proteinaceous components of spargana contained antigens to which the rabbits had responded.

Mueller (1960) observed that precipitates formed on the surfaces of spargana that were put into serum taken from fish that had not been infected with spargana, and he suggested that this was a manifestation of the natural resistance that fish have to spargana. There was, however, no evidence to show whether or not this response was in any way specific.

1.132 The local and general pathological changes induced by spargana

A number of workers have described the pathological changes induced in human beings by naturally acquired infections of spargana (Capron and Brygoo,1960; Houdemer, Dodero and Cornet, 1933; Huang and Kirk, 1962; Read, 1952; Sanders, 1954; Short and Lewis, 1964; Strauss and Manwaring, 1964; Weinstein, Krawczyk and Peers, 1952; Wirth and Farrow, 1961) and to experimentally introduced spargana (Mueller and Coulston, 1941; Mueller and Goldstein, 1939). These descriptions indicated that spargana induce local cellular responses in which neutrophilic leucocytes, macrophages, eosinophilic leucocytes, plasma cells and possibly lymphocytes and giant cells may be involved. The sparga examined frequently were enclosed in fibrous capsules (Mueller and Coulston, 1941; Read, 1952; Tashiro, 1924). In some instances, external signs of infections such as local inflammation, oedema and giant urticaria were seen (Mueller and Coulston, 1941; Swartzwelder, Beaver and Hood, 1964; Tashiro, 1924) but on the other hand, such

signs often were absent (Swartzwelder, Beaver and Hood, 1964). Human beings naturally infected with relatively few spargana generally showed few or no signs or symptoms of systemic pathological changes (Swartzwelder, Beaver and Hood, 1964) but it has been reported that human beings experimentally infected with only two spargana may feel generally ill and depressed especially when it seemed that the spargana were migrating (Mueller and Coulston, 1941). It also has been suggested (Mueller and Coulston, 1941) that the number of eosinophilic leucocytes in the blood of infected human beings rises when spargana migrate. On the other hand, infections of large numbers of spargana may be highly pathogenic (Stiles, 1908) and may cause death (Tashiro, 1924). In such cases spargana may cause extensive necrosis of tissues and an elephantiasis-like condition (Tashiro, 1924).

Rhesus monkeys and rats often produce local inflammatory responses to spargana and may enclose them in fibrous capsules (Mueller, 1938a,b) and it seems that monkeys given sensitising infections of macerated spargana respond to their infections of spargana more strongly than unsensitised monkeys (Mueller and Chapman, 1937). An elephantiasis-like condition may develop in the tissues of Rhesus monkeys chronically infected with numerous spargana (Mueller, 1938a,b).

Some observations on the responses produced by mice to the spargana of the North American form of spirometrid tapeworm were

reported by Mueller (1938a) who stated that he found "extensive galleries filled with caseous material and areas of discoloured necrotic tissues" in mice infected with spargana for more than six weeks whereas mice infected for less than six weeks produced little or no reaction to the spargana that they contained. Mueller (1938a) also observed that mice infected with spargana for less than six weeks showed signs of illness such as emaciation and inflammation of the eyes but that these signs later disappeared. However, observations described in a later paper published by Mueller (1963) contradicted these earlier observations and suggested that spargana are not highly pathogenic to mice provided that they do not damage tissues of vital organs by migrating extensively through them. Some mice infected with as many as 150 spargana were able to survive for more than one year without showing signs of illness provided that the spargana remained in the subcutaneous tissues of the mice (Mueller, 1963). The subcutaneous tissues in which these spargana were located apparently showed few or no signs of local pathological changes and the spargana were either not encapsulated or were enclosed in only flimsy capsules (Mueller, 1963). Furthermore, mice infected with only up to 10 spargana gained weight at an abnormally high rate and showed no signs of becoming emaciated (Mueller, 1963).

Galliard and Ngu (1946) found that spargana of the Oriental form of spirometrid tapeworms usually weaken tadpoles and prevent

them from metamorphosing but Yutuc (1951) succeeded in rearing tadpoles infected with these spargana into frogs.

1.2 DISCUSSION

When the present study was started it was clear that many aspects of the relationships between spargana and their hosts were worthy of further investigation. This appeared to be especially true of the immunological aspects of these relationships. Although little was known about the immunological responses stimulated by spargana in their various hosts, there seemed to be some peculiarities in the manner in which some hosts responded or apparently failed to respond to spargana.

In a review of the literature on the immunological relationships between parasites and their hosts Soulsby (1962) commented that the 'larval stages of cestodes infecting various animals usually evoke a marked antibody response' The immunogenicity of larval tapeworms will be discussed in section 5 of this study. For the present, however, it is sufficient to point out that the results of previous studies have indicated that larval cestodes which inhabit the tissues of mammals are usually highly immunogenic.

Whether or not spargana were immunogenic to their hosts was difficult to estimate. However, some observations made by previous workers at least suggested that spargana seemed to be only weakly immunogenic to some hosts such as mice whereas they were more immunogenic to other hosts such as rats, monkeys and human

beings. This hypothesis was based largely on observations concerning the pathological conditions induced by spargana.

The pathological conditions induced by parasites frequently are associated with the development of an immunological response in the form of a state of hypersensitivity in the host of the parasites (Sprent, 1963, p.26). The immediate form of hypersensitivity is commonly induced in mammals by metazoan parasites (Boyd, 1956, p.125; Sprent, 1963, p.88) and it seems likely that spargana induced this form of response in their mammalian hosts. One characteristic of a parasite induced hypersensitive state is that eosinophilic leucocytes usually accumulate in the tissues around the parasites and that there may be an increase in the number of eosinophilic leucocytes in the blood of the infected animal (see section 3.3). The local cellular responses produced by animals that develop this form of hypersensitivity also usually involve other cells such as plasma cells (Litt, 1964) and giant cells (Bohrod, 1954), the latter being formed by the fusion of groups of macrophages which commonly occur in chronic inflammatory lesions (Florey, 1962). The local cellular responses described by for example previous workers who examined the reactions of human beings to spargana (section 1.132) clearly are of the kind produced when an antigen to which a mammal has developed an immediate form of hypersensitivity is introduced into the tissues of the mammal. Moreover, it has been

found that there is an increase in the number of eosinophilic leucocytes in the blood of infected monkeys and mice (Mueller, 1938b) and rabbits (Ariyoshi, 1930; Toyoda, 1933) so these hosts also may develop an immediate form of hypersensitivity to spargana. The observation that antigens of spargana induce an Arthus response when injected into the skin of sensitised rabbits (section 1.131) further supported the proposition that spargana induce an immediate form of hypersensitivity in rabbits.

An antigen which induces an immediate form of hypersensitivity in a mammal usually stimulates the production of antibodies which can be found in the serum of the hypersensitive animal (Gray,1964, p.73; Boyd, 1956, p.383). Although little was known about the humoral responses stimulated by spargana, it seemed probable that anti-sparganum antibodies were produced by chronically infected monkeys and mice and that they almost certainly were produced by infected rabbits (section 1.131).

On the basis of this somewhat fragmentary evidence, it was provisionally assumed that mammals develop an immediate form of hypersensitivity to spargana. When the appropriate antigen is introduced into the tissues of a hypersensitive animal the tissues usually show signs of pathological changes. Manifestations of these local pathological changes include "excessive cellular aggregations, necrosis, fibrosis, caseation and cavitation" and the affected animal may display general signs of pathological changes

such as fever, internal haemorrhage and it may even die if the reaction is strong enough (Gray, 1964, p.16). Marked local and general signs of pathological changes were seen in human beings infected with many spargana and occasionally in human beings infected with relatively few spargana (section 1.132) although infections of only one or two spargana in human beings sometimes induced only "a mild local allergic response" (Strauss and Manwaring, 1964). Marked local pathological changes also were induced in some infected monkeys (section 1.132). Thus it seemed that spargana were capable of inducing marked pathological changes in human beings and monkeys. Similar though perhaps less intense responses were stimulated in rats. Little direct evidence to show that these pathological changes were the results of immunological responses was produced by previous workers. However, the pathological changes induced by spargana in human beings, monkeys and perhaps rats corresponded to those induced when the appropriate antigen is introduced into the tissues of a hypersensitive mammal so it seemed likely that the pathological changes induced by spargana had an immunological basis. If this was true, then it also seemed likely that spargana could be considered to be highly immunogenic to human beings monkeys and possibly rats. Whether or not spargana were highly immunogenic to most of the other species of animals that could act as their hosts could not be estimated because too little

was known about the immunological responses and pathological conditions induced in these hosts by spargana. The responses stimulated by spargana in mice possibly were exceptional in this context.

According to recent observations made by Mueller (1961,1963) the spargana of the North American form of spirometrid tapeworm seemed to exite little or no reaction when they were located in the sub-cutaneous tissues of mice even when each mouse was infected with up to 150 spargana for a year or more. Equivalent infections in human beings usually induced marked pathological changes and sometimes resulted in death. If therefore spargana were as pathogenic to mice as they were to human beings it seemed reasonable to propose that even when they were restricted to sub-cutaneous tissues in mice they should have induced some clearly recognisable local and perhaps general pathological changes similar to those induced in human beings. However, they apparently did not. Mueller (1963) commented that spargana seemed to be adapted to living in mice because they induced so few pathological changes. If it is reasonable to assume that, with respect to metazoan parasites, low pathogenicity is indicative of low antigenicity, then it seemed that either spargana were not sufficiently immunogenic to mice to stimulate them to produce strong immunological responses or that the responses produced by mice to spargana failed to manifest themselves. Neither of

these explanations of the apparent failure of spargana to induce marked pathological conditions in mice seemed reasonable.

Sprent (1963, p.28) stated that parasites which have been associated with their hosts for long evolutionary periods tend to be less pathogenic to their hosts than parasites which have only recently become associated with a species of host, especially when the parasites and the hosts are in their natural environments. The pathological conditions induced by parasites often are associated with the development of a state of hypersensitivity in the host (Sprent, 1963, p.26). Parasites that induce few and only mild pathological changes in hosts with which they have been associated for long evolutionary periods therefore probably are immunologically adapted to those hosts. A theory to account for immunological adaptation of this kind has been proposed by Damien (1964) and Dineen (1963a,b). The principle of this theory is that strains of a parasite, any antigens of which resemble the antigens of the host, may be at a selective advantage in comparison with strains of the same species of parasite the antigens of which resemble the antigens of the host to a lesser extent or not at all. If the immunological responses produced by the host affect the ability of the parasites to survive and reproduce in the host, then it seems reasonable to assume that natural selection would bring about a reduction in the antigenic disparity between the species of parasite and its host with the result that the host

may respond less vigorously to the parasite. However, this form of immunological adaptation clearly can occur only over a long evolutionary period. It is unlikely that spargana have become immunologically adapted to mice in this way because, according to Mueller (1963), spargana only rarely occur in wild mice.

The observation that spargana are able to live in the tissues of a wide variety of animals further supported the hypothesis that they probably are not adapted to living in the tissues of mice. Animals of different species which are only distantly related phylogenetically are likely to be different antigenically (Boyd, 1956, p.142). Therefore it may be suggested that a species of parasite can become immunologically adapted to only one species or a few closely related species of animals in which the parasite is usually found. Spargana therefore cannot be immunologically adapted to living in every species of animal in which they have been found. It has been proposed that spargana occur in reptiles and amphibians more commonly than they occur in mammals (section 1.122) so it is possible that if spargana have undergone immunological adaptation they may be better adapted to living in reptiles or amphibians than they are to living in mammals such as mice.

If the relationship between mice and spargana cannot be explained in terms of a theory of immunological adaptation, how else can it be explained? Is it possible, for example, that

spargana contain substances that are highly antigenic to mice but that these substances are not released into the tissues of mice and therefore never stimulate marked immunological responses or pathological changes? So little was known about the antigens of spargana that it was impossible to give a definite answer to this question, but it seemed likely that any antigens produced by spargana must eventually enter the tissues of infected mice. Antigenic substances on the surfaces or in the secretions and excretions of spargana clearly must come into contact with the tissues of infected mice and may be widely distributed through the tissues of each mouse when spargana migrate. Moreover, if spargana in mice release fragments of their bodies and these fragments die and disintegrate it is likely that substances from deep in the tissues of the spargana enter the tissues of the mice. Such substances also may be released from the tissues which presumably are damaged when the holdfast and tail of each sparganum separates just before or when the ingested sparganum penetrates the wall of the gut of a newly infected mouse. Any antigenic substance produced by spargana therefore may enter the tissues of infected mice at some time and mice should respond to disintegrating fragments of spargana even if they fail to respond strongly to intact spargana.

The possibility that the spargana examined by Mueller (1963) had induced a state of immunological unresponsiveness in mice

also was considered. There is some evidence which suggested that such a condition may be induced in, for example, calves infected at or near birth with Taenia saginata (Urquhart, 1958; Soulsby, 1963a). However, it was considered to be very unlikely that this explanation of the apparent inability of spargana to stimulate marked responses in mice was valid (see section 3.5).

Finally, it is relevant to point out that the pathological changes induced by parasites often do not have an immunological basis. Some parasites cause pathological conditions by, for example, releasing toxic substances, directly damaging tissues physically or by instigating the production of neoplastic tissues (Sprent, 1963; pp.25-26). Apparently, spargana failed to provide the stimuli necessary for the development of such pathological conditions of a non-immunological nature. Thus there seemed to be no satisfactory explanation for the apparent inability of spargana to stimulate or induce pathological changes in mice.

1.3 AIMS OF THE PRESENT STUDY

The first aim of the present study was to investigate the relationships between mice and spargana to find out if mice develop an immediate form of hypersensitivity to spargana and to try to confirm and explain the observations that spargana seem to induce few or no clearly recognisable pathological changes in mice.

A second aim of the study was to try to compare the immunological

responses stimulated by spargana in mice with those stimulated in amphibians in order to find out if spargana are adapted to living in amphibians any better than they are adapted to living in mice.

SECTION 2 MATERIALS AND METHODS

2.1 THE SELECTION AND MAINTENANCE OF THE ANIMALS USED IN THE STUDY

2.11 Parasites

All the parasites used in the study were derived, unless otherwise stated, from a tapeworm that was produced by feeding one sparganum to a cat. The sparganum was taken from a marine toad (Bufo marinus) which was obtained from Queensland, Australia. During the course of the study four generations of the parasite were completed.

The parasites used could not be identified because the taxonomy of the tapeworms belonging to the genus Spirometra is so confused (see section 1). Spargana and adult spirometrid tapeworms found in Australia have been identified by previous authors as those of the species Spirometra erinacei (Bearup, 1948; Pullar and McLennon, 1949) or S. mansoni (Roberts, 1934, 1935) but both of these species probably are invalid (Iwata, 1933; Mueller, 1966, 1967; Pullar, 1946). Furthermore, even if the species Spirometra erinacei and S. mansoni

are valid, it is difficult to identify them because the characteristics used for the purposes of identification are so variable (Iwata, 1933). Mueller (1966) stated that he would not attempt to identify any spirometrid tapeworm other than one belonging to the species Spirometra mansonoides. This confusion in the taxonomy of the genus Spirometra is comparable with the confusion in the taxonomy of the genus Diphyllobothrium (see Stunkard, 1965), the genus in which spirometrids originally were placed. It was therefore considered to be more reasonable to refer to the parasites used in the present study as the Australian form of spirometrids than to suggest that they belong to a species which probably is invalid and certainly is not clearly defined.

The propagation of parasites in the laboratory is described in section 2.2.

2.12 Mice

Only young albino female mice weighing 20-25gm on arrival in the laboratory were used. These mice were obtained from the Central Animal House of the University of Adelaide. They were kept in cages of the 'Harwell' design (UFAW Handbook, 1957) and were given continuous access to water and food (M164 Mouse cubes, W. Charlick Ltd., Adelaide). Both experimental and stock mice were kept in a room maintained at 72±2°F.

2.13 Amphibians

Marine toads (Bufo marinus) and marsh frogs (Limnodynastes tasmaniensis) were used in the study. The marine toads were obtained

from the Apex Club of Ingham, Queensland or from Mr. H. J. Hohnke of Paddington, Queensland. All the toads received were at least three inches long when measured from the tip of the snout to the end of the urostyle. Wild marsh frogs were obtained from Mr. R. G. Beck of Mount Gambier, South Australia.

Naturally acquired infections of spargana were found in approximately one per cent of the marsh frogs and up to five per cent of the marine toads received. These spargana often were found only after the toads and frogs were dissected; many frogs and toads which harboured spargana showed few or no external signs of infection. These wild amphibians clearly could not be used for experiments in which the responses of amphibians to experimentally introduced spargana were examined because (1) it was possible that some of the toads and frogs had been responding for some time to the naturally acquired spargana they contained and (2) the naturally occurring spargana could have been incorrectly identified as experimentally introduced spargana. It therefore was necessary to breed amphibians in the laboratory.

Attempts to breed marine toads in the laboratory failed. Even though male and female toads often went into amplexus when they arrived in the laboratory, they did not produce fertile eggs spontaneously. Male toads produced sperm when injected with urine from pregnant women (see Bettinger and O'Laughlin, 1950), but female toads did not produce eggs when they were injected with urine from pregnant women or 100-500 international units of gonadotropins

(Pregnyl, Organon Laboratories Ltd., England). Gravid female toads also were injected according to the method described by Hamburger (1942 pp.30-35) with up to 10 pituitary glands obtained from other gravid female toads and went into amplexus with male toads that had been stimulated to produce spermatozoa, but no fertile eggs were produced. No eggs could be obtained by the 'stripping' method described by Hamburger (1942). Attempts to breed toads in the laboratory therefore were discontinued.

I decided to use marsh frogs as experimental animals because they could be obtained readily abd because they laid fertilized eggs spontaneously when brought into the laboratory. Moreover, they have a breeding season longer than that of most other species of Australian frogs and it has been reported that the tadpoles of this species are able to grow and metamorphose into frogs in a shorter time than that required by the tadpoles of other common Australian frogs (Martin, 1965). The observation that some wild marsh frogs contained spargana also showed that frogs of this species can support infections of spargana. However, to be sure that both adult frogs and tadpoles of this species could be infected with spargana in the laboratory, two preliminary experiments were done. In the first experiment, ten marsh frogs were each fed two holdfasts of spargana. When these frogs were killed and dissected three weeks later, eighteen spargana were recovered. In the second experiment, eighteen tadpoles were allowed to remain for 12 hours in a dish of water which contained cyclops infected with procercoids. When these

tadpoles were dissected two weeks later, it was found that each tadpole was infected with 1-10 spargana. The frogs and tadpoles used in these experiments seemed to be unaffected by the spargana that they contained.

To breed frogs in the laboratory eggs of Limnodynastes tasmaniensis were allowed to hatch in large crystallising dishes containing pond water and the young tadpoles were transferred to polythene trays which were 12 inches wide, 20 inches long and three inches deep. These trays contained pond water to a depth of 1-1½ inches. No more than 40 tadpoles were put into any tray because it was found that when the tadpoles were too crowded they grew slowly. Similar stunting of growth in crowded tadpoles of Rana pipiens was noted by Richards (1958). The rate of growth of tadpoles seemed to be normal however when 40 or less tadpoles were placed in one tray. These tadpoles were fed with lettuce leaves that had been frozen and then thawed before being placed in the trays. The pond water in each tray was replaced with fresh pond water two or three times each week.

Frogs that developed from the tadpoles were kept in large ventilated polythene boxes which contained a small quantity of pond water. Each box was placed on an inclined shelf so that the water formed a small pool in one corner of the box. The frogs were fed on flies of the species Drosophila melanogaster. A bottle containing some culture medium and eggs, larvae and adult flies was placed in each of the boxes and supplied sufficient food for

the frogs in the box for one week. In these conditions the frogs survived well.

Frogs and tadpoles were maintained at a temperature of 72±2°F. The water in which the tadpoles and frogs were kept was taken from a small ornamental pond in the grounds of the University of Adelaide and was placed in the constant temperature room at 72±2°F for two days before being used. Although a few cyclops were occasionally found in the debris at the bottom of the pond from which the water was obtained, they were not infected with spirometrid procercoids and care was taken to ensure that no cyclops were present in the water taken from the pond.

The methods used for breeding marsh frogs in the laboratory were successful to the extent that tadpoles were readily available and they usually grew to a length of 30-45mm in approximately three months. Some of the tadpoles began to metamorphose approximately three months after they hatched, but many showed no sign of metamorphosing even when they were 11 months old. For example, out of a total of approximately 600 tadpoles obtained in one breeding season, nearly 400 grew normally but failed to metamorphose within 11 months of hatching and approximately 100 tadpoles died. Why so many tadpoles failed to metamorphose was not clear. However, it is known that even though tadpoles of Limnodynastes tasmaniensis usually begin to metamorphose approximately three and a half months after they hatch, some wild tadpoles which hatch late in the breeding season may not metamorphose for up to a year (Martin, 1965).

Therefore, it is possible that the environmental conditions which prevent many wild tadpoles from metamorphosing may have been created accidentally in the laboratory. But whatever the reason, it was difficult to breed sufficient numbers of frogs.

2.2 THE PROPAGATION OF PARASITES IN THE LABORATORY

2.21 The maintenance of adult spirometrid tapeworms in cats

Adult tapeworms were maintained in cats which were obtained from the Central Animal House of the University of Adelaide. Samples of the faeces produced by each cat during the first seven to nine days that it was in the laboratory were examined for eggs of parasites. If eggs identified as those of cestodes or trematodes were found in any faecal sample, the cat which produced the faeces was not used. To infect each cat spargana were placed in a slit cut in a small piece of horsemeat and this was fed to the cat. Most of the cats used were each given three spargana that had been bred in the laboratory, but the first cat used was infected with one spargana taken from a marine toad (see section 2.11).

Infected cats were maintained in a manner similar to that described by Mueller (1959a). They were given free access to water and were fed on a diet of either a canned animal food called 'Sleigh Dog' (Australian Wide Canneries Ltd., Melbourne) to which was added a daily dose of approximately 0.25ml of a vitamin and mineral supplement called Vi Daylin/M (Abbot Laboratory Pty. Ltd.) or lean fresh horsemeat. Each cat was given a maximum of half a pound of either of these foods each day. Cats from which spirometrid

eggs were being collected were maintained on a diet of horsemeat alone because the spirometrid eggs could be easily separated from the debris in faeces produced by cats on this diet (Mueller, 1959a). No cat was maintained on a diet of horsemeat alone for more than two weeks (Mueller, 1959a) and, when cats were on the alternative diet of 'Sleigh Dog' they were also occasionally given milk and liver. All the cats produced faeces containing many spirometrid eggs after they had been infected for two to three weeks.

2.22 Separation, incubation and hatching of spirometrid eggs

Eggs were separated from faecal debris using methods similar to those described by Mueller (1959a). Faeces freshly deposited in trays provided for the cats were macerated in water and were strained through a stainless steel strainer (20 meshes per inch) to remove coarse debris. The debris and eggs in the filtrate then were allowed to settle for three to five minutes and the supernatant liquid was decanted and fresh water was added. This sedimentation procedure was repeated three or four times. The sediment finally obtained was then filtered through a stainless steel mesh (300 meshes per inch). The sediment obtained from this second filtration was washed into a Petri dish of water. After two to three minutes, the water in the Petri dish was decanted, and fresh water was added. This last procedure was repeated two or three times. Eggs treated in this way were almost completely free of debris. They then were placed in covered crystallising dishes which contained water to a depth of approximately half an inch and were incubated at 72±2°F.

After an incubation period of 14-16 days, 90 per cent or more of the eggs in each dish contained coracidia almost all of which hatched and became active if the dish containing the eggs was placed in strong sunlight.

2.23 The production of stocks of cyclops in the laboratory

Mueller (1959b) described a method of producing cyclops in the laboratory. This method may be briefly described as follows. Cyclops are kept in well-lit glass tanks that are filled with water which is aerated with a stream of fine bubbles. The flow of air into the tubes must be regulated so that the water in the tanks is not excessively turbulent because too much turbulence in the water causes the cyclops to cling to the sides of the tanks and stop feeding. Cyclops are fed on infusions of Timothy or Clover hay.

When this method was tried, very few cyclops were obtained. Although the cyclops that were used to start the cultures usually survived for considerable periods of time, they did not breed. It seems that they may have been given inadequate or unsuitable food even though the hay infusions used were prepared according to the method described by Mueller (1959b). But whatever the reasons for failure, after four abortive attempts to produce cyclops in the above manner, a new method for breeding them was sought.

It was noticed that populations of cyclops occasionally appeared in aquarium tanks that contained stocks of snails belonging to the species Lymnaea lessoni. These snails invariably cause the

water in which they are kept to become turbid and turn to a green-brown colour, i.e. substances released by these snails seem to promote the growth of micro-organisms such as bacteria, protozoans and algae. It was presumed that the cyclops which sometimes appeared in the tanks that contained this turbid water must be feeding on these micro-organisms. However, populations of crustaceans belonging to the genera Daphnia and Cypris also appeared in the tanks and usually replaced the populations of cyclops. It was clear, therefore, that the populations of cyclops represented only one stage in a succession of populations in each tank. On the basis of these observations, I devised the following method of breeding cyclops.

Clean aquarium tanks (30 litre capacity) supplied with aerators and loosely fitting glass covers were filled with rain-water. The tanks were left for 2-3 weeks during which 'blooms' of various micro-organisms appeared. After the 'blooms' had subsided, the tanks developed lining layers of green algae and at this stage the Lymnaea lessoni were introduced. The snails were fed with a continuous supply of lettuce leaves that were frozen and then thawed before being placed in the tanks. The water in the tanks became turbid over a period of 3-5 weeks at the end of which the cyclops were added. After a further period of 3-5 weeks, each tank usually contained enough cyclops to allow harvesting to begin. Populations of cyclops could be maintained in any one tank for only 2-6 weeks after which they were generally replaced by populations

of Daphnia or Cypris. Contamination with these latter organisms was reduced to a minimum by taking care that only cyclops were used to 'seed' the tanks, but some unwanted forms usually appeared. These probably were transferred between tanks along with the snails. It was therefore necessary to set up a series of aquaria at various stages of development so that a more or less continuous supply of cyclops was available. However, although some cyclops were always present in one or more of the culture tanks, the stocks usually were small and never were as large as those produced by Mueller (1959b). The cyclops used in the study were identified as Mesocyclops leuckarti (Claus).

2.24 The infection of cyclops with procercoids and the maintenance of infected cyclops

To produce procercoids cyclops were infected with coracidia in the manner described by Mueller (1959b). Spirometrid eggs that contained mature coracidia were exposed to strong sunlight for 30-60 minutes and the water containing the hatched coracidia was decanted, leaving behind the unhatched coracidium and empty egg-shells. Cyclops then were harvested from the aquarium tanks using a small hand-net and were added to the decanted water. Most or all of the cyclops in any culture readily ate the swimming coracidia, but the older form of cyclops, especially the females, seemed to be almost totally resistant to infection and seemed to defaecate the coracidia. Resistance to infection in older cyclops especially

females was noted also by Mueller (1959b). Moreover, even the younger forms of cyclops seemed to be partially resistant to infection and few contained more than five procercoids each. It was also found that few or none of even the young cyclops in many of the cultures became infected. The procercoids which developed in the infected cyclops seemed to be normal and were active but they rarely were plentiful. Mueller (1965a) experienced similar difficulties when he tried to culture the procercoids of the Oriental form of tapeworms (provisionally called 'Spirometra ranarum') in cyclops of the species Cyclops vernalis. Cyclops of this species yielded only scant harvests of procercoids of 'Spirometra ranarum' whereas they seemed to be excellent hosts for the procercoids of Spirometra mansonoides.

Infected cyclops were maintained at 72±2°F in 500 ml beakers that were half filled with water taken from aquarium tanks which contained Lymnaea lessoni. Care was taken to make sure that the water in these beakers always was turbid; if the water became clear it was replaced by turbid water. In this way the infected cyclops were supplied with adequate amounts of food. Procercoids were harvested when they were 14-19 days old.

To harvest the procercoids the water in the beakers which contained infected cyclops was passed through a small sieve made of nylon netting (100 meshes per inch). This netting retained the cyclops in the cultures but allowed the fine debris in the water to pass. The cyclops were liberally washed with distilled water and

then with a solution of antibiotics in saline (see section 2.38) if they were to be infected into mice.

2.25 Infection of mice to produce stocks of spargana

Each mouse in which stocks of spargana were produced was lightly anaesthetised and then given an intraperitoneal injection of infected cyclops in approximately 0.5ml of physiological saline solution which contained antibiotics (see section 2.38)

Some attempts were made to infect mice with procercoids which had been dissected out of cyclops but these attempts failed because the procercoids adhered to the surfaces of syringes and pipettes even when these surfaces were coated with a layer of silicone or formvar.

Mice were killed and examined for spargana 3-10 weeks after they were infected with procercoids.

2.3 MATERIALS AND METHODS USED IN EXPERIMENTAL STUDIES

2.31 The materials and methods used in serological tests

2.311 The preparation of serum

To obtain blood from mice each mouse was deeply anaesthetised and its thoracic cavity was opened. A Pasteur pipette was inserted into the right ventricle of the heart of the mouse and blood was drawn into the pipette. The blood was transferred to a centrifuge tube in which it was allowed to clot over a period of 4-5 hours. It was then centrifuged at 1500g for 10 minutes and the supernatant serum was drawn off by means of a Pasteur pipette. Sera obtained from the mice used in each experiment were, unless otherwise stated, pooled and again centrifuged at 1500g for 10 minutes.

The supernatant serum was drawn off and either was used immediately or was stored in a refrigerator at -20°C for periods of up to three months. This method was essentially the same as that described by Campbell et al. (1964, pp.19-20, 22-23).

To obtain blood from marine toads the toads were pithed and dissected. Both the aortic arches of each toad were ligatured and a Pasteur pipette was inserted into one of the arches at a point proximal to the ligature. The heart of the toad then pumped blood up into the pipette.

It was difficult to obtain serum from the blood of toads. The blood of some toads failed to clot, and even when the blood clotted the clot produced contracted very little. Only small quantities of serum therefore were obtained. It also was found that the liquid that was obtained after the blood of toads had apparently clotted was not pure serum free from fibrin because it usually clotted when stored at 5°C for only one or two hours. Clots formed in this way did not contract to exude serum. If tubes in which these secondary clots had formed were centrifuged, a small quantity of serum-like liquid sometimes could be obtained, but this liquid often clotted again when stored at 5°C for one or two hours. These observations confirmed some of the observations made by Hackett and Hann (1964) who studied the clotting of blood of amphibians belonging to the species Bufo marinus and Hyla caerulea .

Furthermore, it was found that when serum that had been taken from toads was stored at -20°C for only 24 hours, and then was used

in immunodiffusion tests, some constituents of the serum precipitated in the agar around wells charged with the serum. This precipitation seemed to be a non-specific reaction (see section 2.313). The halos of precipitate produced almost completely obscured any bands of precipitate formed by antigen-antibody complexes. Less dense halos of precipitate sometimes appeared in the agar around wells charged with fresh 'serum' but these halos usually were not dense enough to obscure bands of precipitate formed by antigen-antibody complexes. However, it was clear that immunodiffusion tests could not be carried out with amphibian serum that had been stored, so all tests were done with freshly prepared 'serum'.

2.312 The preparation of extracts of spargana

Extracts of spargana were made by macerating whole spargana in ice-cold physiological saline solution (0.85 per cent NaCl in double- distilled water). Spargana taken from mice were washed in three changes of physiological saline solution, the volume of solution used for each wash being at least 50 times the volume of the spargana. The spargana then were blotted dry with filter paper and were weighed. They were macerated in physiological saline solution in a ground-glass tissue-macerater (A. Gallenkamp and Co. Ltd., London), 1.0ml of saline being used for every 100 mg of spargana macerated. The resulting suspension was centrifuged at 10,000g for ten minutes. The slightly opalescent supernatant liquid was drawn off with a pipette and the dry weight of the dissolved

materials (corrected for added sodium chloride) in some samples of this supernatant liquid was estimated (see Table 2.1). The total amount of nitrogen present in other samples of this supernatant liquid also was estimated (see Table 2.1).

Table 2.1

The total weight of nitrogen and dissolved materials in three extracts of spargana

Estimation	Extract		
	1	2	3
Weight (mg) of dissolved material per millilitre of extract (corrected for added sodium chloride)	17.5	11.1	11.0
Weight (mg) of nitrogen per millilitre of extract	0.952	0.658	0.632
Weight of nitrogen expressed as a percentage of the weight of dissolved material per millilitre of extract	5.4%	5.9%	5.7%

The methods used to estimate dry weight and total nitrogen and details of the results obtained are given in the appendix.

Between five and 15 ml of each of these extracts were left after these estimates had been made. Each of these surplus quantities of extract was diluted with a quantity of physiological saline solution (8.5mg NaCl/ml) sufficient to reduce the concentration of dissolved material (including added sodium chloride) in the extract to 18.5mg per millilitre. These diluted extracts were stored in a refrigerator at -20°C. Although there clearly was some variation in the composition of extracts of spargana prepared in this manner, the variation was considered to be too small to affect significantly the results obtained in the experiments in which the extracts were used. In the following pages, therefore, the extracts were all referred to as the standard extract of spargana (SES).

It was necessary to filute the standard extract of spargana with physiological saline solution in order to prepare solutions to be used in some of the experiments described in sections three and four. Serial two-fold or four-fold dilutions of the standard extract of spargana were made. The concentrations of these diluted extracts were represented by fractions. For example, a solution prepared by mixing one part of the standard extract of spargana with three parts of physiological saline solution was represented by the fraction SES/4.

2.313 The preparation of agar gel

Although Ionagar Number 2 (Consolidated Laboratories,

Chicago, U.S.A.) was used in some tests for precipitating antibodies, Difco Bactoagar (Difco, Detroit, U.S.A.) was used for most of the study. Ionagar may be considered to be superior to Bactoagar for use in immunoelectrophoresis tests because there is less of an electro-osmotic flow of water through gels of Ionagar than there is through gels of Bactoagar (Weime, 1965, p.112). However, the non-specific precipitating action of Ionagar is greater than that of Bactoagar (Weime, 1965,p.112) and it was found in some preliminary trials that the halos of non-specifically precipitated materials which appeared around wells cut in gels prepared from Ionagar and charged with serum tended to obscure some of the weaker bands of specific precipitates produced. This happened especially when serum from amphibians was used. This difficulty was largely avoided if Bactoagar was used; little or no non-specific precipitation of serum occured in gels made from Bactoagar even with amphibian sera.

Ionagar used for gels was not purified in any way, but Bactoagar was washed in a manner similar to that described by Weime (1965, p.105). A five per cent solution of Bactoagar was prepared by dissolving the agar in boiling double-distilled water. The gel which was produced when this solution cooled was cut into cubes of approximately 1ml volume. These cubes were washed in double-distilled water for seven days during which time the water was changed twice each day. The cubes then were dried and were re-dissolved in a sufficient volume of boiling double- distilled water to produce a

two per cent solution of agar. During this last stage of dilution Merthiolate (Eli Lilley and Co. Ltd., England) was added to produce a final concentration of 0.02 per cent (Weime, 1965, p.59). The hot gel then was divided into 10ml samples each of which was stored at 4°C in a 30 ml McCartney bottle. Immediately before each experiment was started, the gel in one McCartney bottle was melted and diluted with an equal volume of barbital buffer at pH 8.2 and 0.1 ionic strength (Crowle, 1961, p.303) to produce a final solution of one per cent agar in barbital buffer at pH 8.2 ionic strength (μ) of 0.05.

2.314 The preparation of plates and tubes to be used in immunodiffusion tests

Clean microscope slides (3" long and 1" or 2" wide) that were used to support agar gels were first coated with a thin layer of dilute agar solution (0.1%) and were dried at 60°C (Weime, 1965, p.59). The hot solution of one per cent buffered agar was poured onto the plates which had been placed on a horizontal surface (Crowle, 1961, p.204). Three millilitres of the agar solution were poured onto the surface of each slide which was 1" wide and 6ml of solution were poured onto each slide which was 2" wide. Wells and troughs to receive reactants were cut into the agar after it had been allowed to solidify for one hour.

Tubes were prepared by first cutting glass tubing (2mm bore) into pieces 2cm long. These short tubes were coated with a thin layer of 0.1 per cent agar solution and were dried at 60°C. They

then were used to punch cylindrical plugs of agar gel out of a previously prepared layer of gel 3-4mm thick. One end of each short tube was pushed vertically down through the layer of gel in order to produce a plug 3-4mm long. This plug then was pushed to a position half way along the length of the tube and was sealed in place with a small quantity of hot agar solution.

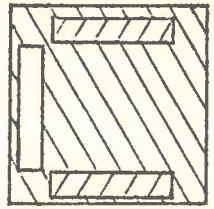
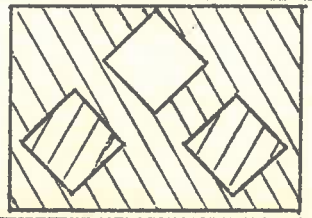
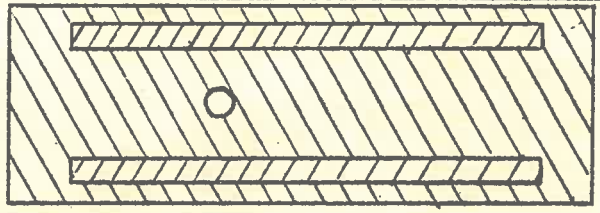
2.315 The immunodiffusion tests used in the study

The kinds of immunodiffusion tests done and the quantities of reactants used in these tests are shown by the diagrams and figures given in Table 2.2. Templates made of 'Perspex' (Crowle, 1961, p.206) were used as guides when the troughs and wells were cut out of agar gels. The quantities of the reactants placed in the wells and troughs were measured with micro-pipettes (Drummond Scientific Company, Broomall, U.S.A.). Gels charged with reactants were placed in a humid atmosphere and were maintained at 20°C to prevent the development of artefacts in the patterns of the bands of precipitates produced (see Crowle, 1961, p.16).

Electrophoretic separation of the constituents of extracts of spargana was carried out using apparatus essentially the same as that described by Grabar (1964). This apparatus is shown in Figure 2.1. To avoid overheating agar gels, electrophoresis was carried out in a refrigerator at 5°C (Crowle, 1961, p.26). The apparatus shown in Figure 2.1 was placed in the refrigerator for one hour before being used. During this time a bridge of polythene tubing filled with buffer solution was placed between the two end-tanks

Table 2.2

The kinds of immunodiffusion tests used in the experimental studies

Name of test	Description of test	Pattern of reservoirs for reactants used in the tests	Amounts of reactants used in each reservoir
(a) Tube test	Double diffusion in one dimension		20µl
(b) Gradient test	Double diffusion in two dimensions		30µl
(c) Ouchterlony test	"		50µl
(d) Immuno-electro-phoresis test	"		Troughs - 150µl Wells - 5µl

 - Agar gel  - Serum  - SES

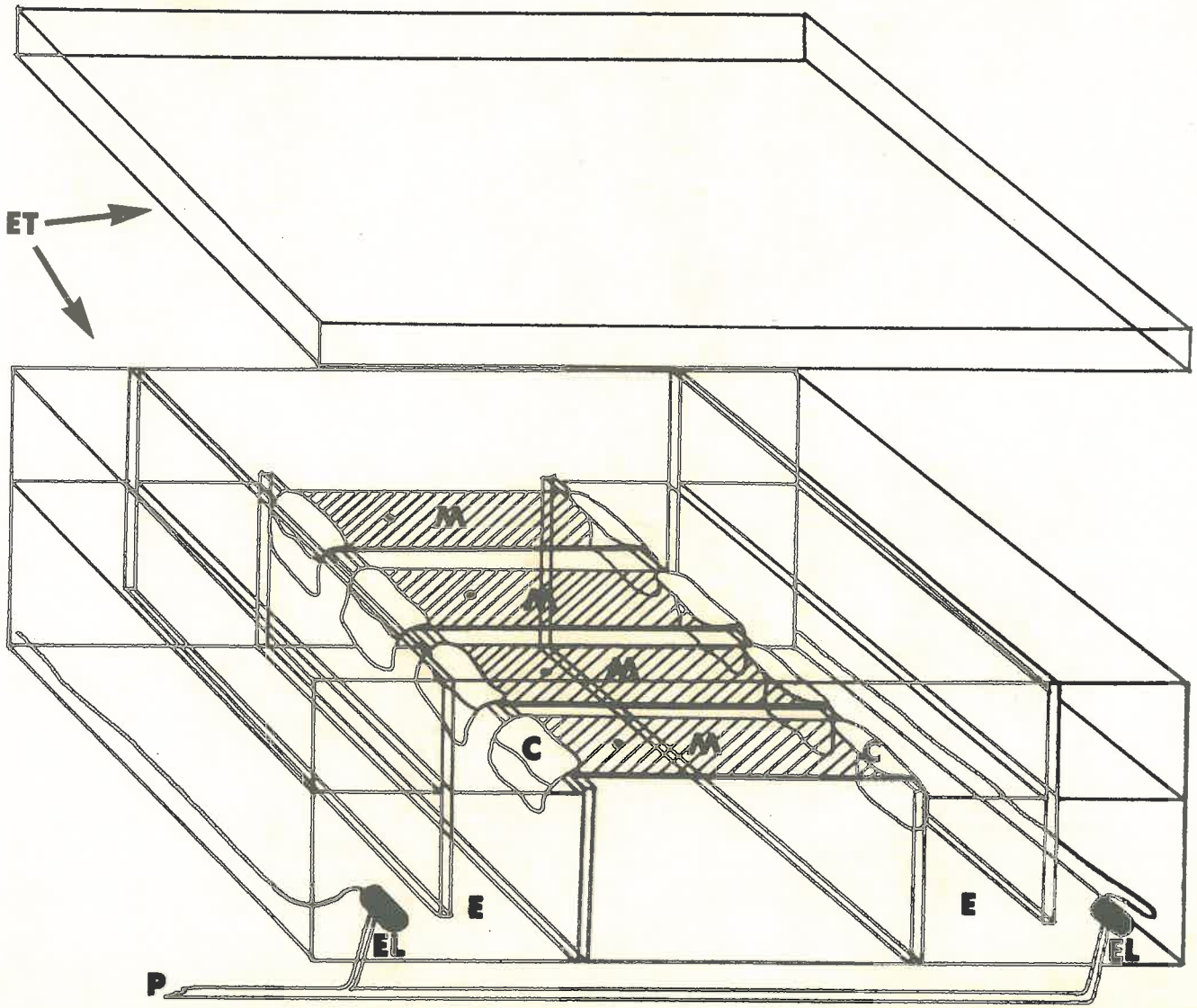
References - Crowle (1961), Grabar (1964), Ouchterlony (1964), Weime (1965).

Legend to Figure 2.1

The apparatus which was used to electrophoretically separate the components of extracts of spargana.

(C - chamois wicks; E - end tanks; EL - electrodes; ET - electrophoresis tank and cover; M - microscope slides supporting agar gels; P - power supply (alternating current to direct current rectifier))

Figure 2.1



of the apparatus to allow the surfaces of the buffer solutions in these tanks to come to the same level. The tanks were charged with barbital buffer solution (pH8.2, ionic strength 0.05). Chamois leather wicks soaked in the buffer solution were used to make electrical contact between the buffer in the end tanks of the apparatus and the agar gel on the microscope slides shown. During the electrophoretic separation of the constituents in the extracts of spargana, the gel on each slide was subjected for one hour to a potential difference of five volts per centimeter and passed a current of five milliamps. Gels used for electrophoretic separations were allowed to attain room temperature (15-20°C) before the troughs in the gels were charged with reactants.

Gels used for immunoelectrophoretic analysis and for two dimensional double-diffusion tests were incubated at 20°C for 24 hours in order to allow bands of precipitate to develop. Tubes used for experiments which involved the double-diffusion of reactants in one dimension were incubated at 20°C for up to three days (Preer, 1956) and were inspected each day.

2.316 The fixing, washing and staining of materials in agar gels used in immunodiffusion tests

Methods described by Crowle (1961, pp. 265-275) were used to fix, wash and stain materials in agar gels.

Electrophoretically separated components of the extract of spargana were fixed in various solutions according to the kind of substance that was to be stained. Proteins were fixed in a two per cent solution of acetic acid in a 60 per cent solution of

ethyl alcohol in water, polysaccharides were fixed in a solution of 95 per cent ethyl alcohol in water and lipids were fixed in a two per cent solution of acetic acid in water. The gels which contained the fixed materials were covered with moist filter paper and were dried at room temperature. They then were stained. Proteins were stained with a one per cent solution of amidoblack 10B (Crowle, 1961, p.304), polysaccharides were stained with alcian blue (Crowle, 1961, p.310) or by means of the p-phenylenediamine oxidation reaction (Crowle, 1961, p.310) and lipids were stained with a saturated solution of oil red O in a 60 per cent solution of ethyl alcohol in water (Crowle, 1961, p.306).

Gels in which bands of precipitate had developed were washed for one week in barbital buffer solution (pH 8.2, μ = 0.05) to wash out excess reactants. They then were covered with moist filter paper and were dried at room temperature. The dried gels were stained with the solutions of the stains described above. The gels stained for proteins were washed free of excess stain with a two per cent solution of acetic acid in water and they were again dried at room temperature.

2.32 Anaesthesia of mice and amphibians

Mice were anaesthetised with ether.

Tadpoles to be anaesthetised were immersed in a solution of one part of an anaesthetic called MS222 Sandoz (Sandoz Ltd., Basle, Switzerland) in 10,000 parts of pond water (see Bove, 1962). The tadpoles became quiescent after being in this solution for less than

one minute and they recovered within five minutes of being transferred to fresh pond water. The solution of MS222 seemed to have no ill effects on tadpoles.

2.33 Intravenous injection of mice

Mice were injected intravenously in the manner described by Campbell et al. (1964, pp.7-8). Each mouse was put into a holder and the tail of the mouse was cleansed with a 70 per cent solution of ethyl alcohol. The materials to be injected were given to the mouse through a 26 s.w.g. hypodermic needle which was inserted into one of the lateral tail veins of the mouse.

2.34 Methods used to introduce spargana and procercoids into mice, tadpoles and frogs

Lightly anaesthetised mice were given procercoids contained in cyclops either through a stomach tube made out of a 19 s.w.g. hypodermic needle the point of which had been removed or through a 19 s.w.g. needle introduced into the peritoneal cavity of each mouse. Infected cyclops which were fed to mice were suspended in the small amount of distilled water whereas cyclops injected into the peritoneal cavity of each mouse were suspended in a solution of antibiotics of physiological saline solution (see section 2.38). The skin of the abdomen of each mouse was cleansed with a 70 per cent solution of ethyl alcohol in water before each injection was given.

Spargana (or their holdfasts) were either forced fed to mice or were injected through a 19 s.w.g. needle into the subcutaneous

tissues of lightly anaesthetised mice (Mueller, 1963). Approximately 0.25ml of a solution of antibiotics in physiological saline solution was injected with each holdfast. Unless otherwise stated the mice used in the experiments described in section 3 were fed with spargana.

Marsh frogs to be infected were fed with holdfasts of spargana or with cyclops infected with procercoids. Holdfasts of spargana were simply placed in the mouth of the frogs and infected cyclops were picked up by means of a Pasteur pipette and also placed in the mouths of the frogs. After they were given infected cyclops or holdfasts of spargana, frogs were placed in dishes of clean pond water and inspected for periods of up to 15 minutes to make sure that they did not disgorge the cyclops or spargana. Some attempts were made to infect tadpoles with procercoids and spargana. The methods used in these attempts are described in section 4.2.

2.35 Histological methods

Histological methods described by Humason (1962) and Cowdry (1952) were used in the study. Tissues to be examined histologically were fixed in a five per cent solution of acetic acid in a saturated solution of mercuric chloride in water. These tissues were embedded in paraffin wax and sectioned. The microtome used was set to cut sections 10 microns thick. These sections were subsequently stained with Weigert's haematoxylin and Picro-ponceau stain (Humason, 1962, pp.126, 165-166) or Jenner-Giemsa's stain (Humason, 1962, pp.225-227).

Smears of blood or impression preparations of cells in infected tissues or on spargana were prepared according to the standard methods described in Cowdry (1952, p.309). These smears were stained with Wright's stain (Humason, 1962, p.219-221).

2.36 Statistical methods

The results of some experiments were assessed statistically using the Student's t-test (Bailey, 1959, pp.43-51). If a level of significance (p) of less than five per cent ($p<0.05$ was obtained when the results of any experiment were analysed, it was considered that the null hypothesis could be rejected (Bailey, 1964, pp.29-30).

2.37 The random selection of mice and procercoids used in experiments to determine the susceptibility of mice to procercoids

It was necessary to do each experiment in which the susceptibility of mice to procercoids was investigated in at least two stages because not enough procercoids were available to do each experiment in one stage. In each of the stages mice were randomly divided into experimental and control groups using tables of random numbers published by Fisher and Yates (1957). The cyclops which contained the procercoids to be given to the mice were examined and the procercoids were counted. The cyclops were divided into groups so that each group of cyclops contained 20 procercoids. The groups of infected cyclops were then randomly distributed amongst the control and experimental mice again by means of tables of random numbers. In each of the stages of the experiments in which the susceptibility of mice to super-infections of procercoids was examined, only 3-4

uninfected and 3-4 infected mice were used. Such small numbers of mice were used at each stage because there was at least a reasonable chance that enough procercoids would be available to be distributed randomly in groups of 20 procercoids to all the mice when the time came for the mice initially infected with spargana to be given a super-infection of procercoids.

2.38 The sterilisation of solutions and equipment used for injecting mice

Equipment used to prepare and store extracts of spargana and to inject spargana and cyclops into mice was boiled for 20 minutes and was dried for 1-2 hours in covered dishes in an oven at 110°C. Physiological saline solution used to prepare extracts of spargana was made with double-distilled water and was heated in an autoclave at 120°C for 30 minutes. Spargana from which the extracts were made were washed in three changes of this sterilised saline before being macerated. Intra-venous injections were given using disposable 26 s.w.g. hypodermic needles and tuberculin syringes sterilised by the manufacturer (Jintan Terumo Co. Ltd., Tokyo, Japan). Saline solution used for injecting cyclops and spargana into mice was sterilised with antibiotics (500 international units of streptomycin (Dista Products Ltd., England) and 500 international units of penicillin (Glaxo Laboratories Ltd., England) per millilitre of saline). A small amount of the saline was injected into the mice along with the spargana or procercoids. The skin at the sites where mice were injected was cleansed with a 70 per cent solution of ethyl alcohol in water.

SECTION 3 THE IMMUNOLOGICAL RELATIONSHIPS BETWEEN MICE AND SPARGANA

The results obtained during the course of the present study supported the hypothesis that mice develop an immediate form of hypersensitivity to spargana. However, it seemed that this response could be considered to be normal in both form and intensity. The evidence on which these statements are based is given in the following pages.

3.1 THE INDUCTION OF ANAPHYLAXIS IN MICE SENSITISED TO SPARGANA

Anaphylaxis is a specific immunological response characteristically produced when an antigen to which an animal has developed an immediate form of hypersensitivity is introduced into the tissues of that animal. General accounts of anaphylaxis have been published by Boyd (1956, pp.390-399), Gladstone (1962) and Gray (1964, pp.76-81).

If a sufficient quantity of an antigen to which an animal has become hypersensitive is introduced into the bloodstream of the animal, a general response described as systemic anaphylactic shock is

produced. The signs of distress associated with this response vary according to the species of the animal, but many shocked animals show signs of laboured breathing, cyanosis, decrease in spontaneous activity, prostration, convulsive kicking movements and they may die, often within one hour of being injected with the antigen. When animals which have died of anaphylactic shock are dissected, characteristic changes in their tissues and organs often are seen. Their lungs may be distended and the gut and skin usually is congested with blood. There also may be failure of the ventricles of the heart, especially the right ventricle, but the auricles may still be beating immediately after the animal dies. Animals that have not developed an immediate form of hypersensitivity to the antigen in question usually do not show these signs of distress when they are intravenously injected with the antigen. Changes similar to those described above sometimes can be produced by injecting animals with substances generally referred to as anaphylactoid agents (e.g. snake venoms) but true systemic anaphylaxis can be distinguished from the anaphylactoid response because systemic anaphylactic shock occurs only in animals that have received a sensitizing injection of the antigen and have passed through a latent period of at least 8-10 days. The anaphylactoid reaction can be induced in animals that have not received a sensitizing injection of the anaphylactoid agent.

Systemic anaphylactic shock may be induced in animals that have been sensitized with antigens (active systemic anaphylaxis) or

with injections of serum taken from animals that have been sensitized with antigens (passive systemic anaphylaxis). A latent period of up to 24 hours must lapse before anaphylactic shock can be induced in passively sensitized animals. Serum from sensitized animals may be rendered incapable of passively sensitizing other animals if the antibody in the serum is allowed to react in vitro with an equivalent amount of antigen to produce insoluble antigen-antibody complexes. If soluble complexes are formed in the presence of an excess of antigen, sensitised animals injected with these soluble complexes may immediately show signs of systemic anaphylactic shock.

Animals which have experienced severe but non-fatal anaphylactic shock pass through a period during which they are insensitive to further injections of the antigen which initially caused the shock. They may be de-sensitized for a period of up to three weeks after which they again become sensitive to the appropriate antigen.

A local form of anaphylaxis also may be produced if small quantities of an appropriate antigen are introduced into the tissues of a hypersensitive animal. This form of response is discussed further in section 3.12.

It is believed that pathological changes associated with both systemic and local anaphylaxis are brought about by the action of pharmacologically active substances such as histamine and serotonin. These substances apparently are released by mast cells when antibodies present on the surfaces of these cells react

with antigens. The permeability of blood vessels in the tissues into which these substances are released increases and the smooth muscle fibres in the tissues contract. Widespread reactions of this kind are sufficient to produce the changes characteristic of systemic anaphylactic shock.

3.11 The induction of systemic anaphylactic shock in mice sensitised to spargana

3.111 Active systemic anaphylaxis

A condition which clearly resembled active systemic anaphylaxis could be induced in mice infected with the spargana used in the present study but not in uninfected mice. This was demonstrated when 25 mice each of which had been infected with two spargana for three weeks and 25 uninfected mice were injected with a quantity of the standard extract of spargana (SES). Each mouse was given an intravenous injection of the extract administered at a dose rate of 0.1ml of extract for each ten grams of body weight (0.1ml SES/10gm body weight). The infected mice all showed signs of severe distress and 21 of them died 20 to 30 minutes after they were injected. The remaining four infected mice recovered and appeared to be normal again within four hours. The signs of distress shown by the living mice and the pathological changes seen when the dead mice were dissected and examined are summarized in Table 3.1. These signs of shock clearly resembled those which normally are seen in mice that have undergone anaphylactic shock (see Weiser, Golub and Hamre, 1941). The uninfected mice

Table 3.1

The signs of anaphylactic shock shown by mice infected with spargana when those mice were injected with the extract of spargana

Stage of shock	External signs	Internal signs
Early stage (2-15 minutes after injection of SES)	Agitation, scratching, dyspnoea, blanching of ears and feet, decrease in activity	
Late stage (5-20 minutes after injection of SES)	Acute dyspnoea, reddening of ears and feet, weakness and prostration, convulsions and death	Marked congestion of skin and gut, mild emphysema of lungs and heart failure (especially right ventricle)

showed no signs of anaphylactic shock when they were injected with the extract of spargana, so it was considered that the mice infected with spargana had not produced an anaphylactoid response to the injections of the extract of spargana.

It was shown that mice which survived severe anaphylactic shock were temporarily desensitized to further injections of the standard extract of spargana. Ten mice each of which had been infected with two spargana for three weeks and had survived severe anaphylactic shock induced in the manner described above, were injected intravenously with some of the extract of spargana. Each mouse was given a second dose of the extract (0.1ml SES/10 gm body weight) given six hours after it had recovered from severe anaphylactic shock. None of the ten mice showed signs of anaphylactic shock on being injected for the second time. These mice were retained for six weeks after which they were again injected intravenously with a dose of the standard extract of spargana (0.1ml SES/10 gm body weight). All ten mice showed signs of severe anaphylactic shock and eight of them died within forty minutes of being injected.

One experiment was done to determine the length of the latent period which lapsed before mice became hypersensitive to spargana. In this experiment, a group of 40 mice was divided into four groups of ten mice each. These were labelled groups A,B,C and D. The mice of group A were fed with two spargana each at the beginning of the experiment, the mice of group B were fed with two spargana

each five days later and the mice of group C were fed with two spargana each after a further five days. No spargana were given to the mice of group D. Five days after the mice of group C had been infected each mouse in every group was given an intravenous injection of a dose of the extract of spargana (0.1 ml SES/10 gm body weight). In addition, ten mice each of which had been infected with two spargana for three weeks were given similar injections. The results obtained in this experiment are summarized in Table 3.2. The signs of shock exhibited by many of the mice used in this experiment were the same as those described in Table 3.1 so it was assumed that these mice had undergone systemic anaphylactic shock. The results of the experiment described above therefore indicated that mice became hypersensitive to the antigens of spargana after they had been infected with spargana for only 10 days.

3.112 Passive systemic anaphylaxis

Although mice not infected with spargana were insensitive to injections of the extract of spargana, they could be passively sensitized to these injections by being injected with serum taken from infected mice. This was demonstrated in the following manner. Thirty mice were divided into two groups each of 15 mice. Each mouse of one of these groups was injected intravenously with a quantity of pooled serum (0.1ml of serum/10 gm body weight) taken from twenty mice each of

Table 3.2

The reactions observed when mice infected with spargana for various periods of time were injected intravenously with the standard extract of spargana

Number of mice injected	Number of spargana per mouse	Duration of infection	Numbers of mice showing signs of shock of the indicated intensity			
			No shock	Moderate shock	Severe shock	Death
10	2	5 day	10	0	0	0
10	2	10 day	0	2	6	2
10	2	15 day	0	0	4	6
10	2	21 day	0	1	5	4
10	0	-	10	0	0	0

which had been infected with two spargana for three weeks. Each mouse of the other group was injected with a similar quantity of pooled serum taken from twenty mice that had not been infected with spargana. Ten hours later each mouse was injected intravenously with some of the standard extract of spargana (0.1ml SES/10 gm body weight). Twelve of the mice that had been passively sensitized

with serum taken from mice infected with spargana showed signs of severe anaphylactic shock and the remaining three mice of the same group showed signs of moderate anaphylactic shock in that their breathing was laboured and they became quiescent. The mice injected with serum taken from uninfected mice, however, showed no signs of shock.

It was stated above that serum which is capable of passively sensitizing animals to injections of antigens may be rendered incapable of sensitizing the animals if the antibody in the serum is neutralized (combined) with an equivalent amount of antigen to form an insoluble antigen-antibody complex. In the present study it was shown that the addition of the correct quantity of the standard extract of spargana to pooled serum taken from mice infected with spargana made that serum partially or completely incapable of passively sensitizing uninfected mice to injections of the standard extract of spargana. It was difficult to determine exactly how much of the standard extract of spargana should be added to a sample of serum to neutralize the antibody in that serum. Mice made up to 10 antibodies to spargana (see section 3.13) and the concentration of each of these antibodies in any sample of serum was unknown as was the concentration of the corresponding antigen present in the standard extract of spargana. It therefore was not possible to add to any sample of serum a quantity of the standard extract of spargana which would contain all 10 antigens at concentrations known to be equivalent to the concentrations of the

corresponding antibodies in that serum. However, by using the immuno-diffusion method described in section 3.13, it was possible to find out how much of the standard extract of spargana was needed to neutralize the more concentrated antibodies in any sample of serum but serum treated in this manner probably contained some soluble antigen-antibody complexes formed in the presence of an excess of antigen. It therefore seemed reasonable to suggest that mice injected with this serum may immediately show signs of anaphylactic shock, but if they survived the effects of this first shock they may be insensitive to later injections of the standard extract of spargana. Evidence which indicated that this suggestion was correct was obtained in the experiment described below.

Some samples of pooled serum taken from fifteen mice each of which had been infected with five spargana for six weeks were used in the gradient type of immunodiffusion test (see section 3.13) in order to determine the amount of the standard extract of spargana which was needed to neutralize the most concentrated of the antibodies in the serum. The test showed that, with respect to the most concentrated antibodies, the serum and a solution of the standard extract of spargana diluted to one sixteenth of its normal concentration (SES/16) were immunologically equivalent. The pooled serum from which these samples were taken then was divided into two lots each of 2ml of serum. To one lot of serum was added 2ml of the

diluted extract of spargana (SES/16) and to the other lot was added 2ml of physiological saline solution. The two solutions were mixed thoroughly and then were incubated at 5°C for 15 hours. They then were centrifuged at 10,000g for 15 minutes and the supernatant liquid recovered was injected into mice that had not been infected with spargana. Ten mice were each injected intravenously with a dose of the serum treated with physiological saline solution only (0.2ml of treated serum/10 gm body weight) and ten mice were each injected intravenously with a similar dose of serum that had been treated with the extract of spargana. Three of the mice injected with the serum which had been treated with the extract of spargana immediately showed signs of mild anaphylactic shock but they seemed to be normal again twenty minutes later. The remaining mice in both groups showed no signs of shock. Ten hours later, every mouse was injected with a dose of the standard extract of spargana (0.1ml of SES/10gm body weight). Within fifteen minutes of being given this injection, the mice which had received the preliminary injection of serum treated with physiological saline solution only showed signs of moderate to severe anaphylactic shock but the mice which had received preliminary injections of serum treated with the extract of spargana showed no signs of shock up until the time when the experiment was ended five hours later. The mice used in the experiment

therefore responded in the predicted manner to the injections that they were given.

3.12 The induction of cutaneous anaphylaxis in mice infected with spargana

If an antigen is injected into the skin of an animal that has developed an immediate form of hypersensitivity to that antigen, a local response known as cutaneous anaphylaxis is produced (Gladstone (1962) and Ovary (1958,1964)). Cutaneous anaphylaxis seems to be a local response similar in nature to the general response produced when animals undergo systemic anaphylactic shock (see Gladstone, 1962). The response can be clearly demonstrated if the animals in which cutaneous anaphylaxis is induced are given intravenous injections of a vital dye such as Evans blue. As a result of the increased permeability of the capillaries in the region of the skin which undergoes local anaphylaxis, the dye is lost from the capillaries and accumulates specifically in the affected tissues (Ovary, 1958).

It was demonstrated that a response which was considered to be cutaneous anaphylaxis developed in the skin of mice infected with spargana when these mice were given intra-cutaneous injections of small quantities of the standard extract of spargana. Twenty mice were divided into two groups of ten mice each and each of the mice in one group was infected with two spargana. Three weeks later the mice were lightly anaesthetized and the hair was carefully clipped from the skin at two sites on the abdomen of each of the infected and the

uninfected mice. Each mouse then was given an intra-cutaneous injection of 0.01ml of the standard extract of spargana at one of these sites and an injection of 0.01ml of physiological saline solution at the other site. These solutions were injected using the method described by Ovary (1958). Immediately after being injected each mouse was given an intravenous injection of an 0.5 per cent solution of Evans blue in physiological saline solution administered at a dose rate of 0.1ml of solution per 10gm of body weight of the mouse (Ovary, 1958). The reaction was allowed to develop for 30 minutes (Ovary, 1958) and the mice then were killed. The underside of the skin at each of the injected sites was examined. An intensely blue patch 8-12mm in diameter was present on the underside of the skin where each of the infected mice had been injected with the standard extract of spargana. Either no blue patches or blue patches less than 1.5mm in diameter were found at the sites in the skin where the infected mice had been injected with saline solution and at the sites where the uninfected mice had been injected with saline solution or the standard extract of spargana. These results indicated that local cutaneous anaphylaxis had been induced at the sites where the extract of spargana had been injected into the skin of mice infected with spargana.

Cutaneous anaphylaxis could be induced in mice infected with spargana for only 10 days. This was demonstrated in the following way. A group of 40 mice was divided into four groups of ten mice

each. The mice of three of these groups were infected with two spargana each in the manner described above. The mice of the fourth group were not infected with spargana. When the mice of the three groups which had been given spargana had been infected for 5, 10 and 15 days respectively, all the forty mice were used in tests for cutaneous anaphylaxis. Themethod used to test for cutaneous anaphylaxis in these mice was the same as that used in the experiment described above. The results of this experiment are summarized in Table 3.3. These results supported the hypothesis that cutaneous anaphylaxis could be induced in mice infected with spargana for 10 or more days and they also indicated that a latent period of 5-10 days was required before cutaneous anaphylaxis could be induced in infected mice.

The results of the experiments described above clearly supported the hypothesis that mice develop an immediate form of hypersensitivity to spargana. During the course of the experiments on active systemic anaphylaxis 55 mice that had been infected with spargana for 15 or more days were injected with the standard extract of spargana. All of these mice underwent severe shock and 39 of them died. Raffel (1961, p.297) pointed out that it is generally difficult to induce fatal anaphylaxis in mice but that with the correct administration of the sensitizing doses of antigens, a mortality rate of up to 70% may be attained. The antigens of spargana therefore seemed to have the

Table 3.3

The demonstration of cutaneous anaphylaxis in mice infected with spargana for various periods of time

Number of mice	Duration of infection	Number of spargana per mouse	Solution injected at test site	Number of sites showing blue spots of the stated sizes			
				<3mm	3-6mm	6-9mm	9mm
10	0 days	0	saline	10	0	0	0
			SES	10	0	0	0
10	5 days	2	saline	10	0	0	0
			SES	9	1	0	0
10	10 days	2	saline	10	0	0	0
			SES	0	1	7	2
10	15 days	2	saline	10	0	0	0
			SES	0	0	4	6

ability to sensitise mice as effectively as most other antigens.

3.2 THE HUMORAL RESPONSES PRODUCED BY MICE TO SPARGANA

It was pointed out in section 1.2 that antigens which induce an immediate form of hypersensitivity in an animal usually stimulate the production of circulating antibodies. Mueller (1961) suggested that serum from mice infected with spargana may contain antibodies to the antigens of spargana. However, he (Mueller, 1961) produced relatively little experimental evidence to support his suggestion. Attempts therefore were made to demonstrate clearly that mice produce circulating antibodies to spargana and to try to estimate the strength of the antisera produced by mice infected with spargana.

3.21 The demonstration of precipitating antibodies in the serum of mice infected with spargana

The gradient form of immunodiffusion test (section 2.315) initially was used to test for precipitating antibodies produced by mice to spargana. Samples of pooled serum from 10 uninfected mice and samples of pooled serum from 10 mice each of which had been infected with five spargana for six weeks were allowed to react with serial fourfold dilutions of the standard extract of spargana (SES, SES/4, SES/16, SES/64, SES/250, SES/1000). All the solutions of the standard extract of spargana except that represented by the fraction SES/1000 reacted with the serum from the infected mice to produce bands of precipitate. The most dense and well defined bands of precipitate appeared in the gel

charged with a solution of the extract of spargana represented by the fraction SES/16 (see Figure 3.1 (a)). This gel clearly contained two bands of precipitate. These two bands of precipitate could not be distinguished in the gels charged with other dilutions of the standard extract of spargana because the bands of precipitate which formed in these gels were broad and diffuse. Kabat and Mayer (1961,p.84) and Ouchterlony (1964) pointed out that when immunologically equivalent solutions of antigens and antibodies are used in immunodiffusion tests, the bands of precipitate formed are sharply defined whereas relatively broad and diffuse bands of precipitate appear if the solutions of antigens and antibodies used are not immunologically equivalent. It therefore seemed that the serum used in the experiment described above and a solution of the standard extract of spargana represented by the fraction SES/16 respectively contained approximately equivalent amounts of the antibodies and antigens which reacted to produce the bands of precipitate described. The pooled serum taken from the 10 uninfected mice did not react with any of the solutions of the standard extract of spargana to produce bands of precipitate (see Figure 3.1 (a)). Essentially the same results were obtained when this experiment was repeated with sera taken from different groups of uninfected and infected mice.

It also was demonstrated that if serum from mice infected with spargana was treated with a sufficient quantity of the standard

Legend to Figure 3.1

(a) A photograph of a gel used to test for precipitating antibodies in sera from mice infected with spargana and uninfected mice (x4).

(b) A photograph of a gel used to demonstrate the absorption of antibody in the serum of mice infected with spargana. The bands of precipitate which appeared in this gel were at a different angle and were less dense than those shown in the photograph in Figure 3.1 (a) because of the dilution of the serum used for the test (x4).

(SIM - Pooled serum from 10 mice infected with spargana;

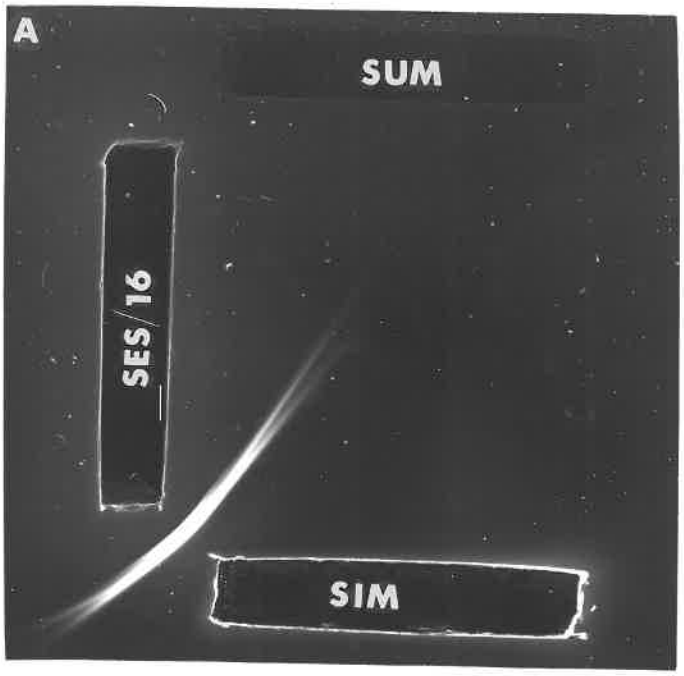
SUM - Pooled serum from 10 uninfected mice;

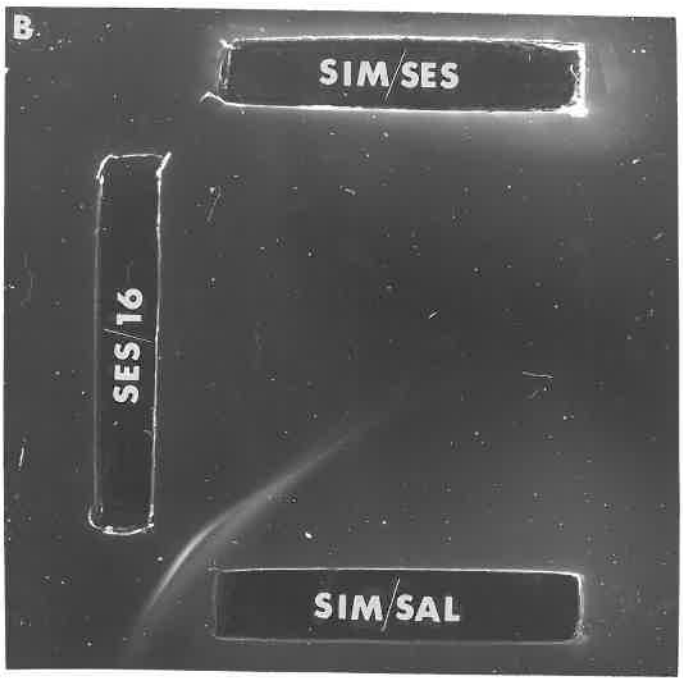
SES/16 - Standard extract of spargana/16;

SIM/SES - Serum mixed with the standard extract of spargana

SIM/SAL - Serum mixed with physiological saline solution)

Figure 3.1





extract of spargana, no bands of precipitate formed when that serum was used in the gradient form of immunodiffusion test. Pooled serum was taken from a group of 10 mice each of which had been infected with five spargana for six weeks. The results of some preliminary immunodiffusion tests showed that this serum reacted with a solution of the standard extract of spargana represented by the fraction SES/16 to produce two well defined bands of precipitate. This solution of the standard extract of spargana and the serum used therefore presumably contained approximately equivalent concentrations of antigens and antibodies respectively. Two 0.5ml samples of the serum then were taken. To one of these samples was added 0.5ml of physiological saline solution and to the other sample was added 0.5ml of SES/16. The two samples of serum were incubated at 5°C for 12 hours and they then were centrifuged at 10,000g for 10 minutes. The results obtained when portions of these two samples of sera were used for the gradient type of immunodiffusion test showed that only the serum treated with the physiological saline solution of the standard extract of spargana (SES/16) to produce bands of precipitate (see Figure 3.1 (b)). Crowle (1961, pp.251-255) pointed out that an antibody can be absorbed out of a sample of serum if a sufficient quantity of the appropriate antigen is added to the serum and that sera from which antibodies have been removed in this way are no longer capable of reacting in

immunodiffusion tests to produce bands of precipitate. The results obtained in the experiments described above therefore clearly supported the hypothesis that mice produce precipitating antibodies to spargana (Mueller, 1961).

3.22 The number of precipitating antibodies produced by mice to spargana

Crowle (1961, p.63) mentioned that each band of precipitate produced when mixtures of antigens and antibodies are used in the gradient type of double-diffusion test usually represents one antigen-antibody system. Because two bands of precipitate appeared when serum from mice infected with spargana was used in the gradient type of immunodiffusion test (see section 3.21) it seemed likely that spargana produced at least two substances that are immunogenic to mice. It was subsequently shown that between two and eight bands of precipitate were produced when sera taken from various groups of infected mice were allowed to react with the electrophoretically separated constituents of the standard extract of spargana. Although no more than eight bands of precipitate appeared in each of the gels used for the immunoelectrophoresis tests, it was found that when a number of different gels were compared, these bands of precipitate were located in 10 distinct positions in the gels. According to Grabar (1964), each band of precipitate formed in gels used for immunoelectrophoresis tests usually represents the complexes formed between one kind of antigen and its corresponding antibody. Therefore it seemed that the standard extract of spargana probably contained

10 substances which could act as antigens to mice but the pooled serum taken from any single group of mice contained little or no antibody to at least two of these antigens. The diagram in Figure 3.2 (a) shows the 10 positions in which thebands of precipitate appeared. A photograph of the bands of precipitate which appeared in one gel is shown in Figure 3.2 (b) and a photograph of a gel in which the constituents of the standard extract of spargana were electrophoretically separated and then stained is shown in Figure 3.2 (c). The photograph in Figure 3.2 (b) shows that the bands labelled M9 and M10 were more dense than the other bands of precipitate produced and it was assumed that these two dense bands of precipitate were equivalent to the two bands of precipitate which appeared in gels used for the gradient type of immunodiffusion test (section 3.21).

The number and the identity of the bands of precipitate which appeared when samples of pooled sera taken from eight groups of infected mice were used in immunoelectrophoresis tests are indicated by the results summarised in Table 3.4. These results clearly showed that there was a considerable variation in the number of detectable antibodies produced by mice to spargana. Moreover, the density and curvature of the bands of precipitate produced in each of the 10 positions described above also varied to some extent. Grabar (1964) pointed out that the density and curvature of the bands of precipitate produced

Legent to Figure 3.2

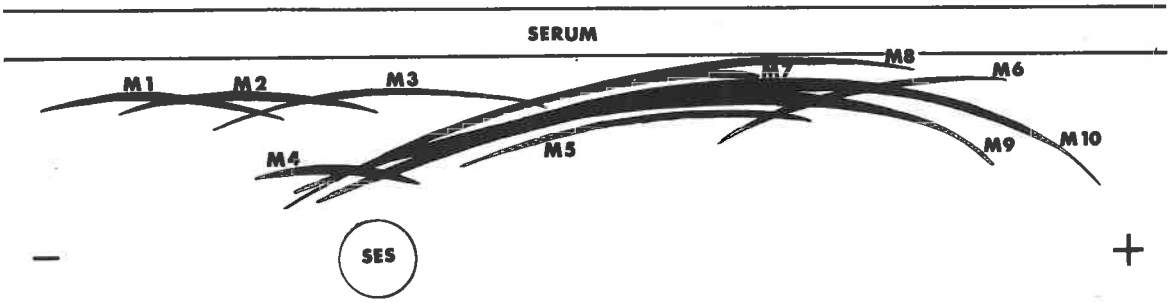
(a) Diagram of the bands of precipitate which appeared when serum from mice infected with spargana reacted with the electrophoretically separated constituents of the standard extract of spargana.

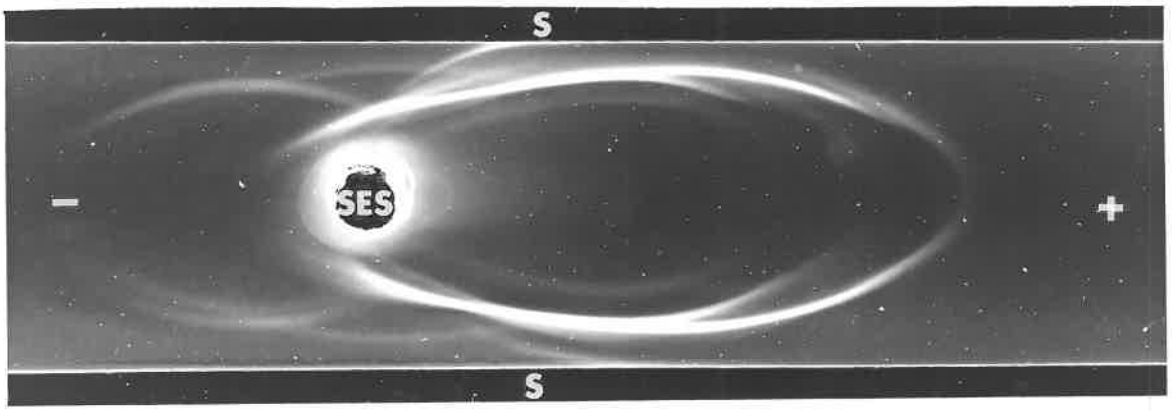
(b) A photograph of one gel in which eight bands of precipitate appeared (x3).

(c) The electrophoretically separated proteins of the standard extract of spargana (Ionagar, pH8.2/μ=0.05; stained with 1% amidoblack 10B) (x3).

(SES - Standard extract of spargana; S-Serum from mice infected with spargana; - - Cathode; - Anode)

Figure 3.2





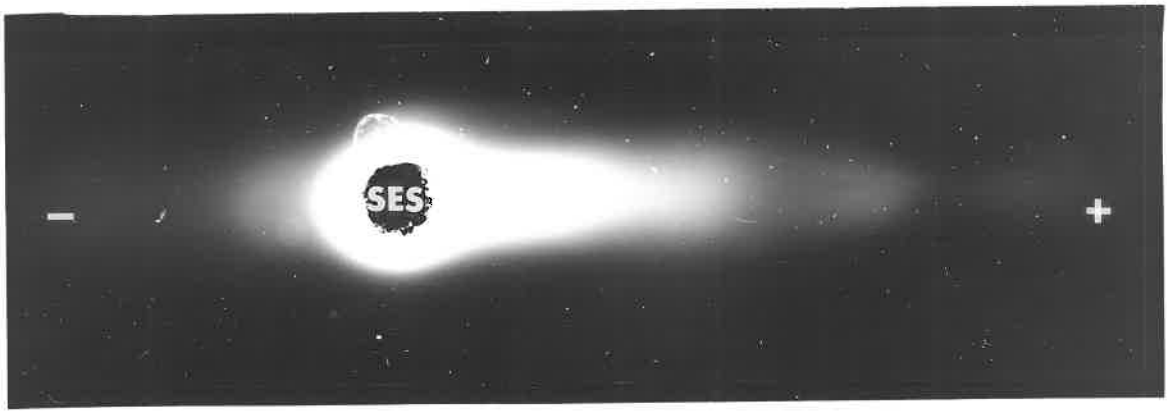


Table 3.4

The number and the identity of the bands of precipitate which appeared when samples of eight different sera were used in immunoelectrophoresis tests

Sample of serum *	Number of mice	Number of spargana per mouse	Duration of infection	Number of bands of precipitate produced	Identity of bands of precipitate
1	10	2	4 weeks	2	M2,M10
2	10	2	8 weeks	2	M2,M10
3	10	2	16 weeks	4	M2,M5,M9,M10
4	10	5	6 weeks	5	M1,M3,M4,M9, M10
5	10	5	6 weeks	7	M1,M2,M3,M5, M6,M9,M10
6	10	5	6 weeks	8	M1,M2,M3,M5, M7,M8,M9,M10
7	10	5	6 weeks	7	M1,M3,M5,M7,M8, M9,M10
8	10	20-70	10 weeks	5	M1,M2,M5,M9,M10

* Serum from mice pooled

in immunoelectrophoresis tests depends partly on the relative concentrations of the antigen and antibody present in the solution used for each test. Because fixed quantities of serum and the standard extract of spargana were used for each immunoelectrophoresis test, it seemed likely that the observed variation in the density and curvature of bands of precipitate produced in gels used for immunoelectrophoresis tests was a manifestation of the variation in the concentration of the antibodies present in the sera examined. Such variations in the number and quantity of the antibodies produced by animals which have been immunised with complex mixtures of antigens is commonly observed (Grabar, 1964).

Some tests were done to determine the nature of the antigens of spargana. Gels containing the electrophoretically separated constituents of the standard extract of spargana and other gels in which bands of precipitate had been produced when these constituents reacted with the serum from infected mice were stained for lipids using oil red O stain and for polysaccharides using alcian blue stain and the p-phenylenediamine oxidation reaction (see section 2.36).

Although polysaccharides and traces of lipids were detected in the gels containing the electrophoretically separated constituents of the extract of spargana, attempts to demonstrate the presence of these substances in bands of precipitate which were formed when these constituents reacted in agar gel with serum from infected mice

failed. It therefore seemed likely that most or all of the antigens produced by spargana were proteinaceous but it is possible that spargana may have released some polysaccharide antigens which failed to become stained with the stains used.

3.23 The amount of precipitating antibody produced by mice to spargana

The absolute amount of precipitating antibody present in any serum can be determined by means of the quantitative precipitation reaction only (see Kabat and Mayer, 1961 pp.23-35). However, it is necessary to use either pure antigens or pure antibodies for this reaction (Kabat and Mayer, 1961, p.35). Evidence which indicated that mice may produce up to 10 different kinds of antibodies to spargana was given in section 3.22. The standard extract of spargana therefore presumably contained at least 10 substances which could act as antigens to mice. It clearly was impractical to try to purify either all 10 of these antigens or the antibodies produced to them in order to do quantitative precipitation analyses, so the absolute amount of antibody produced by mice to spargana could not be determined. Nevertheless, an attempt was made to estimate the relative amounts of antibody produced either by mice infected with various numbers of spargana or by mice infected with spargana for various periods of time. The immunodiffusion test used to made the estimates was similar to that described by Kabat and Mayer (1961,pp.83-85) and Preer (1956) except that short lengths of tubing

which were open at both ends were used in the present study (see section 2.315) wereas Kabat and Mayer (1961, p.83-85) and Preer (1956) described a technique which employed tubes which were closed at one end (test tubes). The procedure followed to make each of the estimates is described below.

Each of the tests was conducted in two stages. First, it was necessary to prepare a solution which contained antigen at a concentration immunologically equivalent to the concentration of the antibody present in the serum to be tested. The correct concentration of antigen to use was determined by allowing samples of the serum to react in agar gels with serial twofold dilutions of the standard extract of spargana. The gels then were inspected to find out which contained the most clearly defined band of precipitate. The solutions used to charge the gel in which the clearly defined band of precipitate appeared was considered to be immunologically equivalent to the serum being tested (see section 3.21). In most instances, it was found that one dense and a number of faint bands of precipitate appeared in the gels but attention was paid to only the dense band of precipitate because the most concentrated antibodies in the serum presumably were incorporated in this band. The number of different kinds of antibodies incorporated in this dense band of precipitate was not determined.

In the next stage of the estimation, serial twofold dilutions of the serum were allowed to react in agar gels with immunologically equivalent solutions of the antigens of spargana to find out how

much the serum could be diluted before it became incapable of producing a band of precipitate. This limit was called the precipitation threshold of the serum.

The results obtained when the relative amounts of antibody present in a number of different sera were estimated are summarised in Table 3.5. Ratios have been used in this table to indicate the precipitation threshold of each of the sera. These results confirmed the suggestion (section 3.22) that there is a considerable variation in the amount of antibody produced by mice to spargana. Nevertheless, it seemed clear that the serum of mice infected with many spargana contained more antibody than the serum of mice infected with relatively few spargana.

The serum taken from mice that had been infected with spargana for only three weeks apparently contained only small quantities of precipitating antibody and no antibody was detected in the serum taken from mice infected with spargana for only two weeks. Similar results were obtained when two pooled sera that were obtained from two groups of 10 mice each of which had been infected with two spargana for three weeks were tested for antibody. No precipitating antibody could be detected in thepooled serum from one of these groups of mice and the serum from the other group had a precipitation threshold of 1:4. It was shown in section 3.1 that mice infected with spargana for

Table 3.5

Estimates of the amount of antibody in sera taken from various groups of mice infected with spargana

(a) Pooled sera

Number of mice	Number of spargana per mouse	Duration of infection	Precipitation threshold*
10	2	6 weeks	1:16
10	5	6 weeks	1:16
10	10	6 weeks	1:16
7	20-25	8 weeks	1:64
10†	25-70	10 weeks	1:128
10	2	2 weeks	No antibody detected
10	2	3 weeks	1:1
10	2	4 weeks	1:4
10	2	8 weeks	1:32
10	2	16 weeks	1:32

* The extent to which the serum could be diluted with physiological saline solution before it became incapable of reacting with antigen to produce bands of precipitate in immunodiffusion tests

† Mice initially infected with procercoids

Table 3.5 (Contd.)

(b) Non-pooled sera

Number of mice	Number of spargana per mouse	Duration of infection	Precipitation threshold
1	5	6 weeks	1-4
1	5	"	1-8
1	5	"	1-8
1	5	"	1-8
1	5	"	1-16
1	5	"	1-16
1	5	"	1-32
1	5	"	1-64

only 10 days became hypersensitive to injections of the standard extract of spargana so it seemed reasonable to propose that if this hypersensitivity was mediated by precipitating antibodies, it should be easy to demonstrate the presence of these antibodies in the serum of mice infected with spargana for 14-21 days. According to Kabat and Mayer (1961, p.299) the form of immunodiffusion test described by Preer (1956) is one of the most sensitive tests which can be used to detect precipitating antibody but this test failed to detect such antibody in samples of the sera taken from two groups of mice infected with spargana for up to three weeks. It seemed that there were two possible explanations which could account for this observation. Firstly, the sera of mice infected with spargana for 10-21 days may contain antibodies in quantities which often may be too small to be detected by means of immunodiffusion tests. Secondly, mice infected with spargana for 10-21 days may produce non-precipitating (reaginic) antibody but little or no precipitating antibody. It has been shown that a number of species of helminthes can stimulate mammals to produce antibodies that resemble human reaginic antibodies in that they have an affinity for skin and they do not form precipitates when they combine with antigens (Dobson, Campbell and Webb, 1967; Ogilvie, 1964; Ogilvie, Smithers and Terry, 1966).

Attempts to use methods other than immunodiffusion techniques to demonstrate the presence of precipitating or non-precipitating

antibodies in serum taken from mice infected with spargana for up to three weeks failed either because practical difficulties were encountered or because negative results were obtained. The passive haemagglutination test (Boyden, 1951) is considered to be one of the most sensitive serological tests which can be used to detect divalent (precipitating) antibody (Kabat and Mayer, 1961, p.299) so it seemed reasonable to use this test to try to demonstrate the presence of antibody in the serum of mice infected with spargana for 2-3 weeks.

The haemagglutin test normally is done with sheep erythrocytes onto which molecules of antigens have been absorbed. Such sensitised erythrocytes normally do not agglutinate until they are mixed with antibody. However, it was found that when erythrocytes of sheep were exposed to the substances present in the standard extract of spargana they agglutinated spontaneously. These erythrocytes clearly could not be used for the passive haemagglutination test. No attempt was made to find out if the test could be done with erythrocytes of animals other than sheep.

Kabat and Mayer (1961, p.299) mentioned that the response described as passive cutaneous anaphylaxis can be used to detect antibody in quantities as small as those which can be detected by means of the passive haemagglutination test. To induce passive cutaneous anaphylaxis, a sensitising dose of serum which contains antibody is injected into the skin of a normal (non-immune)

animal and the dose of shocking antigen is injected intravenously into the animal some time later (Kabat and Mayer, 1961, p.272-273; Ovary, 1964). Evans blue usually is incorporated in the solution of antigen in order to make the reaction more clearly visible (Kabat and Mayer, 1961, p.272). Two experiments were done to find out if this technique could be used to demonstrate the presence of antibodies in the serum of mice infected with spargana for 2-3 weeks. In the first of these experiments, pooled serum that was taken from eight mice each of which had been infected with two spargana for two weeks was tested for antibodies. Twenty uninfected mice were taken and the hair was carefully clipped from the skin at two sites on the abdomen of each mouse (see section 3.12). The skin at one of the sites on each mouse was injected with 0.02ml of physiological saline solution. The other site on each of 10 of the mice was injected with 0.2ml of the serum from the infected mice and the other site on each of the remaining 10 mice was injected with 0.2ml of pooled serum taken from mice that had not been infected with spargana. Six hours later, each of the 20 mice was injected with a solution of 0.5% Evans blue in the standard extract of spargana (0.1ml SES/10gm body weight). Thirty minutes later each mouse was killed and the underside of the skin at the injected sites was examined. Blue spots 1-2mm in diameter were found at all of the sites. Similar results were obtained when, in the second experiment which was done, pooled serum

from 10 mice each of which had been infected with two spargana for three weeks was tested for antibodies. These results clearly failed to demonstrate the presence of antibody in the samples of serum tested. However, this did not necessarily mean that the samples of serum examined contained no antibody. In a discussion of the factors which may affect passive cutaneous anaphylaxis, Ostler (1963) pointed out that estimates of the minimum amount of antibody which can be detected by means of passive cutaneous anaphylaxis have been based on results obtained from experiments in which strong sera were diluted until they were no longer capable of sensitising the skin of recipient animals. He (Ostler, 1963) also mentioned that previous workers have produced evidence which suggests that 'normal' gamma-globulin may interfere with the fixation of antibody to the tissues at the injected site in the skin of the recipient animal and he stated that 'an important consequence of this finding is that the sensitivity of PCA (passive cutaneous anaphylaxis) may be reduced as much as a thousand-fold with weak antisera'. Furthermore, it seems that the amount of antibody required to sensitise passively the skin of rodents such as rats and mice is considerably greater than that required to passively sensitise the skin of most other mammals (Ostler, 1963). It therefore was not surprising that serum of mice infected with spargana for only 2-3 weeks was not able to sensitise passively the skin of uninfected mice. On the other hand,

it was shown that systemic anaphylactic shock could be induced in uninfected mice which had been passively sensitised to the antigens of spargana by being injected intravenously with serum taken from mice infected with spargana for only three weeks (section 3.112) and that active cutaneous anaphylaxis could be induced in mice infected with spargana for only 10 days (section 3.12). However, it was possible to propose some tentative explanations for the apparent disagreement between the results obtained by means of experiments involving passive cutaneous anaphylaxis and those obtained by means of experiments involving active cutaneous anaphylaxis or passive systemic anaphylaxis.

First, it is known that the pathological changes associated with anaphylactic shock are manifested more in some organs of the body than in others, possibly because the especially sensitive organs may contain more histamine, blood capillaries and smooth muscle tissue than the less sensitive organs (Boyd, 1956, p.401). These especially sensitive organs often are referred to as 'shock-organs'. The gut seems to be one of the main shock organs in mice (Kabat and Mayer, 1961, p.287), so it is possible that small quantities of antibody may be able to sensitize passively the tissues of the gut of mice but not the skin. Thus mice injected intravenously with relatively small amounts of antibody may be susceptible to systemic anaphylactic shock whereas it may be difficult to induce cutaneous anaphylaxis at sites in the

skin which have been previously sensitised with small amounts of antibody.

Second, it may be possible to induce active cutaneous anaphylaxis in mice whose serum cannot be used to sensitise passively the skin of other mice. Ostler (1963) pointed out that a quantity of antibody which is represented by approximately 0.5µg antibody nitrogen is required to sensitise passively the skin at one site on a mouse. In most cases it is found to be impracticable to inject more than approximately 0.04ml of serum into the skin of a mouse, so it follows that passive sensitisation of skin can be achieved using serum which contains a minimum of approximately 0.5µg antibody nitrogen per 0.04ml.

Thus serum must contain antibody at a certain minimum concentration before it can be used to passively sensitise skin. If the serum of an actively sensitised mouse contains antibody at a concentration just above the minimum required to sensitise the skin of the mouse, it should be possible to induce active cutaneous anaphylaxis in the mouse. However, it may not be possible to use serum from such a mouse to sensitise passively the skin of another mouse because during the latent period which is required for the antibody to become attached to the tissues in the skin of the recipient, the introduced serum may become diluted by the intercellular fluids of the recipient animal with the result that it is no longer capable of passively sensitising skin.

The results from the experiments involving passive cutaneous anaphylaxis also suggested that mice examined did not produce reaginic antibodies to spargana or that such antibodies could not be detected. Reaginic antibodies have a strong affinity for the tissues of skin which they are able to sensitise passively. Furthermore, skin which has been injected with a small quantity of reaginic antibody remains sensitised for a period longer than that for which skin injected with a similar quantity of precipitating antibody remains sensitive (Boyd 1956, p.417). Thus it is possible to find out if a sample of serum contains reaginic antibody by determining the period for which skin that has been injected with the serum remains sensitive. It was clear, however, that it was not possible to demonstrate the presence of reaginic antibody in the serum of mice infected with spargana for less than three weeks because such serum appeared to be incapable of passively sensitizing the skin of mice even for a short period.

Although the amount of antibody produced by mice to spargana could not be estimated accurately, it nevertheless was possible to propose tentatively that strong antisera were produced by mice infected with spargana for five weeks or more. When immunodiffusion tests were done with serum taken from such mice, dense bands of precipitate were produced. These bands of precipitate often began to appear within 2-3 hours of the start of the tests. Preer (1956) stated that when strong antisera are used in immunodiffusion tests

of the kind used in the present study to estimate the relative amounts of antibody produced by mice to spargana, bands of precipitate may appear within a few hours of the start of the tests whereas when weak antisera are used, bands of precipitate may appear only after a few days. Furthermore, Grabar (1964) pointed out that immunoelectrophoretic analysis can be carried out only with sera which contain high titres of precipitating antibody. When sera of mice infected with spargana for five weeks or more were analysed in this way, at least two dense bands of precipitate appeared in each gel used.

It therefore seemed reasonable to suggest that the sera used to charge these gels contained high titres of at least two precipitating antibodies.

3.3 THE PATHOLOGICAL CHANGES INDUCED IN MICE BY SPARGANA

3.31 The local pathological changes

A short time after the present study was started, it became clear that the local reactions stimulated in mice by the spargana of the Australian form of spirometrid tapeworm differed considerably from the local reactions stimulated by the spargana of the North American form of spirometrid. Observations made by Mueller (1961;1963) indicated that the spargana of the North American form of spirometrid stimulate few or no local response when they are located in the sub-cutaneous tissues of mice even if the mice have been infected for periods in excess of one year. However, the spargana used in the present study stimulated

marked local pathological changes even when they were located in the sub-cutaneous tissues of mice. Almost all the spargana that had been in mice for three or more weeks were enclosed in capsules most of which were located in the sub-cutaneous tissues of the neck and thoracic regions of the mice. For example, when 30 mice were examined three weeks after each of them had been infected with five spargana, eight unencapsulated and 126 encapsulated spargana were recovered. One hundred and seventeen of these spargana were in the sub-cutaneous tissues of the neck and shoulder regions of the mice and the remaining spargana were located in the sub-cutaneous tissues of abdominal or thigh regions.

It was pointed out in section 1.2 that both antigenic and non-antigenic objects can stimulate the formation of a capsule so the observation that mice formed capsules round the spargana used in the present study did not necessarily indicate that these spargana stimulated a specific local inflammatory response. However, evidence obtained when histological sections of capsules were examined supported the hypothesis that the spargana enclosed by the capsules had stimulated specific local inflammatory responses.

Histological sections of 20 capsules each of which was taken from the subcutaneous tissues of the neck of a mouse that had been infected with two spargana for four weeks were examined. These sections showed that the walls of the capsules were composed of

fibres and cells, the fibres being similar to and continuous with the fibres of the connective tissues adjacent to the capsules. The photograph in Figure 3.3 shows the appearance of a section through the wall of a typical capsule. The walls of capsules were 50-500μ thick and they contained fibroblasts and numerous leucocytes which were identified as eosinophilic leucocytes and macrophages. Scattered eosinophilic leucocytes and macrophages also were present in the tissues adjacent to each capsule. A few giant cells and neutrophilic leucocytes were present in the walls of some capsules, but no plasma cells were seen. The figures given in Table 3.6 indicate the relative numbers of neutrophilic leucocytes, macrophages and eosinophilic leucocytes present in walls of 10 of the capsules examined. These figures were obtained by counting the numbers of neutrophilic leucocytes, macrophages and eosinophilic leucocytes which were found in a group of 100 of the leucocytes present in the wall of each capsule. The results obtained clearly show that the majority of the leucocytes in the walls of the capsules examined were eosinophilic leucocytes. The cells present in sections of samples of the sub-cutaneous tissues taken from 10 infected mice also were examined to make sure that the tissues of the mice used in the present study did not normally display a marked eosinophilia. The relative number of eosinophilic leucocytes present in uninfected and infected tissues was estimated

Legent to Figure 3.3

A photomicrograph of a section through the wall of a capsule formed by a mouse around a sparganum (x500)

(C - cavity of capsule; CT - Connective tissue of mouse M - muscle fibres of mouse tissue; Sp - sparganum; W - wall of capsule)

Figure 3.3

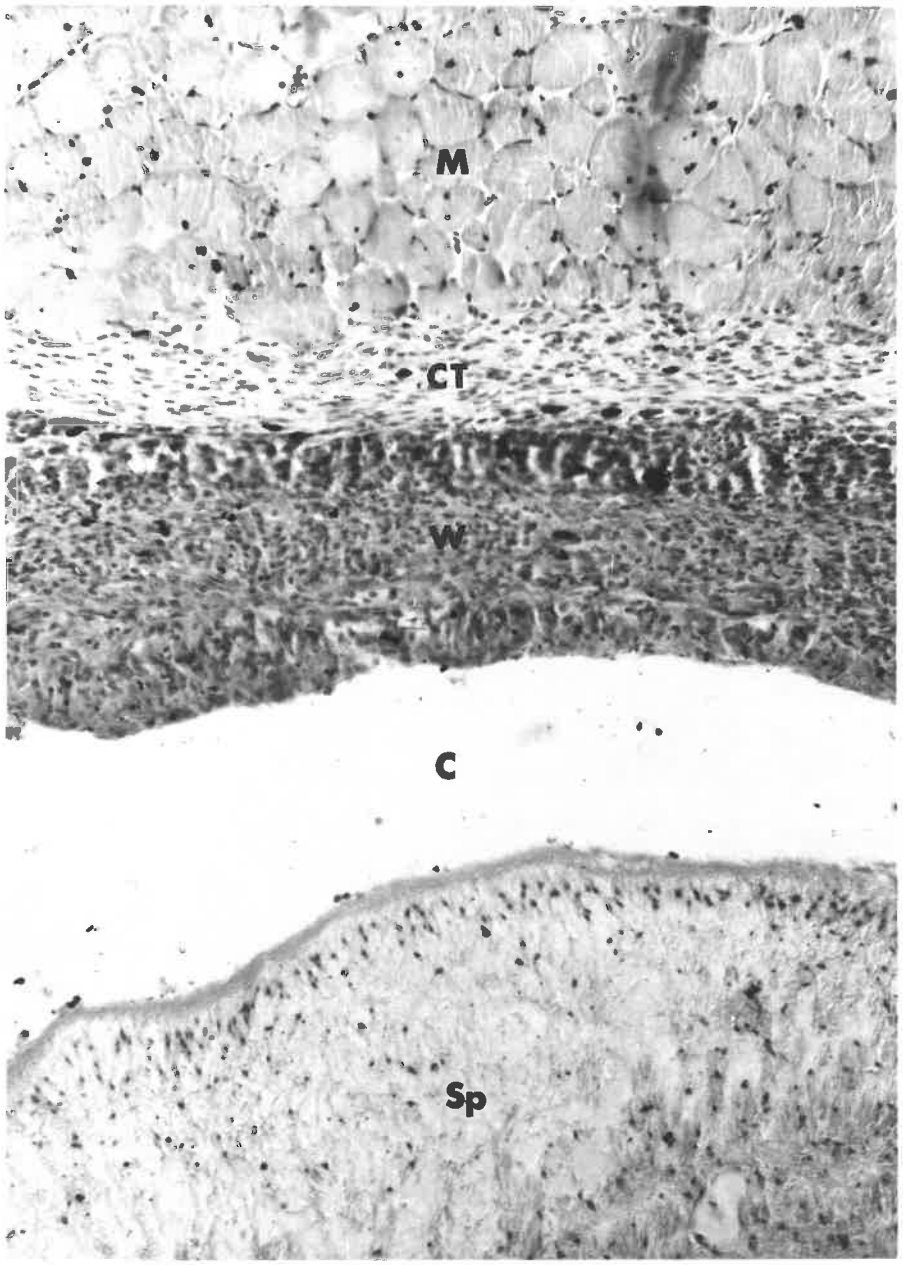


Table 3.6

The relative numbers of macrophages, eosinophilic leucocytes and neutrophilic leucocytes present in the walls of 10 sectioned capsules

(100 of the cells in the wall of each capsule identified and counted)

Capsule	Macrophages	Eosinophilic leucocytes	Neutrophilic leucocytes
1	29	71	0
2	40	59	1
3	37	63	0
4	18	82	0
5	9	91	0
6	20	78	2
7	27	70	3
8	38	61	1
9	29	71	0
10	40	59	1

by counting a number of these cells found in equivalent areas of the sections of the uninfected sub-cutaneous connective tissues and the walls of the 10 capsules mentioned above. An area of each section equal to the field of view of the 40x objective on the microscope used was examined. It was found that this area of sections through uninfected tissues contained 0-7 eosinophilic leucocytes whereas the equivalent areas of sections through the walls of capsules contained 100-300 eosinophilic leucocytes. These results clearly showed that the spargana used in the present study stimulated a marked eosinophilia of the tissues of mice. According to Archer (1963, pp. 91-108) and Litt (1962, 1964), eosinophilic leucocytes are attracted to sites in tissues in which antigen-antibody complexes have been formed, so it seemed likely that the eosinophilia of the tissues of mice infected with the spargana used in the present study was a manifestation of the specific immunological response stimulated by these spargana.

These observations on the local pathological changes induced in mice by spargana clearly were important because they suggested that the spargana used in the present study were more antigenic to mice than the spargana of the North American form of spirometrid tapeworm. However, at the time when it first became apparent that there may be a difference in the immunogenicity of the two forms of spargana, only the preliminary stages of the present study had been completed. Thus it was not

possible to be sure of the form or the intensity of the response stimulated by the spargana used in the present study. I therefore continued experimenting in order to find out whether or not the responses stimulated by these spargana could be considered to be normal. The experiments which were carried out have been described in sections 3.1 and 3.2.

The possibility that the apparent differences between the local responses stimulated by the spargana of the North American and the Australian form of spirometrid tapeworm could be the result of differences between the hosts of the parasites and that the two forms of spargana could be equally immunogenic also was considered. However, a short time after it was discovered that the spargana used in the present study stimulated mice to produce marked local responses, Mueller (1965a,b) published papers in which he stated that mice encapsulate the spargana of the Malayan form of spirometrid tapeworm more firmly than they encapsulate the spargana of the North American form of tapeworm. On the basis of these observations, Mueller (1965a,b) suggested that the spargana of the Malayan form of spirometrid tapeworm are more antigenic than the spargana of the North American form of spirometrid tapeworm. Mueller (1965a,b) clearly assumed, perhaps without justification, that the local changes stimulated by the spargana of the Malayan form of spirometrid tapeworm were manifestations of a specific rather than a non- specific

response. However, it seemed that, if Mueller (1965a,b) was correct, the spargana of the Malayan and Australian forms of spirometrid tapeworms may be similar and that these spargana may be antigenically different from the spargana of the North American form of spirometrid tapeworm. Subsequently, spargana of the kind used in the present study were sent to Mueller who, in a personal communication (Mueller, 1967) stated that the spargana of the Malayan, Formosan and Australian forms of spirometrid tapeworms seemed to be similar to each other but different from the spargana of the North American form of spirometrid tapeworm. Thus it was clear that the difference between the local responses produced by mice to spargana of the North American and the Australian forms of spirometrid tapeworms was not a manifestation of a difference between the kinds of mice which acted as hosts to these parasites. The relative antigenicity of the two forms of spargana is discussed further in section 3.5.

3.32 The general pathological changes

Although the spargana used in the present study stimulated marked local pathological changes in mice, they apparently induced few or no general changes which could be described as pathological. The results obtained from two experiments showed that mice infected with these spargana gained weight at an abnormally high rate. In each of these experiments 20 mice were randomly divided into two groups of 10 mice and the mice in both

these groups were weighed. The mice of one group in the first experiment were infected with five spargana each and the mice of one group in the second experiment were infected with 10 spargana each. Ten weeks later, the mice were again weighed. The infected mice were killed and dissected and the spargana recovered from each mouse were weighed. The weight of the spargana in each mouse was subtracted from the weight of the live mouse to find out how much weight each infected mouse had gained. The results obtained are given in Table 3.7. When the Student's t-test was applied to the results it was found that in each of the experiments there was no difference between the average starting weights of the mice in the two groups ($p \Rightarrow 0.5$) but at the end of the experiment the infected mice were significantly heavier than the uninfected mice ($p = \langle 0.05$). Thus mice infected with either the spargana of the kind used in the present study or with the spargana of Spirometra mansonoides gained weight at an abnormally high rate (see section 1.132). Mueller (1965b) found that the spargana of the Malayan form of spirometrid tapeworm also stimulated mice to gain weight at an abnormally high rate. To explain these observations, it has been proposed that spargana secrete a substance or substances which have properties similar to those of insulin (Harlow, Mertz and Mueller, 1964, 1967). It therefore was clear that no pathological changes which resulted in emaciation were induced in mice infected

Table 3.7

The results of two experiments to determine the effect of spargana on the rate at which mice gain weight

Experiment 1				Experiment 2			
Weights of uninfected mice (gm)		Weights of mice infected with 5 spargana each* (gm)		Weights of uninfected mice (gm)		Weights of mice infected with 10 spargana each* (gm)	
Start of expt.	Finish of expt.	Start of expt.	Finish of expt.	Start of expt.	Finish of expt.	Start of expt.	Finish of expt.
23.4	26.5	26.8	32.8	18.2	30.0	20.5	34.4
24.0	22.7	21.4	31.7	18.0	20.0	20.0	34.9
19.9	26.4	21.9	25.0	21.4	28.5	20.1	32.7
21.3	25.7	22.2	35.1	19.5	30.6	21.0	30.8
19.1	28.2	22.5	35.3	20.6	29.5	18.8	32.5
25.4	30.4	20.9	33.6	19.0	28.4	18.5	32.2
23.6	31.2	21.5	39.5	18.0	29.5	19.5	28.9
17.6	31.4	22.6	31.6	21.4	30.0	18.5	33.2
23.6	29.4	18.2	34.0	19.6	24.3	19.7	29.7
22.3	30.0	19.5	31.6	19.1	32.0	20.0	28.7

* Weights corrected for weights of spargana

with relatively small numbers of the spargana of either the Oriental or the North American forms of spirometrid tapeworms.

The numbers of spargana and procercoids required in order to determine the pathogenic effects of infections of large numbers of spargana became available at too late a stage in the study, so no controlled experiments concerning this aspect of the relationships between spargana and their hosts were done. However, it so happened that the large numbers of spargana which were produced at the end of the study were found in a group of 10 mice which had been injected with infected cyclops. When some of these cyclops were inspected before the batch of cyclops used was injected into the mice few procercoids were found, so it was assumed that the mice would become infected with relatively few spargana. None of these mice showed any signs of illness. Ten weeks after they had been infected, the mice were killed and examined. The number and total weight of the spargana recovered from each of the mice is shown by the figures given in Table 3.8. All except 19 of the spargana found were encapsulated and it was found that there was more antibody per millilitre of the pooled serum taken from these mice than there was in any other sample of serum tested (see Table 3.5). The spargana therefore had stimulated the mice to produce immunological responses which could be considered to be normal or even strong, but they apparently were not highly pathogenic to the mice.

It is relevant at this point to consider some of the factors which determine whether or not a parasite such as a sparganum may be

Table 3.8

The numbers and weights of sparganum found in 10 heavily infected mice

Mouse	Number of spargana recovered from mouse	Total weight of spargana (gm)	Number of spargana encapsulated
1	70	1.96	68
2	25	0.65	20
3	60	1.95	54
4	56	2.13	55
5	37	0.50	34
6	26	0.47	26
7	71	1.31	70
8	47	0.70	47
9	54	0.95	50
10	26	0.44	26

pathogenic to its host. According to Gray (1964,pp.11-18) parasitic organisms may induce local or general pathological changes in the five different ways described below:-

(1) They may release substances that are toxic to their hosts

(2) They may produce substances which suppress the phagocytic activity of the cells of the host.

(3) They may compete with the cells of the host for available nutrients.

(4) They may cause local non-specific inflammation which may interfere physically with the functioning of vital organs.

(5) They may stimulate the production of local or general specific inflammatory responses such as those normally associated with the hypersensitive (allergic) state.

If spargana contained any substances that were highly toxic to mice, it seemed reasonable to suggest that any mouse weighing say 20gm should have shown signs of the effects of toxins such as weakness, diarrhoea and possibly non-specific shock (see Gray,1964, p.13) when it was injected intravenously with 0.2ml of the standard extract of spargana. The uninfected mice used in the experiments described in section 3.11 were injected with such doses of the standard extract of spargana, but these mice showed no signs of illness

so it seemed that the standard extract of spargana either contained no toxic substances or that any toxic substances it contained were at too low a concentration to be effective. This clearly suggested that living spargana produced no substances that were highly toxic to mice or that any toxic substances they produced were present in the extracts of spargana in quantities too small to affect mice extensively.

Whether or not spargana produced anti-phagocytic substances was of relatively little importance because spargana were too large to be phagocytosed.

It seemed clear that any competition between spargana and the cells of infected mice for available food materials did not affect the mice because infected mice increased weight at an abnormally high rate so their cells apparently received an adequate supply of food materials.

Spargana caused local non-specific inflammatory responses but because the spargana used in the present study occurred exclusively in sub-cutaneous tissues the local responses that they stimulated could not interfere with the functioning of vital organs.

The observations made during the present study therefore suggested that if spargana are able to induce any pathological changes in mice they would do so by releasing antigenic substances to which the mice had become hypersensitive (allergic). Such antigenic substances could and did induce local pathological changes in the tissues near spargana, but it was unlikely that they could

have induced general pathological changes such as systemic anaphylactic shock in mice. The reasoning behind this statement can be explained by describing what is generally believed to be the probable mechanism of systemic anaphylactic shock (see Gray, 1964, pp.78-79). The pharmacologically active substances which mediate anaphylaxis (histamine, serotonin etc.) are found in the mast cells of the connective tissues. It seems that these cells also may have antibodies bound to their surfaces and when these antibodies react with antigens, they cause a disruption of the cells and the release of the pharmacologically active substances which mediate local and systemic anaphylaxis. It therefore followed that the local form of anaphylaxis may be induced when mast cells are disrupted by the local release of antigens whereas systemic anaphylaxis may be induced when antigens are widely disseminated throughout the body of the hypersensitive animal and cause widespread disruption of mast cells. The dissemination of antigens in an animal can be accomplished most readily if the antigens are released into the bloodstream of the animal. However, the blood of most hypersensitive animals contains some antibody. Antigens released into the blood therefore react with this antibody to produce antigen-antibody complexes. If such complexes are formed with an excess of antibodies, they apparently are unable to cause the disruption of mast cells. This means that the antibody in the blood of a hypersensitive animal must be

neutralised with an excess of antigen before the antigen can cause widespread disruption of mast cells and systemic anaphylactic shock. Thus it appeared that spargana would have had to release antigens in quantities in excess of those necessary to neutralise the antibodies present in the blood of infected mice and these antigens would have had to have been widely disseminated round the bodies of the mice in order to induce systemic anaphylactic shock.

Whether or not antigens released by either encapsulated or unencapsulated spargana ever entered the bloodstream of mice was difficult to determine. If suitable materials and equipment had been available, it may have been possible to use some of the immunofluorescence techniques described by Nairn (1962) to determine the distribution of antigens in the tissues of mice infected with spargana. However, this could not be done with the available equipment. Nevertheless, it seemed reasonable to suggest that no antigens escaped from the capsules surrounding spargana and entered the bloodstream of any infected mouse in quantities sufficient to neutralise all the antibody present in the blood of the mouse. All the sera taken from mice infected with spargana for four weeks or more contained antibodies detectable by means of immunodiffusion tests. These antibodies clearly were not combined with an excess of antigens because they were able to react <u>in vitro</u> with antigens to produce bands of precipitate.

Antibodies combined with an excess of antigens would not have reacted in vitro with antigens to produce bands of precipitate (see section 3.21). Therefore, all the sera tested contained an excess of antibody even though they may have contained some antigen combined with antibody. It therefore seemed unlikely that free antigens of spargana could naturally become dispersed readily enough through the bodies of infected mice to cause systemic anaphylactic shock. It may have been that the antigens were so restricted by local responses that they rarely diffused out of capsules provided that the spargana enclosed by the capsules did not migrate. If all the spargana present in any mouse had migrated at the same time, the mouse may have shown signs of anaphylactic shock induced by the widespread release of any free antigens which may have been present in the capsules which enclosed the spargana. Such a reaction sometimes is produced, for example, when hydatid cysts rupture and release antigens into the tissues of infected human beings (Soulsby, 1962). However, no responses of this kind were seen in mice infected with spargana so it seemed that either the capsules contained little or no free antigen or any antigens present in capsules were not released into the tissues of mice in sufficient quantities to cause marked general pathological changes.

3.4 THE SUSCEPTIBILITY OF MICE TO INFECTION WITH PRIMARY AND SUPERINFECTIONS OF PROCERCOIDS AND SPARGANA

Although it has been shown that mice can be infected with

procercoids and spargana of spirometrid tapeworms (see section 1.22), no previous workers attempted to obtain quantitative data in order to determine whether or not mice showed any degree of natural or acquired resistance to procercoids or spargana. The results described in sections 3.1 and 3.2 suggested that the hypersensitivity induced in mice by the spargana used in the present study could be considered to be at least normal in intensity. It therefore was possible that infected mice may display an acquired resistance to procercoids or spargana. Experiments therefore were carried out to try to determine susceptibility of mice to primary and superinfections of procercoids and spargana.

3.41 The susceptibility of mice to orally and parenterally administered procercoids and orally administered spargana

Before the experiments concerning the acquired resistance of mice to spargana are described, it is necessary to point out that mice displayed a natural resistance to orally administered procercoids of the kind used in the present study.

Mueller (1963) reported that many spargana usually were found in mice each of which had been fed with cyclops that contained a total of more than 100 procercoids of the North American form of spirometrid tapeworm. At first, therefore, I tried to produce stocks of spargana by feeding mice with cyclops infected with procercoids. During a period of a few months at the beginning of the study, 30 mice were each fed with cyclops that

contained a total of 20-25 procercoids. The procercoids used all were 14-16 days old. When these mice were dissected three weeks after they had been infected, no spargana were found. According to Mueller (1959), procercoids of the North American form of spirometrid tapeworm which were maintained at approximately 76% became infective to mice when they were nine days old. It therefore seemed unlikely that the 30 mice mentioned above failed to become infected because they were fed immature procercoids. All the procercoids used appeared to be mature and some had already lost their cercomas.

The possibility that the procercoids were unable to emerge from the cyclops used also was considered. Because of the difficulties mentioned in section 2.25, it was not possible to determine the susceptibility of mice to infection with procercoids which had been dissected out of cyclops, but it was found that no spargana developed in 10 mice each of which was fed with 20 procercoids enclosed in dead cyclops the exoskeletons of which had been extensively damaged with a needle. It therefore seemed unlikely that no spargana developed in these mice because the procercoids used to infect the mice were unable to emerge from the damaged cyclops.

It was discovered later that mice could be infected if they were injected with cyclops infected with procercoids. In one experiment a total of 30 mice were randomly divided into two groups

of 15 mice each. The mice of one group were each fed with cyclops containing a total of 20 procercoids and the mice of the other group were each given an intra-peritoneal injection of cyclops containing a total of 20 procercoids. When the mice were killed and examined three weeks after they had been given procercoids no spargana were found in the mice that had been fed with procercoids but a total of 59 spargana were recovered from the mice that had been injected with procercoids. The mean and standard error of the mean of the numbers of spargana taken from these mice was 3.8± 0.8SE. These results clearly showed that the mice used were more susceptible to intra-peritoneally administered than to orally administered procercoids of the kind used in the present study.

Figures which show that the spargana used in the present study were highly infective when fed to mice were given in section 3.3. In this section it was stated that 134 spargana were recovered from 30 mice each of which initially had been fed with the holdfasts of five spargana. Therefore, approximately 89 per cent of the spargana fed to these mice became established in the tissues of the mice. A similar proportion of the spargana used to infect mice for each of the experiments done during the course of the study also became established in the tissues of the mice. It is relevant to point out that the statements concerning the number of spargana in each of the mice used for the experiments described in sections 3.1, 3.2 and 3.3 indicate the number of spargana fed

to the mice rather than the number of spargana recovered from them. The mice were so consistantly susceptible to spargana that it clearly was reasonable to indicate the numbers of spargana present in mice by stating the numbers of spargana initially given to the mice.

3.42 The susceptibility of mice infected with spargana to superinfections of spargana or procercoids

Mice infected with up to 10 spargana for 10 weeks were unable to resist superinfections of mature spargana. This was demonstrated by the results obtained in three experiments in which mice each of which had been infected with two, five or 10 spargana for ten weeks were given further infections of five spargana each. The spargana of both the primary and the superinfections were fed to the mice. The numbers of spargana recovered when these mice were killed and examined three weeks after they had been given the superinfections of spargana are shown in Table 3.9.

Although it was not possible to distinguish between the spargana of the primary and the superinfections, it was clear that most or all of the spargana of the superinfections had become established.

Two experiments were done to determine the susceptibility of mice to superinfections of procercoids. In the first of these experiments, a total of 22 mice were used. These mice were randomly divided into groups of 11 control and 11 experimental mice. Each of the

Table 3.9

The susceptibility of mice to super-infections of spargana

Number of mice	Primary infection (10 weeks duration)	Super-infection (3 weeks duration)	Total spargana recovered
10	2 spargana per mouse	5 spargana per mouse	64
10	5 " " "	5 " " "	87
10	10 " " "	5 " " "	131

11 experimental mice was given two spargana and, three weeks later, all the mice were injected intra-peritoneally with a dose of 20 procercoids each. After a further three weeks, all the mice were killed and examined. It was found that the spargana which developed as super-infections in the experimental mice were considerably smaller than the spargana of the primary infections in these mice so the spargana of the primary and the super-infections could be identified easily. The second experiment done was similar to the first except that in the second experiment only 10 control and 10 experimental mice were used and the super-infections of procercoids were given six weeks after the experimental mice had been infected with spargana. The figures given in Table 3.10 indicate the numbers of procercoids which became established as spargana in the experimental and the control mice used in the experiment described above. When the Student's t-test was applied to these results, it was found that there was a statistically significant difference between the numbers

Table 3.10

The results of two experiments to determine the susceptibility of mice to primary and super-infections of procercoids

Experiment	Group of mice*	Number of mice in group	Number of procercoids which became established as spargana in each mouse										
			1	2	3	4	5	6	7	8	9	10	11
1	Control	11	1	2	2	3	4	5	5	5	6	8	8
	Experimental	11	0	0	1	1	1	2	2	2	3	3	4
2	Control	10	1	1	2	4	5	5	6	7	8	9	-
	Experimental	10	0	0	0	0	0	0	0	0	1	1	-

*See text for details of experiments

of spargana which developed from the procercoids given to the control and the experimental mice in both of the experiments($p=<0.01$). These results clearly showed that the infected mice used in these two experiments were partially or completely able to resist the establishment of super-infections of procercoids. According to Mueller (1965a), mice infected with the spargana of the North American form of spirometrid tapeworm also may be partially or completely able to resist super-infections of procercoids but not mature spargana. However, Mueller (1965a) did not give the details of the evidence on which he based his statement so it was not possible to relate the

results he obtained with those obtained in the present study.

3.43 The mechanisms of the acquired resistance developed by mice to procercoids

A number of previous workers have produced evidence which indicates that humoral factors may be important mediators of the acquired resistance displayed by animals such as rabbits, rats and mice to the larvae of various cestodes (Hearin, 1941; Kerr, 1935; Miller and Gardiner, 1934; Weinmann, 1966). This involvement was demonstrated by passively inducing resistance to infection in normal animals by injecting them with serum taken from infected animals. Unfortunately, this technique could not be used to find out if the antibodies produced by mice to spargana were responsible for the acquired resistance to super-infections displayed by mice because this aspect of the immunological relationships between mice and spargana was considered at a late stage in the study and too few procercoids were available. However, it was found that when procercoids were immersed in serum taken from mice that were infected with spargana their appearance changed in a characteristic manner whereas the appearance of procercoids immersed in serum from uninfected mice remained unchanged. This difference between the effects of serum from uninfected and infected mice was first observed when 20 procercoids that had been dissected out of cyclops were placed in approximately 0.03ml of pooled serum taken from 10 uninfected mice and a further 20 procercoids were placed in a similar quantity of pooled serum taken from 10 mice each of which had been infected with

two spargana for three weeks. These sera were maintained at 20°C in the cavity of welled microscope slides onto which cover glasses were sealed with petroleum jelly in order to reduce the loss of water by evaporation.

It was found that within 30 minutes of the start of the experiment, the procercoids in the serum from the infected mice had an appearance distinctly different from that of the procercoids in the serum from the uninfected mice. Each spirometrid procercoid is surrounded by a membrane that normally is thin and is closely applied to the surface of the procercoid (see Li, 1929). The membranes surrounding the procercoids in the serum from uninfected mice seemed to be in the normal state but the membranes surrounding the procercoids in the serum from the infected mice were distended to many times their normal thickness (see Figure 3.4). Whether or not this effect was a result of the action of antibodies was not certain. However, it seemed reasonable to suggest that if antibodies caused the observed changes in the surface membranes of procercoids, it may be possible to neutralise these antibodies with antigens of spargana and thereby prevent them from affecting procercoids. An experiment to test this hypothesis therefore was done.

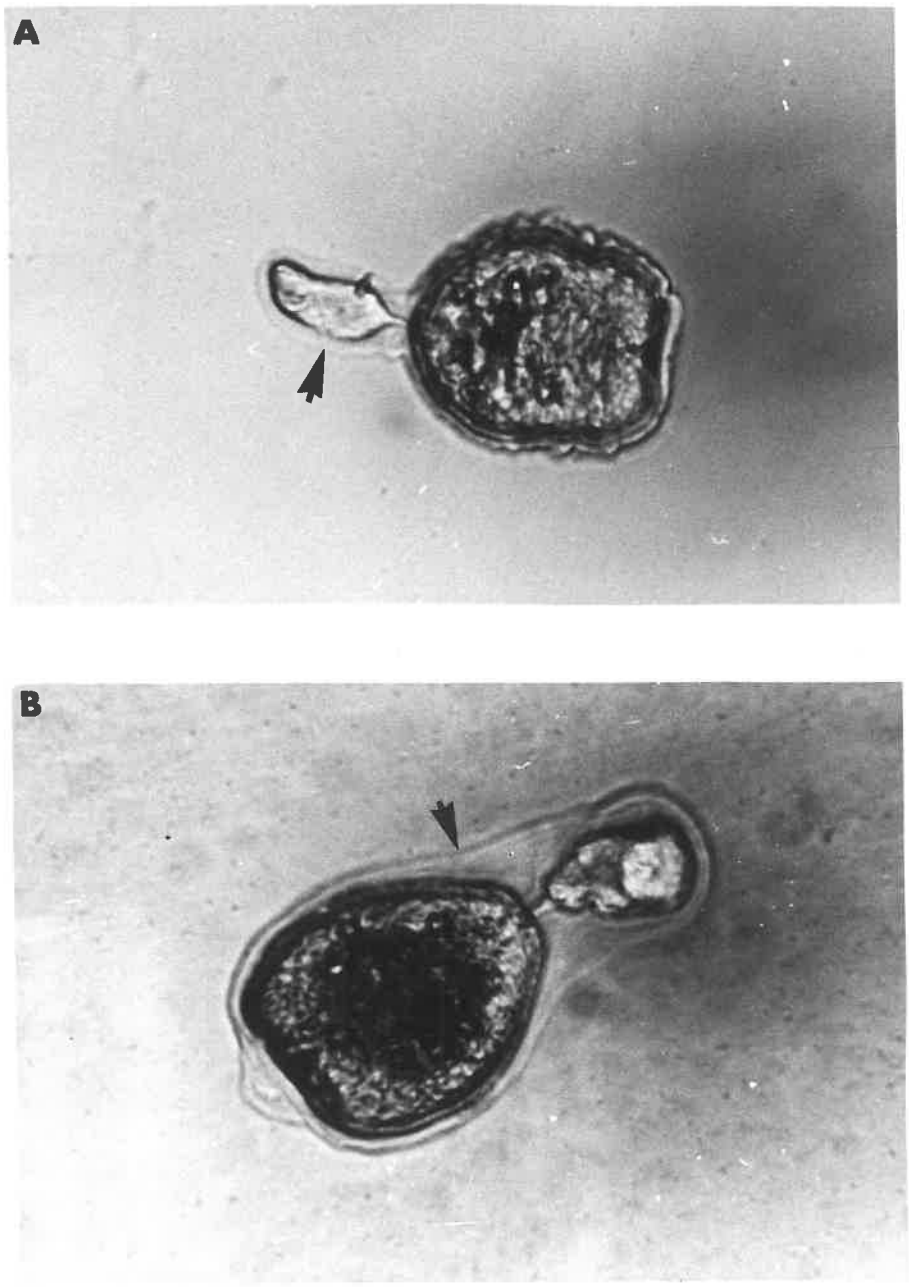
Two 0.5ml samples of pooled serum taken from 10 mice each of which had been infected with two spargana for three weeks were obtained. To one of these samples was added 0.5ml of physiological

Legend to Figure 3.4

(a) A photomicrograph of a procercoid immersed for 30 minutes in serum taken from mice which were not infected with spargana.
Note the thin membrane surrounding the procercoid (arrowed) (X1000)

(b) A photomicrograph of a procercoid immersed for 30 minutes in serum taken from mice infected with spargana. The outer surface of the distended membrane around the procercoid is indicated by the arrow (X1000)

Figure 3.4



saline solution and to the other was added 0.5ml of the standard extract of spargana. The two mixtures were incubated at 20°C for three hours and then were used in an experiment similar to that described above, 20 mature procercoids being placed in a small sample of each mixture. Thirty minutes later the procercoids were inspected and it was found that the membranes on the procercoids in the serum mixed with the standard extract of spargana appeared normal but the membranes on the procercoids in the serum mixed with physiological saline solution were distended. When this experiment and the experiments described previously were repeated with different groups of procercoids, results essentially the same as those already described were obtained. These results clearly were consistant with the hypothesis that antibodies formed by mice to spargana induced the observed changes in the surface membranes of procercoids. More convincing evidence may have been obtained if it had been possible to prepare an extract of procercoid which could have been tested for antigens by means of immunodiffusion tests, but this could not be done with the numbers of procercoids available.

The formation of membranes around cestode larvae or immature cestodes has been observed by other workers. Weinmann(1966) found that a membrane formed around immature tapeworms of the species Hymenolepis nana which were immersed

for four hours in an extract of the intestinal mucosa from mice that were immunised against this species of parasite. He(Weinmann,1966) commented that the response was reminiscent of the cercarienhullen-reaction which is observed when schistosome cercariae are placed in immune serum(Anderson, 1963; Vogel and Minning, 1949). Weinmann(1966) found that the mucosal extract had an adverse effect on the young tapeworms whereas serum taken from immunised mice seemed to have little effect on the young tapeworms or the eggs of H. nana . However, Silverman(1955) found that membranes and precipitates formed around activated hexacanths of Taenia saginata and T. pisiformis Which were placed in immune serum from calves or rabbits

Whether or not the effects of cells enable mice to resist superinfections of procercoids was difficult to determine. It was possible that procercoids could have stimulated marked inflammatory responses in the tissues of sensitised mice with the result that changes in the condition of the micro-environment around the procercoids caused the death of the procercoids. For example, it is known that the pH of the fluids in tissues may fall when the tissues become inflammed(Dittrich, 1962). Such a change may be sufficient to kill procercoids if not spargana. On the other hand, it is possible that the procercoids used to test for acquired resistance in mice did not penetrate into the tissues of

the mice. If cells were in any way responsible for the death of procercoids given as superinfections to mice, it was possible that the procercoids were killed by sensitised cells present in the body cavity of each infected mouse. Soulsby (1962, 1963b) pointed out that when larvae of Ascaris suum are placed in the peritoneal cavities of sensitised rabbits they became covered with leucocytes the majority of which are eosinophilic leucocytes. This phenomenon was called immune adhesion by Soulsby (1963b). It was clear that it would be difficult to find procercoids in the body cavities of mice infected with relatively few of these parasites, so no experiments on the immune adhesion of leucocytes to procercoids in super-infected mice were done. However, it was shown that the cells in the body cavities of infected mice were sensitive to mature spargana. In one experiment, 16 mice were divided into two groups of eight mice and each of the mice in one group was fed with two spargana. Three weeks later, each of the mice in both groups was fed with three spargana. The mice were killed 90 minutes after they had been fed with these spargana and an attempt was made to find the holdfasts of the spargana in the body cavities of the mice. Two or three spargana were recovered from the body cavity of each of the mice. It was immediately clear that many leucocytes had adhered to the surfaces of the spargana recovered from themice which had been infected for three weeks whereas relatively few leucocytes were present on the surfaces of spargana recovered from the mice that had not been

infected previously (see Figure 3.5). Smears of the cells attached to these spargana were prepared and counts of the various kinds of cells in a group of 250 of the cells present in each smear were made.

Because so few cells were attached to the spargana recovered from the unsensitised mice, it was necessary to count almost all thecells present in some of the smears prepared from these spargana. On the other hand, many more than 250 cells were found in each of the smears prepared from the spargana recovered from the sensitised mice. The results obtained were given in Table 3.11. These results clearly show that eosinophilic leucocytes were relatively more abundant amongst the cells taken from the surfaces of spargana recovered from sensitised mice than they were amongst the cells taken from the surfaces of spargana recovered from the unsensitised mice. This experiment was repeated and essentially the same results were obtained. In this second experiment, smears of the cells present in the body cavity of each of the sensitised and unsensitised mice also were prepared by pressing the duodenum of each mouse onto a microscope slide. The results obtained when the numbers of the various kinds of leucocytes present amongst groups of 250 of the cells present in each of the smears were counted are given in Table 3.12. These results indicate that the mice infected with spargana had developed an eosinophilia of the peritoneal cavity. This form of response often is produced by animals that develop an immediate form of hypersensitivity (Bohrod, 1954).

Legend to Figure 3.5

Photomicrographs to show the difference in the appearances of the surfaces of spargana recovered from the body cavities of rice infected with spargana for three weeks and previously uninfected mice.

(a) A sparganum which was fed to a previously uninfected mouse and then was recovered from the body cavity of the mouse 90 minutes later (x400)

(b) A sparganum which was fed to a mouse that had been previously infected with two spargana for three weeks. The spargana was recovered from the body cavity of the infected mouse 90 minutes after it had been ingested (x400)

(C - cells adhering to the surface of the sparganum
Sp - sparganum)

Figure 3.5

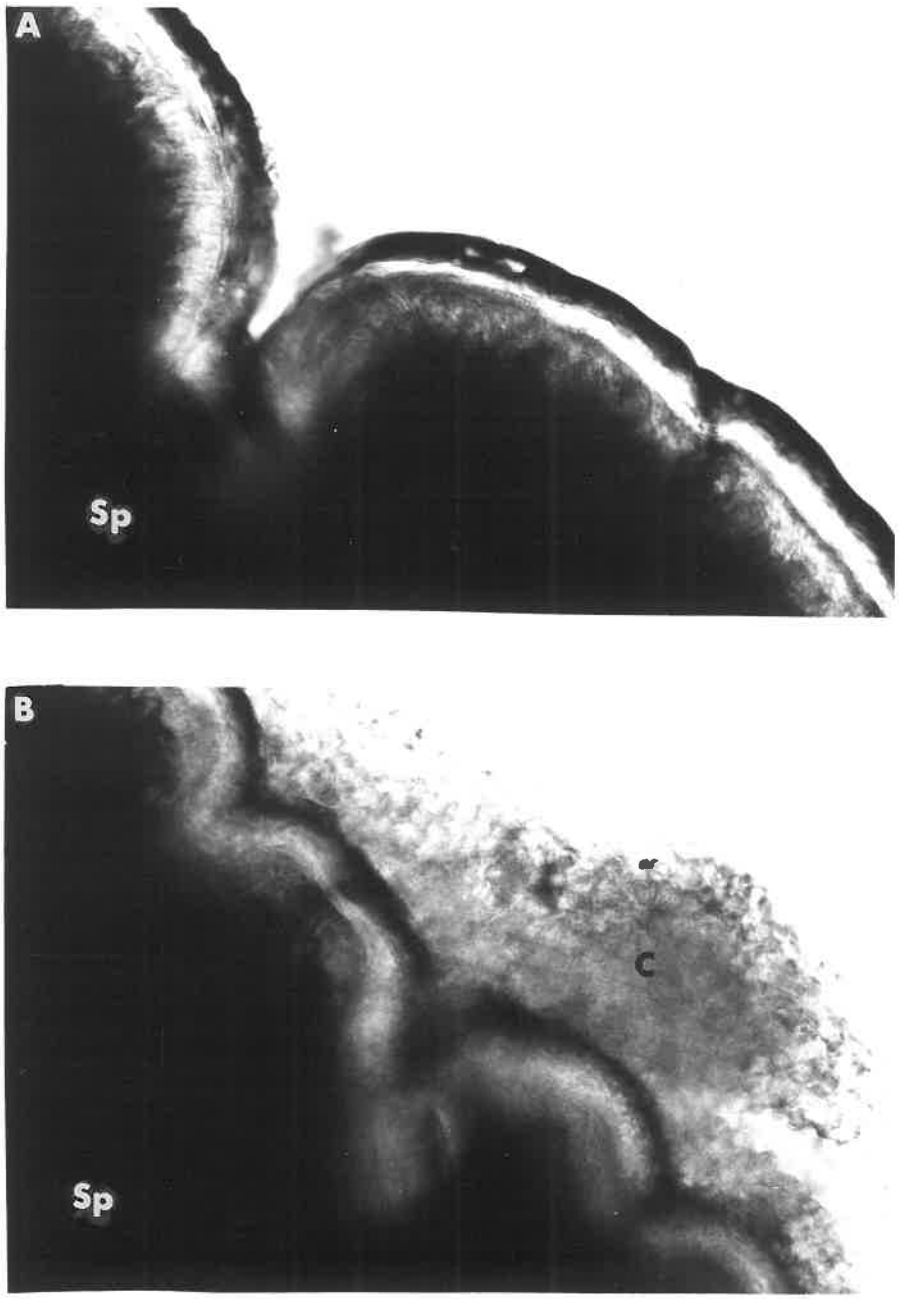


Table 3.11

The identities and numbers of the various kinds of leucocytes found amongst 250 of the cells present in each of the smears of cells taken from spargana recovered from the peritoneal cavities of sensitised and unsensitised mice

	Identity of cells	The number of cells found in the eight smears examined							
		1	2	3	4	5	6	7	8
Cells on spargana recovered from unsensitised mice	Macrophages	243	244	250	246	239	248	248	248
	Eosinophilic leucocytes	1	2	0	1	7	0	0	0
	Neutrophilic leucocytes	2	0	0	0	1	0	0	0
	Basophilic leucocytes	0	0	0	0	0	0	1	1
	Lymphocytes	4	4	0	3	3	2	1	1
Cells on spargana recovered from sensitised mice	Macrophages	208	194	121	176	185	215	201	199
	Eosinophilic leucocytes	37	41	106	66	59	34	40	42
	Neutrophilic leucocytes	3	15	23	6	6	0	6	2
	Basophilic leucocytes	0	0	0	0	0	0	0	0
	Lymphocytes	2	0	0	2	0	1	3	7

Table 3.12

The identities of the various kinds of leucocytes found amongst 250 of the cells present in each of the smears of cells taken from the peritoneal surfaces of the duodena of uninfected mice and mice infected with spargana

	The identity of the cells	The numbers of cells found in the ten smears examined									
		1	2	3	4	5	6	7	8	9	10
Uninfected mice	Macrophages	242	241	244	243	247	249	243	249	242	237
	Eosinophilic leucocytes	6	4	6	6	2	0	7	1	7	10
	Neutrophilic leucocytes	0	4	0	1	0	1	0	0	0	0
	Basophilic leucocytes	2	1	0	0	1	0	0	0	1	3
	Lymphocytes	0	0	0	0	0	0	0	0	0	0
Infected mice	Macrophages	236	229	217	197	237	223	231	210	234	210
	Eosinophilic leucocytes	12	16	32	50	13	25	18	35	14	40
	Neutrophilic leucocytes	0	3	0	0	0	1	1	5	0	0
	Basophilic leucocytes	2	2	1	3	0	1	0	0	2	0
	Lymphocytes	0	0	0	0	0	0	0	0	0	0

It therefore seemed likely that relatively large numbers of eosinophilic leucocytes were attached to the spargana recovered from sensitised mice because the peritoneal cavities of sensitised mice contained a relatively large number of these cells and not because the eosinophilic leucocytes present in the peritoneal cavities of infected mice adhered to spargana more strongly than macrophages.

The results described above therefore suggested that the peritoneal cavities of mice infected with spargana contained sensitised cells which reacted rapidly to super-infections of spargana, but whether or not they were able to react to procercoids in this way and possibly kill them was not certain. These cells clearly were not able to kill mature spargana.

3.5 DISCUSSION

On the basis of the evidence given in sections 3.1-3.4 it seemed reasonable to me to conclude that the spargana of the Australian form of spirometrid tapeworm are sufficiently immunogenic to mice to be able to stimulate them to produce immunological responses that can be considered to be normal in form and intensity. How the immunogenicity of the spargana of the Australian form of spirometrid tapeworm compared with the immunogenicity of the spargana from other parts of the world could not be estimated with confidence because so little is known about the immunological responses stimulated by spargana found in other parts of the

Australasian region or in North America. However, it seems likely that the spargana of the spirometrid tapeworms found in the various parts of the Orient may be equally immunogenic and they possibly are more immunogenic than the spargana of the North American form of spirometrid tapeworm. This hypothesis was first proposed in a paper published by Mueller (1965a). However, a copy of this paper unfortunately was not available to me until 1966 by which time I had independantly discovered that the spargana of the Australian form of spirometrid tapeworm seemed to be more immunogenic than the spargana of the North American form of spirometrid tapeworm. Thus it was clear that the Australian spargana could not be used to find out how it is that the spargana of the North American form of spirometrid tapeworm apparently fail to stimulate strong immunological responses in mice. The experiments on the relationships between mice and the spargana used in the present study therefore were continued only until evidence which seemed to be sufficient to support the hypothesis that these spargana stimulated normal immunological responses was obtained and some explanation of the relative non-pathogenicity of infections of large numbers of these spargana could be proposed. I thought of trying to produce laboratory-bred stocks of the spargana of the North American form of spirometrid tapeworm in order to compare the responses stimulated by these spargana with those stimulated by the spargana used in the

present study but this was not done because it seemed likely that insufficient time would be available to complete the study of the responses stimulated by the North American spargana even if these spargana could be bred successfully. It also was possible that I may not have been able to produce sufficient numbers of these spargana. Instead, I decided to compare the responses stimulated by the spargana of the Australian form of spirometrid tapeworm in mice with those stimulated by these spargana in amphibians. This was done mainly to try to obtain evidence which would support or disprove the hypothesis that the spargana of the Australian form of spirometrid tapeworm may be better adapted to living in amphibians than to living in mice. This seemed to be a reasonable hypothesis to propose because it has been suggested that amphibians are the most common hosts of spargana (Mueller, 1963, 1965a) and some workers have suggested that amphibians are essential second intermediate hosts in the life cycles of spargana (Bonne, 1942; Galliard and Ngu, 1946; Huang and Kirk, 1962). On the other hand, infections of spargana are relatively uncommon amongst wild mammals such as mice (Mueller, 1963). The observations made on the immunological responses produced by amphibians to the spargana of the Australian form of spirometrid tapeworm are described in section 4.

Although the immunological relationships between the spargana of the North American form of spirometrid tapeworm and mice were not

investigated, it seemed reasonable at least to consider some possible explanations which could account for the apparent feebleness of responses stimulated by these spargana when they are located in the sub-cutaneous tissues of mice. Firstly, it is necessary to point out that the responses stimulated by these spargana in mice have not been investigated thoroughly so no reliable estimates of the intensity of these responses have been made. Nevertheless, Mueller (1965a) described the cellular and humoral responses stimulated by the laboratory - bred spargana that he used as feeble. If it is provisionally assumed that this suggestion is correct, it seems that either the substances on the surfaces or in the secretions and excretions of the spargana are only weakly antigenic to mice or that these spargana release highly antigenic substances to which mice fail to produce strong immunological responses.

It was pointed out in section 1.2 that a species of parasite which is associated with a species of host for a long evolutionary period may become immunologically adapted to the host with the result that the disparity between the antigens of the host and those of the parasite may be reduced. These parasites may stimulate only feeble responses in hosts to which they have become immunologically adapted. However, Mueller (1963) stated that spargana are rarely found in wild mice so it seemed clear that spargana could not have become adapted to mice in this way. In this context, it is interesting to note that in an early paper, Mueller

(1938a) stated that he found areas of necrotic tissue and 'galleries' full of caseous material in the tissues of mice infected for up to six weeks with the spargana of the North American form of spirometrid tapeworm whereas in more recent papers, Mueller (1963, 1965a,b) stated that these spargana generally seem to be inert and they excite little local reaction in mice. Furthermore, in this earlier paper Mueller (1938a) mentioned that infected mice may show signs of illness such as emaciation and inflammation of the eyes whereas in the later papers he (Mueller, 1963, 1965a) stated that infected mice usually show few or no signs of illness. In the interval of time between the publications of the earlier and the later papers, Mueller succeeded in rearing the spargana of the North American form of spirometrid in the laboratory, using mice as the hosts of the spargana. It therefore was tempting to propose that the spargana had become immunologically adapted to mice during the relatively short period for which the spargana were maintained in the laboratory. This possibility was partly suggested by observations published by Solomon and Haley (1966) and Wescott and Todd (1966) who obtained evidence which indicated that the strain of the nematode Nippostrongylus brasiliensis which normally occurrs in rats and which usually is not highly infective to mice can become adapted to mice in the laboratory with the result that the infectivity of these parasites to mice increases. Nevertheless,

it seemed unreasonable to suggest that mice fail to produce marked immunological responses to the laboratory-bred spargana of the North American form of spirometrid tapeworm because these spargana have become immunologically adapted to the mice. The time for which these spargana have been bred in the laboratory probably is insufficient to allow such extensive adaptation to occur.

The hypothesis that spargana may release highly antigenic substances into mice but the mice fail to respond to these substances initially was considered because it was thought that spargana may induce a form of immunological unresponsiveness in mice. Two forms of immunological unresponsiveness in otherwise immunologically normal animals have been described (see Raffel, 1961, pp.108-114) and are generally called immunological tolerance and immunological paralysis. Immunological tolerance is a term used to describe a state of unresponsiveness induced by introducing antigens into embryonic or neonatal animals which then grow into adult animals that are partially or completely unresponsive to the antigens. Immunological paralysis, on the other hand is a state of unresponsiveness which can be induced in immunologically competent animals, by, for example, giving the animals large doses of substances which in small doses, are highly antigenic to these animals e.g. the pneumococcal polysaccharides. It was once thought that the mechanisms involved in these two forms of immunological unresponsiveness

may be different, but the results obtained in recent studies indicate that the two forms of unresponsiveness may not be as different as they originally seemed to be (Dresser and Mitchison, 1968).

Previous workers have obtained evidence which indicates that it is possible to induce rats to develop an immunological tolerance to the antigens present in the fluid from the hydatid cyst of Echinococcus granulosus (Moriarty, 1966, 1967) and to the nematode Nippostrongylus brasiliensis (Barth, Jarret and Urquhart, 1966). Whether or not the nematode N. brasiliensis induces a state of true immunological tolerance is debatable because, as Jarret, Jarret and Urquhart (1966) pointed out, the infection of very young rats with these parasites does not prevent the rats from forming reaginic antibodies to the parasites. Furthermore, Dineen and Wagland (1966) suggested that infections of Haemonchus contortus in sheep may induce a state of immunological exhaustion which could be considered to be a form of immunological paralysis. Thus some parasites apparently can induce some form of immunological unresponsiveness in their hosts. However, the hypothesis that the spargana of the North American form of spirometrid tapeworm induce a state of immunological paralysis in mice seemed to be unacceptable if it is true that antibodies to the antigens produced by these spargana can be found in the serum of chronically infected mice (Mueller,

1961, 1965a). Some of the antigens produced by these spargana may have been able to induce a state of immunological paralysis but it appeared that some of them did not.

Finally, the possibility that spargana may secrete some immuno-suppressive substance was considered. The ability of an animal to produce an immunological response may be reducedin a number of ways. According to Dresser and Mitchison (1968), general suppression of the immune response may be induced in an animal by treating it with ionising radiations, immuno-suppressive drugs or substances such as serum gamma-globulins or by treating the animal in such a manner that the population of lymphocytes in it becomes depleted. If the spargana of the North American form of spirometrid tapeworm are able to suppress immunological responses in any way it seems most likely that they do so by secreting some substance that acts as an immuno-suppressive agent. For example, they may secrete a substance which has properties that resemble those of cortisone. It has been postulated that some parasites of plants or animals may secrete substances which resemble some of the hormones normally found in the hosts of the parasites (Fisher, 1963), and it has been proposed that spargana secrete some substance or substances the activity of which resembles that of insulin (see section 3.22) so it seems possible that spargana could produce some substance the activity of which resembles that of cortisone in that it may suppress immunological responses. In this context, it is relevant

to point out that cortisone seems to be especially effective in suppressing the inflammatory responses associated with allergic reactions but it seems to be less effective in suppressing the formation of antibodies and it apparently does not prevent the reaction of antigens with antibodies (Goodman and Gilman, 1955 pp.1662-1663). An animal treated with cortisone therefore may form antibodies to a substance which normally induces a hypersensitive reaction in the animal but the injection of the sensitizing substance into the tissues of the sensitized animal may not stimulate the formation of a local inflammatory response. Furthermore, it has been found that cortisone is able to suppress the immunological response produced by rats to some nematodes (Cross and Duffy, 1963; Ogilvie, 1965) so it seems that the immunological responses stimulated by parasites may be as susceptible to the effects of cortisone as the responses evoked by other allergenic substances. The hypothesis that spargana secrete some cortisone-like immuno-suppressive substance therefore could account for some of the observations made on the immunological responses stimulated by spargana, especially if it is postulated that the sparganа of the North American form of spirometrid tapeworm secrete more of this substance than the spargana of the Australian forms of spirometrid tapeworms. However, there is one major objection to this hypothesis. The action of cortisone apparently is non-specific in that it seems to suppress

all or most of the local cellular responses which an animal is capable of producing. Thus an animal which is treated with cortisone or adreno-cortico-tropic-hormone may be especially sensitive to any pathogenic injury or infection which normally would be controlled by local inflammatory responses in an untreated animal (Goodman and Gilman, 1955, pp.1683-1684). Thus if spargana secrete a general immuno-suppressive substance it seems likely that mice infected with more than say 20 spargana would be especially susceptible to infection with pathogens other than spargana and they may be debilitated or they may die as a result of the pathological changes induced by any concurrent infections. However, it seems that infections of many more than 20 spargana do not adversely affect mice provided that the spargana do not migrate extensively and damage vital organs (Mueller, 1963, 1965a). This observation clearly suggests that the immunological systems of mice are not generally suppressed by any substance secreted by spargana.

At present therefore, it seems that there is no acceptable hypothesis which can be postulated in order to account for the observation thatthe spargana of the North American form of spirometrid tapeworm seemed to stimulate only feeble immunological responses in mice.

SECTION 4 THE IMMUNOLOGICAL RESPONSES PRODUCED BY AMPHIBIANS TO SPARGANA

Before the results obtained in the study of the immunological responses produced by amphibians to spargana are given, it is necessary to summarise briefly some of the characteristics of the immunological responses produced by amphibians. Because the leucocytes of anuran amphibians morphologically resemble the leucocytes of mammals, they have been given names the same as those applied to mammalian leucocytes (see, for example, Andrew, 1965 and Hildeman and Haas, 1962). Furthermore, it has been observed that the non-specific inflammatory responses produced by amphibians closely resemble the equivalent responses produced by mammals and that neutrophilic leucocytes and macrophages (monocytes) are involved in both reactions (see Boyden, 1963; Clark and Clark, 1920; Clark, Clark and Rex, 1936). There is also evidence which indicates that the lymphocytes of amphibians may be concerned with

the specific rejection of homografts in tadpoles (Hildeman and Haas, 1962). The rejection of homografts in mammals is considered to be mediated by lymphocytes (Gowans, 1962) so mammalian and anuran lymphocytes may be functionally as well as morphologically similar. The available evidence therefore at least suggests that the monocytes (macrophages), lymphocytes and neutrophilic leucocytes of amphibians may have functions similar to those of the analogous cells of mammals. Apparently, no work has been done to find out if amphibian eosinophilic leucocytes are functionally similar to the equivalent mammalian cells.

A number of workers have shown that amphibians can produce humoral responses (antibodies) to introduced antigens. Agglutinating antibodies to experimentally introduced Salmonella have been found in the serum of adult amphibians (Diener and Nossal, 1966; Evans, 1963; Evans and Horton, 1961; Kruger and Twedt, 1962) and tadpoles (Evans et al , 1965) and adult amphibians have been shown to produce agglutinating antibodies to bacteriophage X174 (Uhr, Finkelstein and Franklin, 1962) and Pseudomonus fluorescens (Bisset, 1948a,b). Furthermore, it seems that adult amphibians are able to produce precipitating antibodies to egg albumen (Austin and Nace, 1962) the components of the serum of rabbits (Austin and Nace, 1962; Evans and Horton, 1961) goldfish (Cooper, Pinkerton and Hildeman, 1964; Cooper and

Hildeman, 1965) bullfrogs and crayfish, (Cooper and Hildeman, 1965) and bovine serum albumen (Cooper and Hildeman, 1965; Evans, 1963). Evidence which indicated that tadpoles can synthesize precipitating antibodies to the components of the serum of goldfish also was obtained by Cooper, Pinkerton and Hildeman (1964). The antibodies produced by amphibians have been found in a fraction of amphibians serum analogous to the gamma-globulin fraction of human serum (Evans and Horton, 1961; Urh, Finkelstein and Franklin, 1962).

It therefore seemed reasonable to suggest that if amphibians produced any immunological responses to spargana, these responses at least should be recognisable and they may resemble the responses produced by mammals to spargana. Evidence to support this suggestion was obtained in a preliminary study of the responses produced by marine toads (Bufo marinus) to naturally acquired infection of spargana.

4.1 THE IMMUNOLOGICAL RESPONSES PRODUCED BY MARINE TOADS (BUFO MARINUS) TO NATURALLY ACQUIRED INFECTIONS OF SPARGANA

4.11 The local cellular responses

Responses of marine toads to spargana were seen first when toads were dissected for teaching purposes. A few of the toads dissected were infected with spargana and it was found that these toads had encapsulated at least some of the spargana they contained. This clearly showed that the toads had produced local

responses to the spargana. Subsequently, 1000 marine toads obtained from Ingham, Queensland were examined and 37 toads infected with spargana were found. These toads were carefully dissected and examined. Each toad contained both unencapsulated and encapsulated spargana. All of the unencapsulated spargana were embedded in the tissues of muscles whereas the capsules were attached to the outer surfaces of the fascia of muscles. The capsules were spherical or ovoidal, the spherical capsules being 2-8mm in diameter and the ovoidal capsules being 5-20mm long and 3-15mm wide. The figures given in Table 4.1 indicate the numbers of unencapsulated spargana and the number of capsules found in the 37 toads and the distribution of the spargana and the capsules in the bodies of the toad. These figures clearly show that the spargana had accumulated mainly

Table 4.1

The distribution of unencapsulated spargana and capsules formed around spargana in 37 toads (Bufo marinus)

(a) Unencapsulated spargana

Toads		Numbers of spargana found in various locations					
Sex	Number	Thigh muscles	Pelvic muscles	Abdominal muscles	Pectoral muscles	Viscera	Miscellaneous
Male	26	45	5	1	11	1	1
Female	11	21	8	2	3	0	0

Table 4.1. contd.
(b) Capsules

Toads		Numbers of capsules found in various locations					
Sex	Number	Thigh muscles	Pelvic muscles	Abdominal muscles	Pectoral muscles	Viscera	Miscellaneous
male	26	51	18	16	10	5	0
female	11	20	7	2	4	0	3

in the thighs of the toads. This confirmed the observation of Galliard and Ngu (1946), Sanders, (1953), Kobayashi (1930, 1931) and Yutuc (1951) who previously found that spargana congregated in the thighs of amphibians.

Although living muscles infected with spargana did not seem to be inflamed, the appearance of stained sections of these muscles showed that the spargana in the muscles had stimulated the production of a local inflammatory response. When sections of infected muscle-tissues taken from 10 toads were examined, it was found that the sparganum in each sample was surrounded by accumulations of leucocytes whereas few or no leucocytes were present in the tissues more than 2mm away from each sparganum. The photograph in Figure 4.1 illustrates the appearance of a section of an infected muscle that was representative of the 10 samples of infected muscle-tissue examined.

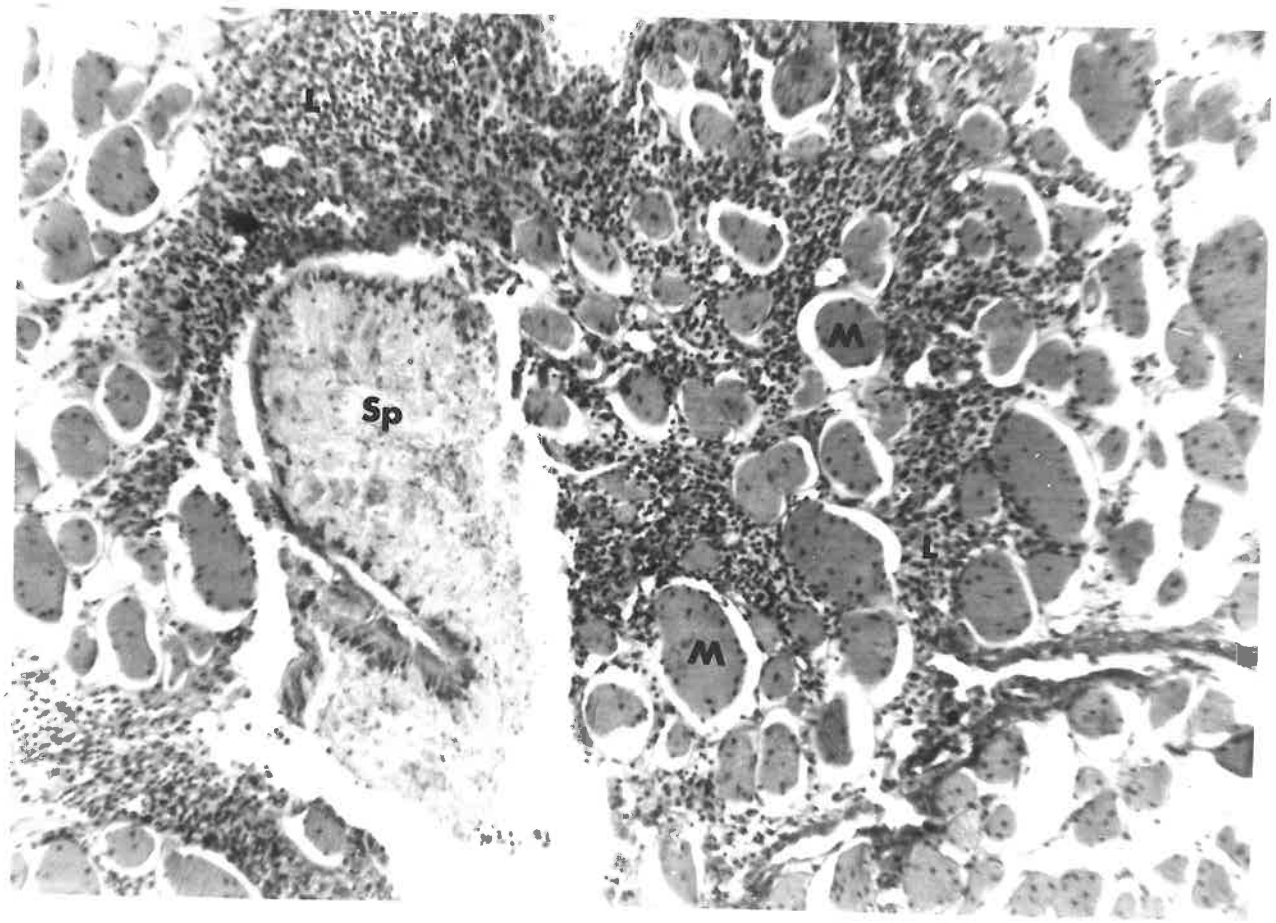
It was difficult to identify most of the cells that had accumulated in the tissues near the unencapsulated spargana. However, between 10

Legend to Figure 4.1

A photomicrograph of a section of infected muscle tissue taken from a toad (*Bufo marinus*) (X200)

(L - leucocytes of the inflammatory response; M - muscle fibres; Sp - sparganum)

Figure 4.1



and 50 per cent of these cells contained conspicuous eosinophilic granules and therefore, were considered to be eosinophilic leucocytes. The remaining cells seemed to be either neutrophilic leucocytes or macrophages but they could not be identified with certainty.

It was possible to identify and estimate the relative numbers of cells present near spargana more accurately by examining smears of cells prepared by pressing spargana which had been taken from muscles onto microscope slides (see section 2.35). Ten of these smears were prepared using spargana taken from ten toads and 100 of the cells in each smear were identified and counted. This number of cells was found in an area of each smear equivalent to two or three fields of view of the high power objective on the microscope used. The results obtained are given in Table 4.2 and a photograph of a representative area of one of the smears was shown in Figure 4.2. Whether or not the figures in Table 4.2 were valid estimates of the true proportions of eosinophilic leucocytes, neutrophilic leucocytes and macrophages that had accumulated around unencapsulated spargana was difficult to determine, but they at least confirmed the observation that approximately 10-50 per cent of the cells near unencapsulated spargana were eosinophilic leucocytes and that the remaining cells were mostly if not only neutrophilic leucocytes and macrophages.

The cellular response to encapsulated spargana was examined in a manner similar to that described above. Stained sections of 25

Table 4.2

Counts of cells in smears prepared by pressing unencapsulated spargana onto microscope slides

(100 cells each smear identified and counted)

Smear	Eosinophilic leucocytes	Neutrophilic leucocytes	Macrophages
1	25	57	18
2	32	49	29
3	36	20	44
4	14	27	59
5	30	22	48
6	20	7	73
7	33	5	62
8	6	63	31
9	39	35	26
10	25	34	41

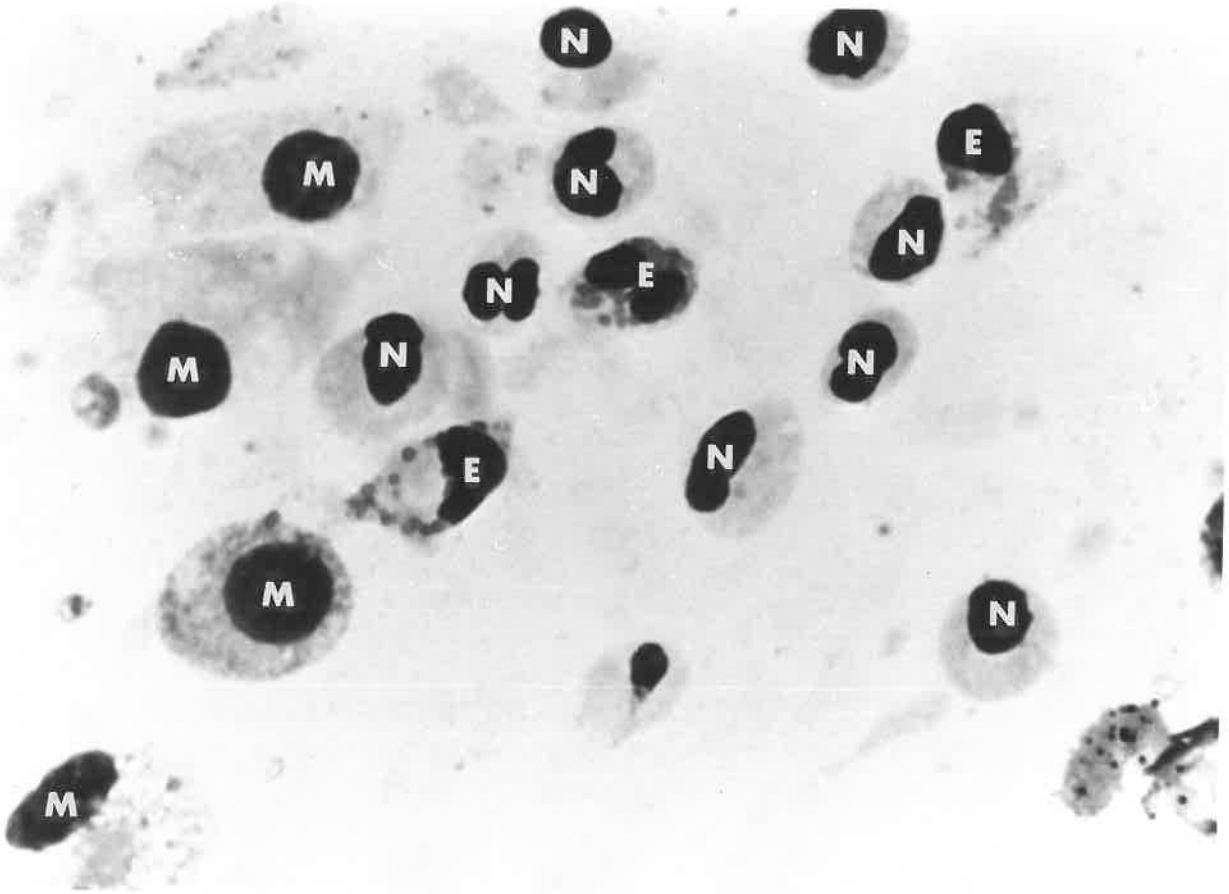
of the capsules and smears of the cells are either present on the surfaces of spargana taken from capsules or in the fluid present in capsules that contained no spargana were prepared. To prepare smears of the cells present in cavities of the capsules that contained no spargana a small drop of the fluid from each capsules was smeared onto a slide in the manner normally employed to prepare films of

Legend to Figure 4.2

A photomicrograph of cells in a smear prepared from an unencapsulated sparganum taken from a marine toad (Bufo marinus) (X2000)

(E - eosinophilic leucocytes; M - macrophages;
N - neutrophilic leucocytes)

Figure 4.2



blood (see Cowdry, 1952, p.47-48). Smears of cells present in 25 capsules were prepared. The results obtained when these preparations were examined are summarised below.

The sections showed that the capsules are composed of fibres and cells. When sections of capsules with walls more than 200 thick were examined it was found that the fibres in the walls of these capsules were arranged in such a manner that four more or less distinct layers of fibres could be identified. These were called the A, B, C and D layers (see Figure 4.3). Some of the characteristics of these layers and of the fibres in them are described in Table 4.3. Although all of the 25 sectioned capsules had walls in which therewere layers of fibres similar to those described, one or more of the layers were missing from the walls of many of the capsules. In Table 4.4 the capsules have been classified according to the structure of their walls. The figures given in this table suggest that the A, B, C and D layers of fibres were formed in sequence on the walls of the capsules, the A layers being formed first and the D layers being formed last. Figure 4.4 shows photographs of the walls of sectioned capsules arranged to show the way in which it was believed that the walls of capsules were formed. It seemed, therefore, that toads first lightly encapsulated spargana and then proceeded to deposit layers of fibres on the inner wall of the capsules until the cavity of each capsule was almost or completely filled with fibres. Moreover it

Legend to Figure 4.3

Photomicrograph of a section through the wall of a capsule formed by a marine toad (Bufo marinus) around a sparganum or a fragment of a sparganum (x500)

(A, B, C, D - layers in the wall of the capsule;
▼ - outer surface of the capsule)

Figure 4.3

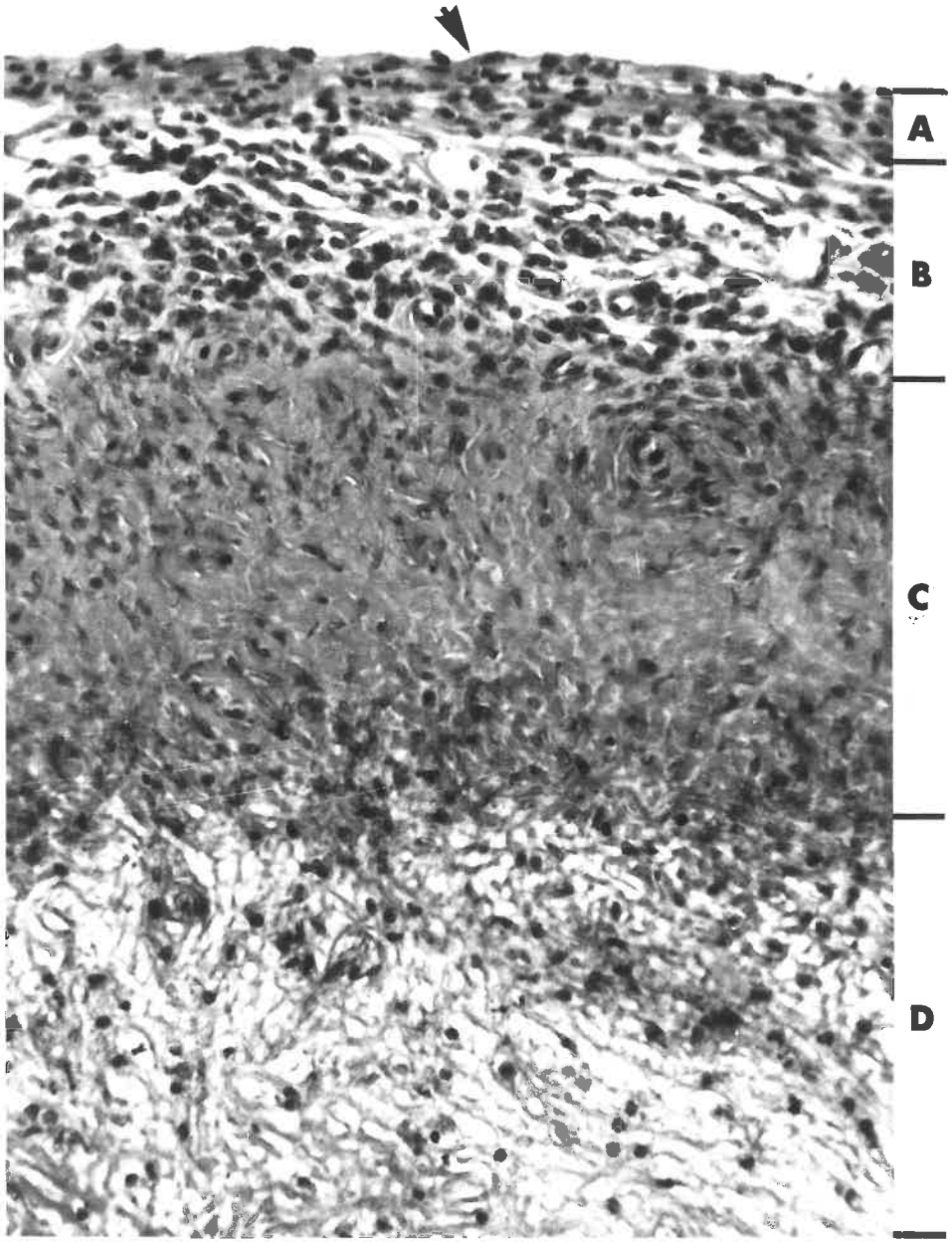


TABLE 4.3

Some structural characteristics of the layers and of the fibres in the layers in the walls of capsules formed around spargana by toads

Layer	Total thickness	Width of fibres in layer	Arrangement of fibres	Packing of fibres	Whorls of fibres*
A	5-15μ	1-5μ	Parallel to surface of capsule	Dense	None
B	10-50μ	1-5μ	"	Loose	None
C	80-100μ	5-10μ	Random	Dense	Some
D	Fills capsule	1-10μ	Variable	Loose	Many

*Whorls of fibres - small groups of fibres concentrically arranged around a focal point not in the centre of the capsule (see Dhaliwal and Griffiths, 1963)

Table 4.4

Classification of the 25 capsules examined according to the layers present in the walls of each capsule

Layers present in the wall of each capsule	Number of capsules
A layer only	1
A and B layers only	9
A and B layers complete, C layer partly formed	4
A, B and C layers completed, D layer partly formed	11
A, B, C and D layers completed	0

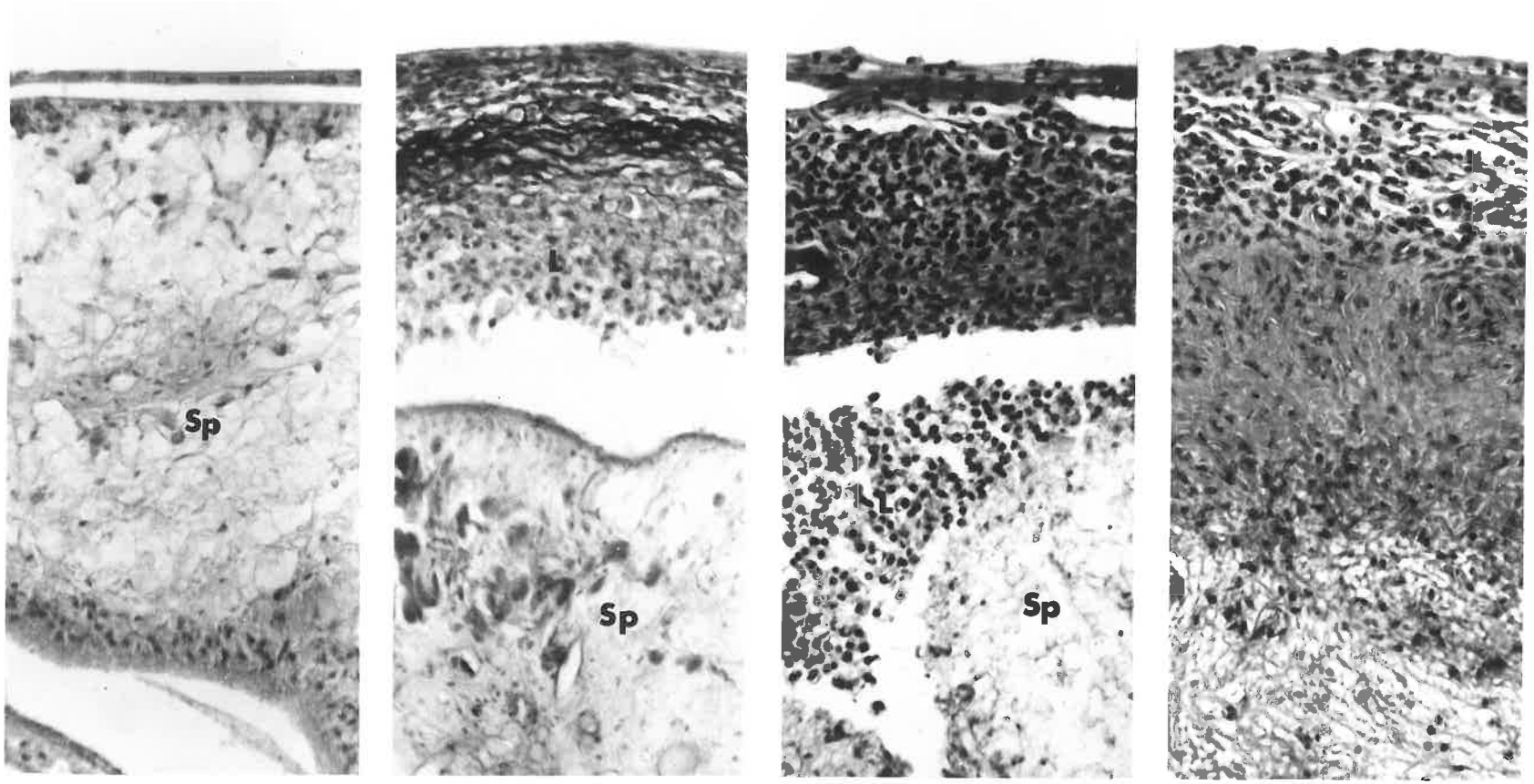
seemed that at one stage in the formation of each capsule, some temporary change occured with the result that the fibres produced at that time were usually thick and closely packed. These fibres formed the C layer described above. It perhaps was significant that all the spargana present in capsules the walls of which contained no C layer seemed to be more or less intact (see Figure 4.4(a) and (b)), whereas the spargana in capsules the walls of which contained a C layer had partially or completely disintegrated (see Figure 4.3 (c)). The C layers therefore may have been formed in the walls of capsules when the spargana in the capsules began to

Legend to Figure 4.4

Photomicrographs of sections of the walls of four capsules showing the proposed sequence of events in the formation of capsules around spargana (x300)

(The letter below each photograph indicates the layer of fibres which was being formed when the capsules were fixed for histological study; L - leucocytes of the inflammatory response; Sp - sparganum)

Figure 4.4



disintegrate markedly, perhaps releasing large quantities of antigens or toxins into the cavities of the capsules.

Leucocytes were present in the walls and cavities of all the capsules apart from three of the capsules whose walls consisted of an A layer on an A and an incompletely formed B layer. Leucocytes were present mainly in the A, B and D layers of the walls of capsules. They also formed a layer of up to 25μ thick on the inner walls of a number of capsules (See Figure 4.4 (b)) and they were distributed throughout the cavities of five of the capsules (See Figure 4.4(c)). Although it was not possible to be sure of the identity of all the leucocytes present in the sectioned capsules all the cells other than the fibroblasts present seemed to be eosinophilic leucocytes or macrophages. Eosinophilic leucocytes were present in the walls and cavities of capsules in which no C layer had been formed whereas these cells were found almost exclusively in the A and B layers of the walls of capsules in which a C layer had been formed. The cavities of the latter capsules contained cells almost all of which were identified as vacuolated macrophages. Giant cells, presumably formed by the fusion of macrophages (see Florey, 1962) were present in the walls and cavities of all the capsules that contained leucocytes (see Figure 4.5 (a)). Dhaliwal and Griffiths (1963) found similar giant cells in lesions caused by fungi in the tissues of Bufo melanostictus and these authors also described whorls of fibres

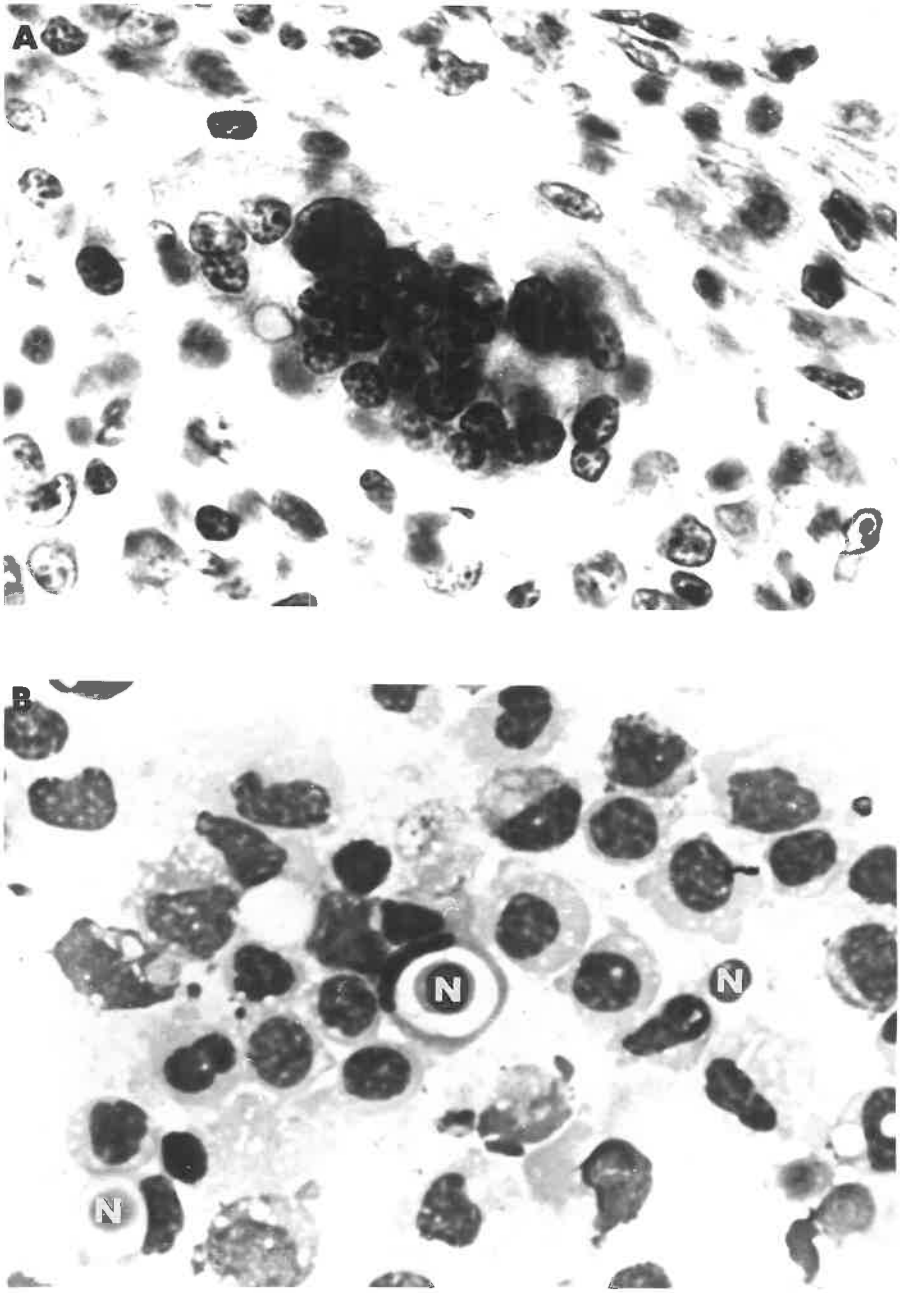
Legend to Figure 4.5

(a) A photomicrograph of a giant cell found in the wall of a sectioned capsule (x1500)

(b) A photomicrograph of macrophages and neutrophilic leucocytes found in a smear of cells taken from an encapsulated sparganum (x1500)

(N - neutrophilic leucocytes)

Figure 4.5



(see Table 4.3) which were formed by the toads around the fungal hyphae.

Results which were consistent with the observations reported above were obtained when the smears of cells in capsules were examined. The figures given in Table 4.5 show the numbers of the different kinds of leucocytes found in groups of 100 of the cells present in each of the smears examined. Although many of the cells in these smears could not be identified because they were too degenerate, it was clear that the smears contained an abundance of macrophages and relatively few recognisable neutrophilic and eosinophilic leucocytes. Twelve of the smears contained no eosinophilic leucocytes or granules of eosinophilic leucocytes so the cells in these smears must have been macrophages and neutrophilic leucocytes or macrophages only. Almost all of the macrophages in these 12 smears were vacuolated. Small fragments of cytoplasm containing eosinophilic granules were found in all the smears that contained eosinophilic leucocytes and it was assumed that these fragments had been released by the eosinophilic leucocytes. A few macrophages that had phagocytosed some of these fragments of cells or whole but apparently dead cells were seen (see Figure 4.5 (b)). An amorphous basophilic debris was present in three of the smears that contained macrophages only.

While the smears of cells from the cavities of capsules were

Table 4.5

The numbers of the different kinds of leucocytes found amongst 100 of the cells present in each of 25 smears prepared from encapsulated spargana or the fluid from capsules in marine toads

Smear	Macrophages	Neutrophilic leucocytes	Eosinophilic leucocytes	Unidentified*
1	43	5	27	25
2	43	7	32	18
3	70	4	16	10
4	85	0	9	6
5	86	0	5	19
6	37	18	14	31
7	53	2	3	40
8	84	1	5	10
9	80	0	2	18
10	65	5	4	26
11	73	0	0	27
12	95	0	0	5
13	88	0	2	10
14	83	1	0	16
15	76	2	0	22
16	69	0	4	27
17	86	0	0	14
18	75	0	0	25
19	90	1	0	9
20	85	0	0	15
21	88	0	1	11
22	86	2	0	12
23	84	0	0	16
24	80	0	0	20
25	79	0	0	21

* Cells too degenerate to be recognisable.

being prepared it was noticed that only seven of the capsules examined contained spargana with recognizable holdfasts. Sixteen of the capsules contained what seemed to be 'headless' spargana and two capsules contained no spargana. Moreover, the sixteen spargana without holdfasts were considerably smaller than most of the unencapsulated spargana found. All of the sixteen headless spargana taken from capsules were less than 20mm long whereas all the unencapsulated spargana found were between 40 and 250mm long. It therefore seemed reasonable to suggest that at least 16 of the 25 capsules examined may have contained fragments of spargana rather than complete spargana.

4.12 The humoral responses

Samples of serum taken from 10 toads in which no spargana was found and 10 toads each of which was infected with 2-5 spargana were separately tested for antibodies using the Ouchterlony form of immunodiffusion test. The appearance of one of the gels used for these tests is shown by the photograph in Figure 4.6 (a). This photograph shows that a band of precipitate appeared between the wells charged with the serum from the infected toad and the extract of the spargana respectively but no band of precipitate appeared in the wells charged with the serum from the uninfected toads and the extract of spargana. Similar results were produced in all of the tests which were carried out. These results clearly suggested that the infected toads had produced precipitating antibodies

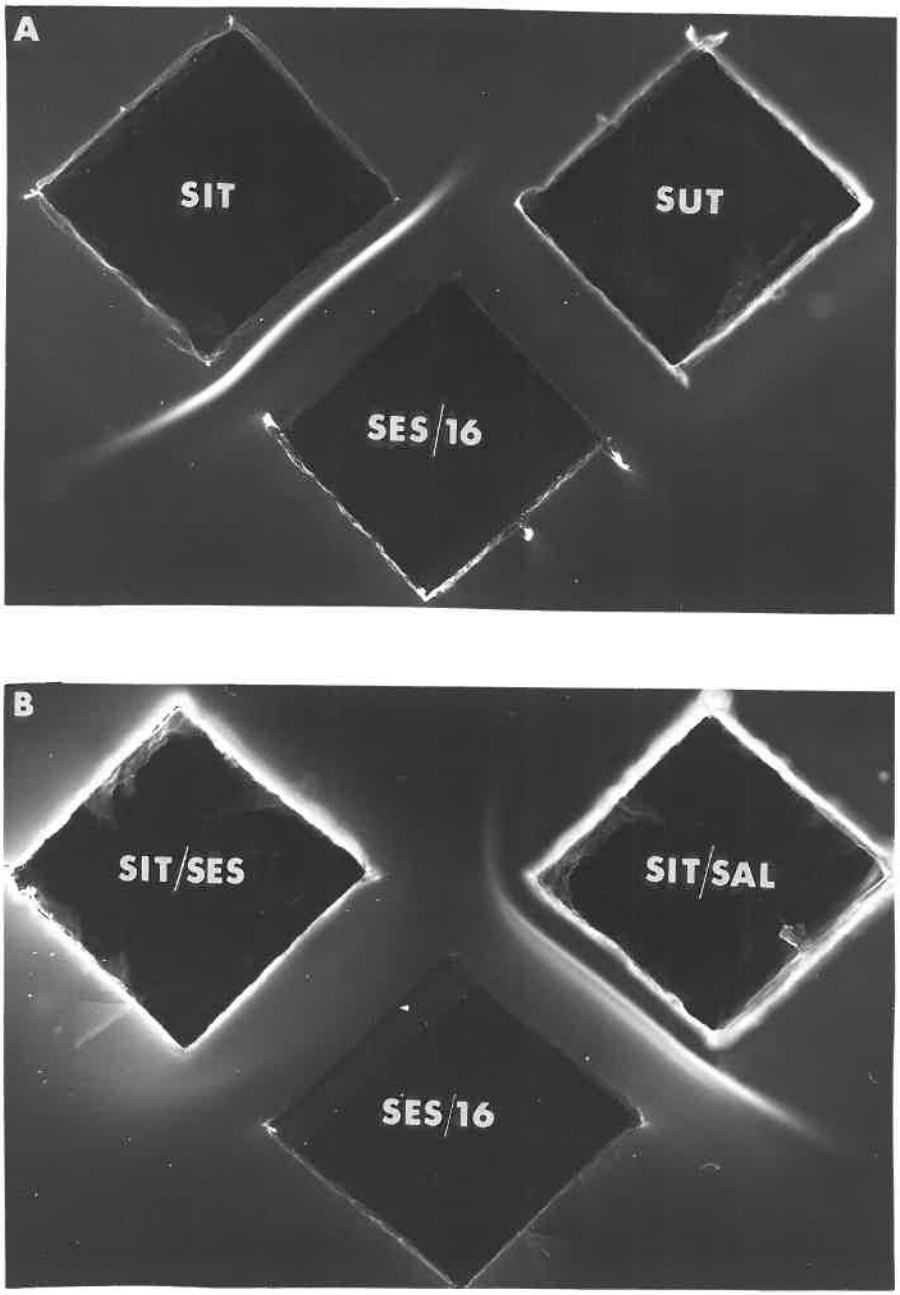
Legend to Figure 4.6

(a) A photograph of an Ouchterlony plate used to test for antibodies in serum of one toad infected with spargana and a second toad in which no spargana could be found (x5)

(b) A photograph of an Ouchterlony plate used to demonstrate that the addition of some of the extract of spargana to serum from toads infected with spargana rendered that serum incapable of reacting with the standard extract of spargana in immunodiffusion tests to produce bands of precipitate (x5)

(SES/16 - standard extract of spargana/16;
SIT - Serum from toad infected with spargana;
SUT - Serum from uninfected toad;
SIT/SES - Mixture of 3 parts of serum from an infected toad and one part of the standard extract of spargana;
SIT/SAL - Mixture of 3 parts of serum from an infected toad and one part of physiological saline solution)

Figure 4.6



to the spargana with which they were infected.

It seemed reasonable to propose that if the properties of any anti-sparganum antibodies produced by toads resembled the properties of anti-sparganum antibodies produced by mice, it should be possible to neutralize the anti-sparganum antibodies produced by toads by treating the serum taken from infected toads with a quantity of the standard extract of spargana which contained an excess of antigens. When serum from infected mice was treated in this manner, it was rendered incapable of reacting in agar gel with the standard extract of spargana to produce bands of precipitate (section 3.21). Some preliminary attempts to neutralize anti-sparganum antibodies in serum from infected toads by allowing a mixture of this serum and the standard extract of spargana to stand in a refrigerator at 5°C for 12 hours failed because the samples of serum 'clotted' (see section 2.311) and no 'absorbed' serum could be extracted from the clots. Subsequently, it was demonstrated that a freshly prepared mixture of one part of the standard extract of spargana and ~~two~~ three parts of serum from an infected toad was incapable of reacting in agar gel with the standard extract of spargana to produce a band of precipitate whereas serum mixed with an equivalent quantity of saline solution reacted with the excess of spargana to produce bands of precipitates (see Figure 4.6 (b)). This experiment was performed with serum taken from 5 infected toads and essentially the same results were

obtained from each test. These results clearly suggested that the serum from infected toads contained anti-sparganum antibodies which could be neutralized with antigens present in the standard extract of spargana.

Samples of serum taken from a further 10 toads each of which were infected with 2-5 spargana were used in the immunoelectrophoresis form of immunodiffusion tests. Serum from each of these toads was tested separately and, in the first five tests one of the troughs in each of the gels used was charged with serum taken from mice infected with spargana. Bands of precipitate appeared in all of the gels. The positions of the bands of precipitate which appeared in the serum from infected toads reacted with the electrophoretically separated constituents of the extract of spargana are shown in the diagram in Figure 4.7 (a). However, not all of these bands of precipitate appeared in each of the gels used. This is clearly shown by the photographs in Figures 4.7 (b) and (c). The frequency with which bands appeared in the gels used to test the ten sera is shown by the figures in Table 4.6. The figures in the column on the right of this table are the code-numbers of the bands of precipitate (see Figure 4.7 (a)) which appeared in the gels used to test each of the sera.

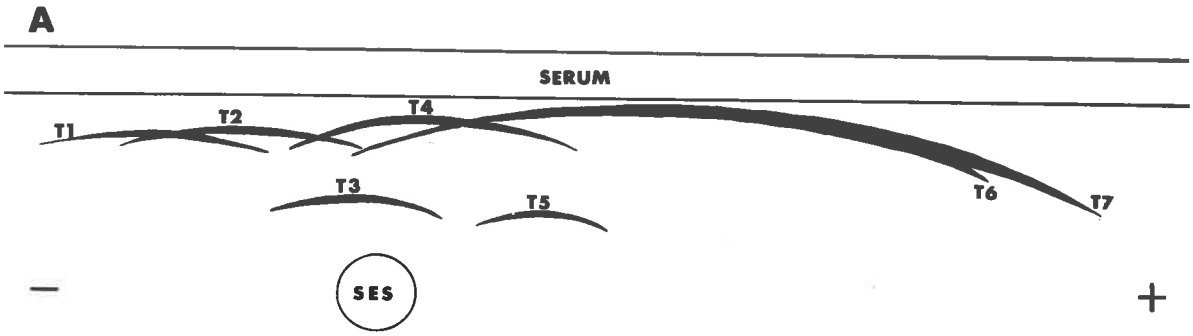
The Ouchterlony form of immuno-diffusion test (see Table 2.2 (c)) was used to find out if any of the substances of spargana to which mice produced antibodies also stimulated toads to produce antibodies.

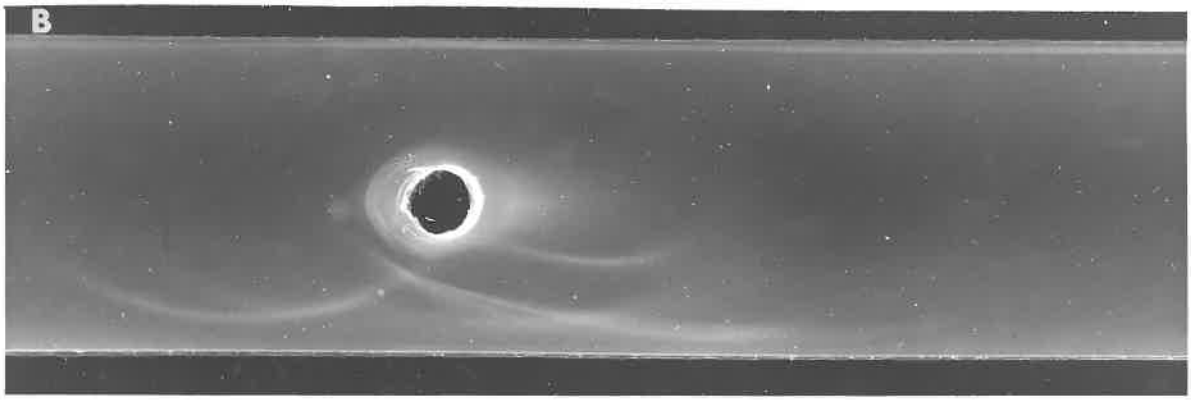
Legend to Figure 4.7

(a) Bands of precipitate formed when sera from toads infected with spargana reacted with the electrophoretically separated constituents of the extract of spargana. Composite diagram showing the positions of all the bands detected

(b) and (c) Photographs of two gels in which the electrophoretically separated constituents of the extract of spargana reacted with sera from two toads infected with spargana and pooled serum taken from 10 mice infected with spargana. Upper bands in lower photograph formed with serum from mice, lower bands in both photographs formed with serum from toads (Bactoagar, pH.8.2, μ = 0.05; stained with 1% amidoblack 10b) (x3)

Figure 4.7





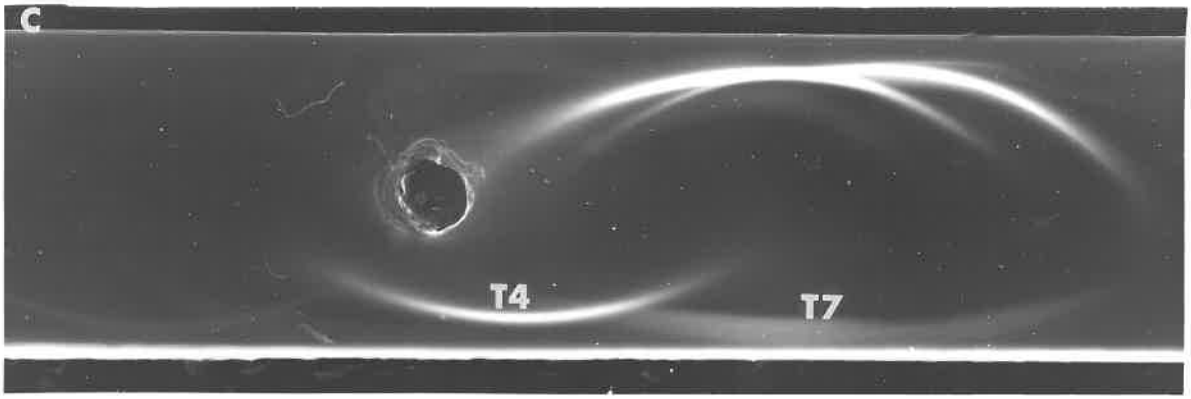


Table 4.6

A summary of the results obtained when the sera taken from ten toads infected with spargana were separately tested for antibodies

(Immunoelectrophoresis test)

Toad	Bands of precipitate which appeared in immunoelectrophoresis test (see Figure 4.7)
1	T1, T2, T4, T5, T7
2	T1, T2
3	T4, T6
4	T1, T2, T4, T5, T6
5	T2, T4
6	T3, T4, T7
7	T1, T2, T4, T5, T7
8	T1, T2, T4, T7
9	T1, T4
10	T1, T2, T3, T4, T6

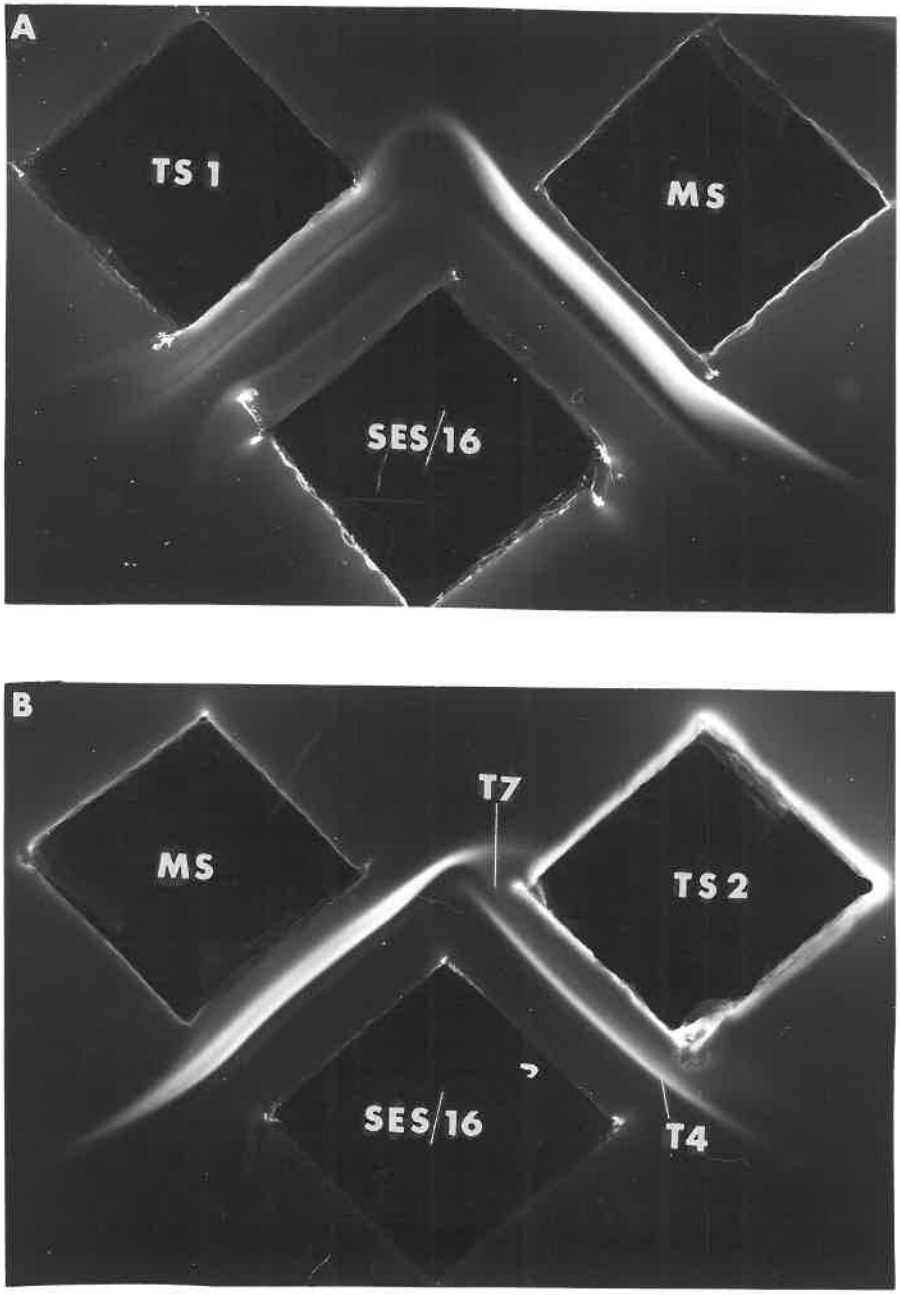
In each of the tests done the wells in the agar gel used were charged with reactants in the manner shown in Figure 4.8. Samples of serum taken from five infected toads were tested separately in this way. In each of the tests the sera from both mice and toads reacted with the extract of spargana to produce bands of precipitate (see, for example, Figures 4.8 (a) and (b)). It seemed reasonable to assume that the dense band of precipitate produced when serum from infected mice reacted with the extract of spargana in these tests represented the dense bands of precipitate labelled M9 and M10 produced in immunoelectrophoresis tests (see Figure 3.2 (a)). In four of the Ouchterlony tests done, this dense band of precipitate curved around to fuse with one of the bands of precipitate produced when the serum from infected toads reacted with the extract of spargana (see, for example, Figures 4.8 (a) and (b)). According to Ouchterlony (1964), fusion of bands of precipitate in this manner indicated that both of the sera used in each of the tests contained antibodies to one of the antigens in the extract of spargana. It therefore appeared that spargana contained some substance or substances that stimulated the production of antibodies in both mice and toads, and that the antibodies produced by mice reacted with these substances to produce bands M9 and M10 shown in Figure 3.2 (a). Bands T6 and T7 shown in Figure 4.6 (a) were in positions similar to those of bands M9 and M10 so it seemed likely

Legend to Figure 4.8

Photographs of two gels used to compare the antibodies present in serum taken from two toads infected with spargana with the antibodies present in pooled serum taken from 10 mice infected with spargana (x5)

(MS - Serum from infected mice; TS1 and TS2 - Sera from two infected toads; SES/16 - standard extract of spargana/16)

Figure 4.8



that one or both of the antigens incorporated in bands M9 and M10 also were incorporated in bands T6 and T7 respectively. This suggestion was supported by the results of immunoelectrophoresis and Ouchterlony tests done with the same sera were compared. For example, Figures 4.7 (c) and 4.8(b) are photographs of bands of precipitate produced in two different tests using the serum from one toad and one group of mice. It can be seen that in each of these tests, the serum from the toad reacted with the extract of spargana to produce one sharp, dense band of precipitate and one broad, diffuse band of precipitate. These bands of precipitate were tentatively identified as bands T4 and T7 respectively. In the Ouchterlony test the broad diffuse band identified as band T7 fused with the dense band of precipitate (M9 and M10) produced when the serum from mice reacted with the extract of spargana. The band of precipitate identified as band T4 also seemed to fuse with one of the faint bands of precipitate produced when the serum from mice reacted with the extract of spargana, but the fusion was not so clearly visible in this case.

4.13 Discussion

The aim of this part of the study was to find out if marine toads produced recognisable responses to spargana and if these responses resembled the responses produced by mammals to spargana. The results obtained clearly showed that the toads produced recognisable responses to spargana. Moreover, there was at least

a superficial resemblance between the cellular responses produced by toads and mice to spargana and there appeared to be a more definite resemblance between the humoral responses produced to spargana by these two hosts. However, some observations suggested that the relationships between toads and spargana may be slightly different from the relationships between mice and spargana. For example, spargana were encapsulated after they had been in mice for only 3-4 weeks but it seemed likely that many spargana were not encapsulated even after they had been in toads for many months. It was proposed by Galliard and Ngu (1946) and Mueller (1965a) that amphibians are in the form of tadpoles when they first become infected with spargana by eating cyclops infected with procercoids. These infected tadpoles then metamorphose into frogs or toads which retain their infections of spargana (see Corkum, 1966; Yutuc, 1951). This seemed to be the most reasonable way to explain how amphibians become infected with spargana. Adult frogs and toads normally prey on small terrestrial and flying invertebrate animals (Goin and Goin, 1962, pp.139, 165-167; Noble, 1954, pp.415-418) none of which have been shown to harbour spirometrid procercoids or spargana. It therefore seemed to be unlikely that the toads examined were adult when they first became infected with spargana. If these toads were in the form of tadpoles when they first became infected with spargana, they probably had been infected for more than a year.

All the infected toads examined were three inches or more long. Straughan (1965) found that marine toads grew to approximately three inches in two years, so the toads used in the present study probably were at least 18 months old. Nevertheless, they had failed to encapsulate many of the spargana with which they were infected even though they had encapsulated what appeared to be fragments of spargana. Some of the toads found contained only one or two unencapsulated spargana with recognisable holdfasts but up to six capsules that contained fragments of spargana. There seemed to be two ways in which these observations could be explained. First, it was possible that some spargana were more susceptible to the responses of toads than others and that these susceptible spargana succumbed while the less susceptible spargana survived. Second, it was possible that spargana migrated continuously or intermittently through the tissues of the toads and sometimes lost fragments from their tails while migrating. These fragments then became encapsulated and degenerated. Because it is known that spargana migrate and lose fragments from their tails in mammals, it seemed likely that the second of these two explanations was the correct one. Whole spargana may have been encapsulated, but they also may have been able to break out of capsules that were in the early stages of formation, possibly leaving fragments behind in the partly formed capsules. This was not observed in mice used in the present study.

Whether or not spargana were in any way immunologically adapted to living in toads was difficult to estimate, but the results obtained in this study indicated that they were not. The responses produced by toads to spargana did not seem to be especially feeble and spargana certainly did not induce a state of immunological unresponsiveness in toads. This was not surprising because the association between spargana of the Australian form of spirometrid tapeworm and marine toads probably originated only recently. Marine toads were introduced into Australia for the first time in 1935 (Straughan, 1966) and it is likely that they have been infected with the spargana of the Australian form of spirometrid tapeworm only since that time. It is unlikely that these spargana could have become adapted to living in marine toads in such a short time. On the other hand the spargana may have become adapted to living in amphibians belonging to one of the species native to Australia. I therefore attempted to study the relationships between the spargana used in the present study and native Australian frogs (Limnodynastes tasmaniensis). It was hoped that such a study would provide quantitative data which would show whether or not spargana are adapted to living in these frogs.

4.2 THE RELATIONSHIPS BETWEEN SPARGANA AND AMPHIBIANS OF THE SPECIES LIMNODYNASTES TASMANIENSES

The study of the relationships between spargana and laboratory-bred tadpoles and frogs of the species L. tasmaniensis was unsuccessful because neither frogs nor tadpoles could be consistantly infected with controlled numbers of spargana. Furthermore, most of the frogs that became infected died after they had been infected for less than three weeks. Examples of some of the experiments done with frogs and tadpoles are given below.

In one experiment, 15 frogs were fed with 10-day-old spargana taken from mice. Each frog was given one sparganum. Eight of the frogs died eight to twelve days after they were infected. Each of the dead frogs was infected with a sparganum but the six frogs that stayed alive were not infected. I thought that perhaps it would be better to try to infect frogs with spargana taken from tadpoles but this method also was unsuccessful. Thirteen frogs were each fed with two small spargana that were taken from tadpoles. Approximately two weeks later three of the frogs died. These three frogs contained two spargana each. The remaining frogs were killed and examined. It was found that seven of them contained no spargana and four were infected with one sparganum each. Because there was this difficulty in infecting laboratory bred marsh frogs with spargana,

I decided to try to infect wild marsh frogs to find out if results similar to those obtained in the preliminary experiment mentioned in section 2.13 could again be obtained. Thirty-seven wild marsh frogs each were infected with the holdfasts of two spargana. Within five days these frogs defaecated 24 holdfasts. All the frogs were killed and examined three weeks after they had been fed with holdfasts. Seven were infected with one sparganum each, but the remaining thirty frogs were uninfected. Because attempts to infect marsh frogs with controlled numbers of spargana by feeding mature spargana to them were unsuccessful, I tried to infect these frogs with orally administered procercoids. In one experiment nine frogs each were fed with a group of cyclops containing a total of twenty procercoids. Two weeks later, these frogs were killed and examined. Three of the frogs contained no spargana and the remaining six frogs were infected with a total of twenty-eight spargana, each frog containing 2-7 spargana. This experiment was repeated with a further ten frogs each of which was infected with forty procercoids. When these frogs were killed and examined six weeks after they had been given the procercoids, it was found that two of the frogs contained no spargana, five frogs were infected with 1-3 spargana each and the remaining three frogs were infected with 5-6 spargana each. Marsh frogs therefore were partially susceptible to orally administered procercoids but there was a considerable variation in the numbers of spargana which became established in each frog.

If sufficient numbers of frogs had been available, it may have been possible to design experiments in such a way that the frogs could have been used for experimental purposes even though their susceptibility to infection with procercoids was variable. However, relatively few frogs were available so correctly designed experiments could not be done. Furthermore, it was found that frogs often disgorged some of the infected cyclops with which they were fed. This made it difficult to even try to estimate the degree of susceptibility of marsh frogs to procercoids because whether or not each frog had ingested all the procercoids which it had been given was uncertain. The frogs bred in the laboratory were rarely more than 1.25cm long and it was not possible to administer infected cyclops to such frogs by means of a stomach tube or a parenterally introduced hypodermic needle. Thus there seemed to be no way in which controlled numbers of procercoids could be administered to marsh frogs so the infection of these frogs with spargana for experimental purposes was abandoned.

Poor results also were obtained when attempts were made to infect tadpoles with spargana or procercoids. Although the eighteen tadpoles used in the preliminary trial (see section 2.13) became infected with spargana when they were placed in a dish containing infected cyclops, it was found that when a further eighteen tadpoles were used in a similar experiment, only four of these tadpoles became infected. Each of the tadpoles contained

1-3 spargana. In these experiments no attempt was made to give a specific number of procercoids to each tadpole so it was possible that some tadpoles did not become infected merely because they did not eat any infected cyclops. Therefore, an attempt was made to feed tadpoles with known numbers of procercoids. Tadpoles isolated in small dishes of water that contained cyclops infected with known numbers of procercoids did not eat the cyclops. It seemed, therefore, that the only way to infect tadpoles with controlled numbers of procercoids was to force-feed them. Tadpoles to be forced-fed were first anaesthetised in a solution of MS222 (see section 2.331) and then were transferred to a dish of clean pond water. When each tadpole began respiring strongly, a living cyclops which contained a known number of procercoids was released near its mouth. If the cyclops was released at the correct time, it usually was ingested by the tadpole. After the cyclops had been ingested, a small amount of freshly macerated lettuce-leaf was released near the mouth of each tadpole. The macerated tissue usually drifted into the mouth of the tadpole, causing it to swallow. This was done in order to stimulate the tadpole to swallow the cyclops. Each tadpole then was left to recover in a dish of clean pond water. Approximately fifteen minutes later, the pond water containing the tadpole was inspected to see if the tadpole had disgorged the cyclops. If any disgorged cyclops were

found the cyclops and the appropriate tadpoles were discarded. A total of twenty tadpoles each were fed with cyclops containing two procercoids and a further twenty tadpoles each were fed with cyclops containing five procercoids. These tadpoles were kept until most of them had metamorphosed into frogs. Four tadpoles failed to metamorphose and these tadpoles all were infected with one to three spargana each. Eight of the frogs developed from the remaining 36 tadpoles were infected with one to three spargana each but the remaining frogs were uninfected. Spargana were found in only seven of the 20 tadpoles or frogs initially given five procercoids each and in only five of the tadpoles or frogs initially given two procercoids each. One important observation was that even though some of the tadpoles were infected with only one or two spargana each, they seemed to be stunted and they failed to metamorphose. This suggested that it would have been futile to try to increase the percentage of tadpoles that could be experimentally infected with spargana by giving more procercoids to each tadpole. Tadpoles infected with larger numbers of spargana may not have been able to metamorphose and may have died. It therefore seemed pointless to continue trying to infect tadpoles with controlled numbers of spargana by feeding them with living cyclops infected with procercoids. However, there was a chance that the tadpoles used in the experiment described above possibly did not become infected because the procercoids that they were given

could not emerge from the cyclops quickly enough to move into the tissues of the tadpole before being defaecated. It therefore seemed reasonable to try to infect tadpoles with procercoids contained in cyclops the exoskeletons of which were damaged. The reason for damaging the exoskeletons of the cyclops was to allow the procercoids to emerge easily. Twenty tadpoles were each each fed in the manner described above with a cyclops the exoskeleton of which had been pierced with a needle. Each cyclops contained two or three procercoids. These tadpoles were killed and dissected four weeks after they had been given the cyclops. No spargana were found.

In a final attempt to produce controlled infections of spargana in tadpoles, some tadpoles were fed with spargana. These tadpoles were anaesthetised in a solution of MS222 and were then transferred to clean pond water. Each tadpole was infected with a holdfast of a small sparganum which was introduced into the mouth of the tadpole. The tadpole then was isolated in a dish containing clean pond water. Fifteen minutes later the water in the dish was examined to see if the tadpoles had disgorged the sparganum. No disgorged spargana was found. Five months later, six of the tadpoles had died, seven had metamorphosed into frogs and the remaining seven showed no signs of metamorphosis. The living and the dead tadpoles and frogs all were dissected and examined and it was found that they all were infected. This at least seemed to be a way in which

tadpoles could be consistently infected with spargana even if they seemed to be adversely affected by their infections. To confirm this result, the experiment was repeated with a further twenty tadpoles each of which was infected with a holdfast taken from a ten day old sparganum. Six weeks later, the tadpoles were killed and examined. No spargana were found. The spargana used in this experiment were taken from mice. It was thought that perhaps better results would be obtained if spargana taken from frogs were used to infect tadpoles. Twenty tadpoles were each infected in the manner described above with a holdfast of a sparganum taken from a frog. Six weeks later these tadpoles were killed and examined and it was found they were uninfected. No more attempts were made to infect tadpoles or frogs with controlled numbers of spargana.

The immunological relationships between marsh frogs and spargana therefore were not examined because these hosts could not be infected consistently. It was considered that there was little point in examining the immunological relationships between spargana and the few frogs and tadpoles that became infected and survived because these frogs and tadpoles may have been atypical. Because frogs or toads of other species which may have been more susceptible to infection with spargana could not be bred conveniently in the laboratory, the study of the immunological relationships between laboratory bred amphibians and spargana had to be abandoned.

4.3 DISCUSSION

It is clear that the results described in sections 4.1 and 4.2 were insufficient to show conclusively whether or not the spargana used in the present study were better adapted to living in amphibians than they were to living in mice. However, these results suggested that the intensity of the immunological responses stimulated by these spargana in marine toads was comparable to the intensity of the responses stimulated by the spargana in mice. All the spargana present in the infected tissues of toads which were examined were surrounded by marked inflammatory responses and walls of the capsules formed by toads around spargana or fragments of spargana were generally denser and thicker than those formed by mice. Furthermore, it seemed reasonable to suggest that the serum taken from most of the toads examined could be considered to be strong antisera. When samples of these apparently strong antisera were used in immunodiffusion tests, bands of precipitate generally started to appear within 2-4 hours and these bands of precipitate often were quite dense. It was pointed out in section 2.32 that mammalian sera which react in this way can be considered to be strong antisera so it seemed likely that the toad sera which were tested also would be considered to be strong antisera. On the other hand, it was possible that the observed responses of infected toads were

the results of prolonged immunological stimulation. It therefore was possible that the spargana may have been less immunogenic to toads than they were to mice, but the responses produced by toads seemed to be as intense as those produced by mice mainly because the toads had been infected for a longer time. Nevertheless, it seemed that the spargana found in naturally infected toads could not be described as weakly immunogenic to the toads and there appeared to be no reason to believe that these spargana were especially adapted to living in toads.

Although nothing can be said about the immunological relationships between the spargana used in the present study and amphibians of the species Limnodynastes tasmaniensis, it seemed unlikely that these spargana were especially adapted to living in these amphibians. If the spargana had been adapted to living in marsh frogs (L. tasmaniensis) it seems reasonable to propose that it should have been relatively easy to infect these frogs or their tadpoles with consistant numbers of spargana. However, it was not. Some previous workers also found it difficult to infect frogs with procercoids (Mueller, 1938a; Li, 1929) and some attempts to infect adult frogs with mature spargana were only partially successful (Li, 1929; Mueller, 1938b). Thus it seems that even though spargana occur more commonly in wild amphibians than in wild mice, they may not be especially adapted to living in amphibians. Up until the present, therefore, no

evidence which shows that spargana are especially adapted to living in any wild animals which can act as their hosts has been produced. The laboratory-bred spargana of the North American form of spirometrid tapeworm seem to be adapted to living in laboratory mice, but how such adaptation could have occured is, as yet, unexplained.

SECTION 5 GENERAL DISCUSSION

It was explained in sections 3.5 and 4.3 that the immunological responses stimulated in mice and in an amphibian(Bufo marinus) by the spargana used in the present study could not be considered to be feeble or abnormal. The main aim of the present study which was to find out if spargana possess some mechanism which enable them to suppress or to avoid stimulating the immunological systems of their hosts therefore could not be achieved by means of the spargana I used Such an aim clearly could be achieved only by using spargana such as those of the species Spirometra mansonoides which seem to be only weakly immunogenic to hosts such as mice and hampsters

In previous sections of this thesis I referred only infrequently to studies of other workers who examined the immunological responses stimulated by cestode larvae other than spargana. Instead, I referred to publications in which the responses that are typically produced by mammals to antigens which induce an immediate form of hypersensitivity were described. I hoped to show in this way that the responses stimulated by

the spargana I used could be described as normal. This approach was adopted because it often was not possible to make valid comparisons between the observations made by other workers who studied the responses stimulated by cestode larvae and the observations recorded in this study. Nevertheless, it seemed pertinent to make such comparisons where possible if only to try to obtain further support for the proposition that the responses induced by the spargana I studied were normal in form and intensity.

Much of the evidence which supports the proposition that cestode larvae are highly immunogenic to their hosts has come from studies on the susceptibility of infected or immunised animals to subsequent infections (see the review by Weinman,1966). It has been shown that a number of species of cestode larvae stimulate a marked or almost absolute degree of resistance to re-infection in their hosts. For example, a high degree of resistance to reinfection may be developed by mice against Hymenolepis nana (Hunninen, 1935), by rats against Taenia taeniaeformis (Miller, 1931) by cattle against Taenia saginata (Penfold, et al., 1936) and rabbits against Taenia pisiformis (Kerr, 1935). Furthermore, it has been shown that, for example, mice develop a marked resistance to reinfection with Hymenolepis nana in less than two days (Hearin, 1941; Heyneman, 1962). On the other hand, experimental infections of Echinococcus granulosis

in sheep seem to induce only a moderate level of acquired resistance, even after the sheep were given initial infections of large numbers of eggs and were challenged nine months later (Sweatman et al., 1963). The experiments on resistance to reinfection in mice that were done during the present study were not strictly comparable with those done by other workers because most other workers challenged the animals they used with onchospheres given per os whereas I gave mice either intraperitoneal injections or procercoids in cyclops or oral infections of spargana Nevertheless, the observations that mice infected with spargana for six weeks developed a marked degree of resistance to reinfection with procercoids at least suggested that the immunogericity of spargana in mice was greater than that of Echinococcus granulosis in sheep but less than that of Hymenolepis nana in mice or Taenia pisiformis in rabbits. Nothing more definite can be stated mainly because it clearly is hazardous to assume that if equal proportions of procercoids and hexacanth larvae fail to develop as a result of the effects of immunological responses then the immunological responses directed against the two kinds of larvae are of equal intensity. Too little is known about the effects of immunological responses on procercoids or hexacanth larvae to justify such an assumption. Some attempts have been made to study the effects of antibodies on hexacanth larvae or eggs of tapeworms by incubating the larvae or

eggs *in vitro* in immune serum (Chen, 1950; Heyneman and Welsh, 1959; Silverman, 1955; Weinmann, 1966). According to Chen (1950) immune serum has no apparent effects on the hexacanth larvae of *Taenia taeniaeformis*. Weinmann (1966) also failed to detect any effect of immune serum from mice on the morphology or the infectivity of the eggs of *Hymenolepis nana*. Heyneman and Welsh (1959) however found that the immune serum of artificially immunised rabbits caused changes in the morphology of *Hymenolepis nana* eggs and reduced their infectivity. Silverman (1955) also showed that precipitates and membranes formed around activated hexacanth larvae of *Taenia saginata* and *Taenia pisiformis* which were incubated in immune calf or rabbit serum. Results obtained in the present study suggested that antibodies induce a change in the morphology of spirometrid procercoids but it was not possible to determine if these morphological changes were correlated with a change in the infectivity of the procercoids. Such evidence clearly does not show conclusively that spirometrid procercoids and the hexacanth larvae of tapeworms other than spirometrids are equally susceptible to the effects of antibodies.

The resistance of mice to superinfections of procercoids may have resulted in part from the effects of cellular responses. Some previous workers obtained evidence which at least suggested that cellular reactions may be partly responsible for the resistance to re-infection of, for example, rabbits to

Taenia pisiformis (Leonard, 1940) and mice to Hymenolepis nana (Bailey, 1951) but the evidence was inconclusive. The effects of cellular responses on the procercoids of spirometrid tapeworms have not been investigated. Therefore it is not possible to determine if these procercoids are any more or any less susceptible to the effects of cellular responses than are the larvae of other cestodes.

The humoral responses stimulated by the antigens of cestodes have been investigated by a number of workers (see, for example, Chordi and Kagan, 1965; Coleman, 1963; Coleman, Carty and Graziadei, 1968; Coleman and Fotorny, 1961; Coleman and de Sa, 1964; Cramer and Dewhurst, 1965; Hadano, 1959; Kajiwara, 1960; Kravtsov,1966; 1967b; Madison, Whittle and Elsen-Dew, 1961; Nakabayashi, 1961; Norman, Kagan and Chordi, 1964; Wilhelmi, 1940). However, there are reasons which suggest that it is invalid to compare the results obtained by these workers with those obtained in the present study in order to try to assess the relative antigenicity of spargana. The relative antigenicity of two antigens or organisms can be assessed only when quantitative or semi-quantitative methods are used to estimate the intensity of the humoral responses stimulated by the antigens or organisms. Furthermore, the method used to estimate the antigenicity of one antigen must be the same as that used to estimate the antigenicity of the other antigen. Most previous workers who studied the humoral responses stimulated

by cestode larvae either did not attempt to obtain quantitative or semi-quantitative results or they used methods that were different from those used in the present study. In addition, many workers used hosts other than mice and a number of workers studied responses induced by injecting animals with extracts of adult or larval cestodes. None of the workers who previously examined the humoral responses induced by the antigens of cestodes used methods and hosts the same as those used in the present study. Consequently the results obtained by those workers were not comparable to those obtained in the present study.

Whether or not spargana produced fewer or more antigens than other kinds of cestode larvae also could not be determined with certainty. The results obtained by previous workers who used animals other than mice to try to determine the number of antigens produced by cestode larvae clearly cannot be compared with the results obtained in the present study. Different species of animals respond differently to complex mixtures of antigens. It therefore cannot be assumed that the substances in spargana to which mice produced antibodies also will stimulate other species of animals to produce antibodies. Moreover, it cannot be assumed that substances in spargana that were non-antigenic to mice also will be non-antigenic to other species of animals. A valid comparison between the number of antigens present in two species of parasites can be made only if one

species of host and standardised procedures and techniques are used to test for the antigens produced by both species of parasites. The procedures and techniques used by each previous worker who studied the antigens of larval cestodes differed to a greater or lesser extent from those used in the present study. Thus the results obtained by all previous workers were not validly comparable to the results obtained in the present study.

Little also can be said about the origin or the nature of the antigens produced by the spargana that I studied. The observation that the antigens were proteinaceous was consistent with the observation of Nakabayashi(1961) who suggested that proteins were responsible for the sensitisation of rabbits to spargana but these observations gave little indication of the nature or the origins of the antigens. Chordi and Kagan(1965) found that there were some carbohydrate and lipid components associated with some of the antigens present in the fluid taken from hydatid cysts found in sheep. However, no such components were found associated with the antigens of spargana to which mice responded. It is perhaps worth noting that some of the antigens produced by spargana may be present in the secretions and excretions of these parasites. Mueller(1961) found that precipitates formed on the surfaces and around the excretory pores of spargana of the species Spirometra mansonoides which were placed in serum taken from infected mice. A similar response was observed by

Shults and Ismagilova(1962) who described the precipitates that formed on the live scolices of Echinococcus granulosus which were placed in hyper-immune serum from rabbits or guinea pigs or the serum of infected human beings or sheep. The secretions and excretions of both spargana and hydatid scolices therefore may contain antigenic substances.

The pathological conditions induced by infections of the spargana used in the present study in mice seemed to be similar in some ways to those induced by other kinds of larval cestodes in other species of hosts. However, the local pathological changes induced by other cestode larvae seemed to be variable. Silverman and Hullard(1961) stated that some calves showed little reaction to Cysticercus bovis infections whereas other similarly infected animals reacted very strongly. In some cases adjacent parasites in a single muscle evoked very different local reactions. Cysts found in skeletal muscle one month after infection were surrounded by a layer of dense connective tissue which enclosed and blended with a layer of loose connective tissue that contained lymphocytes, macrophages and some neutrophils. A crescentic zone of necrotic cells and debris was found near the parasite. Eosinophils and giant cells were not detected. The variation in the response produced to the parasites apparently was in the severity of the atrophy of the muscles immediatly outside the capsule, the thickness of the capsule wall and the thickness of the granulation tissue

associated with the capsule. In general, the responses seen around cysticerci in the tongues and hearts of calves were similar to those seen around cysticerci in the skeletal muscle except that in some instances there was little or no host reaction to cysticerci in the heart and the heart muscle displayed acute lytic changes. An excess of neutrophils and some eosinophils were present around larvae in the tongue whereas relatively few neutrophils and some giant cells were seen around one degenerating larva in the heart. The reactions seemed to increase in severity but changes relatively little in nature during the second and third month of infection. Silverman and Hullard(1961) also stated that some degenerating cysts occurred at every stage following infection from three weeks onwards. The onset of degeneration was marked by an intensification of the inflammatory response and the loss of integrity of the larval cuticle followed by an invasion of granulocytes of the kind normally found in chronically inflammed tissue. They(Silverman and Hullard, 1961) also commented that the absence of an intense inflammatory response around mature Cysticercus bovis cysts was so striking that they decided to investigate the responses induced by other species of cysticerci that develop in skeletal muscles. They found that Cysticercus cellulosae cysts in pig muscle and Cysticercus ovis cysts in sheep muscle elicited reactions similar to those observed with C. bovis in calves. Voge and Berntzen(1963) stated that the local responses evoked

in the dog by cysts that seemed to resemble those of the species Multiceps serialis were similar to those described by Silverman & Hullard (1961). Leonard (1940) reported that infections of Cysticercus pisiformis in rabbits caused marked degenerative changes in liver tissue and within three days of infection the larvae was surrounded by a zone of necrotic tissue which in turn was surrounded by a layer of macrophages. By the end of the tenth day of infection the larvae were enclosed in fibrous capsules which were lined with lymphocytes and epitheloid cells and contained some necrotic cells. This response was accelerated in immunized rabbits. Death of some larvae in non-immunized rabbits and many larvae in immunized rabbits was observed. These observations seemed to support the proposal that the death of Cysticercus pisiformis cysts in immunized rabbits may be due to the effects of local inflammatory reactions. Cellular reactions consisting of mainly polymorphs with possibly some eosinophils apparently were induced by the cysticercoids of Hymenolepis nana in mice but no encapsulation occurred (Bailey, 1951). Second infections of these parasites induced an accelerated response. The local reactions produced by cestode larvae have been examined by Berezautsev (1962) who pointed out that the fibrous and granulomatous cysts produced around cestode larvae usually are more vascular than the capsules formed around foreign bodies. It has been reported that the

larvae of Multiceps serialis in the brains of mice induced responses that in general were marked but nevertheless were variable (Larsh, Race and Esch, 1965).

The intensity of the local inflammatory responses induced in mice by the spargana used in the present study seemed to be intermediate between that of the most severe and that of the least severe response produced by other kinds of animals to other cestode larvae. There was clearly no reason to believe that the spargana I studied induced local inflammatory responses that were less intense than those induced by all other cestode larvae. The responses I observed seemed to be a little more severe than those described by Berezantsev (1962) who studied the inflammation induced by the spargana he identified as those of the species Spirometra erinacei in hampsters. However, they seemed to be less intense than the responses observed by other workers who studied the reactions of human beings and monkeys to spargana (see section 1.132).

It also is relevant to point out that the local responses observed in the vicinity of spargana probably were induced by the spargana only and not by bacteria or other micro-organisms which the spargana may have carried with them from the lumen of the gut and into the tissues. Feng and Hoppli (1936) found that spargana they identified as those of the species Spirometra erinacei carried bacteria with them from the gut and into the peritoneal cavities of hampsters but the spargana were more or

less sterile when they arrived in the sub-cutaneous tissues. Apparently, no serious pathological conditions were induced by the bacteria introduced into the peritoneal cavities of the hampsters as a result of the penetration of spargana.

The general pathological conditions induced by cestode larvae other than spargana also seemed to be very variable. Schiller (1949) observed that infections of large numbers of cysticerci of Taenia taeniaeformis have no apparent effect on mice and that the growth rate of the mice was not related to the degree of infection. Mild to marked signs of general illness such as erratic behaviour and emaciation have been observed in sheep the brains of which have been infected with coenurus cysts (Fankhauser, Hintermann and Valette, 1959), in a dog the brain of which was infected with Cysticercus cellulosae (Mayaudon, Garcia and Merino, 1958), in mice infected with the larvae of Multiceps serialis (Larsh, Race and Esch, 1965) and in a dog infected with larvae that resembled those of Multiceps serialis (Voge and Berntzen, 1963). In section 1.132 it was pointed out that the general pathological changes induced by spargana varied according to the degree of infection and the species of host. In some hosts such as human beings they induced clearly recognisable and often marked signs of pathological changes whereas they seemed to induce few or no signs of such changes in mice and hampsters. From this point of view, therefore, spargana seemed to be little

different from a number of other kinds of cestode larvae. Whether or not the increased rate of growth stimulated by spargana in mice and hampsters (Mueller, 1965b) can be considered to be a pathological change is a matter for speculation. The mechanism which brings about this increase in weight at an abnormal rate is not understood at present. Sadun, et al., (1965) found that mice infected with the spargana of the North American spirometrid (Spirometra mansonoides) had elevated blood-glucose levels and depressed levels of serum alkaline phosphatase and total protein but these workers were reluctant to speculate about the reasons for these changes except to say that it was 'tempting to attribute the elevated serum glucose levels to a relative or absolute deficiency of insulin'. It also has been shown that these spargana can cause an increased rate of growth in rats that have had their growth experimentally suppressed by treatment with propylthiouracil to induce hypothyroidism (Mueller and Reed, 1968) and in rats which were thyroidectomised or hypophysectomised (Mueller, 1968). As Mueller and Reed (1968) pointed out, spargana clearly affect the biochemical and endocrine systems of their hosts but Mueller (1968) commented that ' the idea that the worms can produce on demand insulin, thyroxine or pituitary growth hormone seems a bit too good to be true' and he (Mueller, 1968) seemed reluctant to offer any explanation for the effects of spargana on mice or rats. The growth of rats which have been

treated with propylthiouracil can be stimulated also by spargana from Australia, Malaya and Taiwan though these spargana seem to have less of an effect than the spargana of Spirometra mansonoides (Mueller, 1968). This aspect of the relationships between spargana and their hosts clearly must be studied further before it is understood, but it seems reasonable to suggest that the growth stimulating effect of spargana on mice, rats and hampsters cannot be described as a pathological effect. There have been no other reports of growth stimulating effects of other cestode larvae.

It seemed to me therefore that when it was possible to make valid comparisons between the immunological responses stimulated by the spargana used in the present study and the responses stimulated by other cestode larvae the spargana stimulated responses that were similar in general form and intensity to the responses stimulated by at least some other cestode larvae. In other words, the responses described in the present study possibly could be considered to be normal responses of the kind induced by cestode larvae. This conclusion seemed unsatisfactory because it is vague, but I consider that a more positive statement could not be justified. The difficulties encountered when attempts were made to compare the responses described in the present study with those described by other workers perhaps serve to show that it may be necessary for parasitologists to adopt a more unified approach and more standardised methods in order to achieve a clear

understanding of the immunological aspects of the relationships between cestode larvae and their hosts.

The results obtained in the present study were disappointing firstly because they gave no indication that the spargana used were able to suppress or to avoid stimulating the immunological systems of their hosts and secondly because they seemed to contribute little or nothing to our general understanding of the immunological aspects of the relationships between parasites and their hosts. The observation that these spargana stimulated apparently normal immunological responses in mice added to our knowledge but it did not help to clarify any general aspect of the host-parasite relationship. The same can be said of the observation that marine toads naturally infected with spargana produced immunological responses that seemed to be generally similar to the responses produced by mice. It was pointed out in section 4 that the results obtained by other workers who studied the immunological systems of amphibians suggested that there is a resemblance between the immunological responses produced by amphibians and mammals. The results described in the present study tended to confirm this hypothesis and perhaps extended it slightly. To the best of my knowledge, no previous worker has examined the immunological responses produced by amphibians such as marine toads to tissue-inhabiting metazoan parasites. Thus it has not been shown

previously that there is any resemblance between the immunological responses produced by amphibians and mammals to such parasites. The observations on the responses induced by spargana in marine toads therefore were interesting but could not be used as a basis for extensive speculation. It clearly would be hazardous to base any speculation related to, for example, the phylogeny of the immune response or the immunological adaptation of metazoan parasites to amphibians on some observations of the responses induced by one kind of parasite in naturally infected amphibians of one species. The results of further experimental work will be required before it will be possible to state with confidence that the immunological responses of amphibians to metazoan parasites are similar to the responses of mammals to such parasites. The results of more experimental work also will be needed before it will be possible to state with confidence that the immunological systems of amphibians have reached a level of development comparable to that reached by the immunological systems of mammals. When more work is done, it may be found that neither of these hypotheses is correct.

One further point related to the general significance of the observations described in this thesis is worthy of mention. It has been suggested that the cuticles and the walls of the cysts of larvae such as cysticerci may prevent the release or exposure of some antigens and they thereby reduce the extent of the antigenic

stimulus provided by the parasites (Sprent, 1962). Spargana, on the other hand, seem to have no structures that permanently prevent the release or exposure of their antigens (see section 1.2). If spargana can survive and live in reasonable harmony with their hosts even though they ~~do~~ possess no barriers which permanently prevent the release of at least some of their antigens, why is it that other cestode larvae have developed such barriers? This question cannot be answered at present because so little is known about the immunological relationships between cestode larvae and their hosts. It has yet to be shown that an important function of the cuticle and the cyst of a cysticercus is to prevent the release or exposure of antigens. Perhaps the cysts around some cestode larvae are freely permeable to the antigens of the parasites. Antigens of host origin have been found in the fluid taken from hydatid cysts in human beings and sheep (Chordi and Kagan, 1965) so it is likely that the walls of these cysts are permeable to at least some and perhaps many of the antigens of the parasites.

An important question to ask in the present context is what benefit is gained by the larval cestode if it possesses structures which prevent the release of one or more kinds of antigen into the tissues of its host? Again, no satisfactory answer can be given to this question at present. If it is assumed that antibodies can adversely affect larval cestodes, it is reasonable

to propose that a larval cestode may be at an advantage if it restricts the release of its antigens and thereby fails to evoke a marked humoral response. However, little is known about the effects that antibodies may have on cestode larvae so there is little justification for assuming that antibodies can have a marked effect on these parasites. It seems that antibodies may have a direct adverse effect on the cysticercoids of Hymenolepis nana (Heyneman and Welsh, 1959) but whether or not antibodies have a direct adverse effect on other kinds of cestode larvae has not been determined.

It is also possible that the failure of a cestode larva to release some kinds of antigens may result in a reduction of the inflammatory response induced by the larva. If it is assumed that this is of benefit to the larva, it also must be assumed that inflammatory responses can adversely affect larval cestodes. It has been observed that the degeneration of the cysts of Cysticercus bovis in calves was preceeded by an intensification of the inflammatory responses stimulated by these larvae (Silverman and Hullard, 1961) so it is possible that these larvae are susceptible to the effects of inflammatory responses. Other cestode larvae may also be susceptible to effects of inflammatory responses but no conclusive evidence to support this proposition has been obtained. It nevertheless seems likely that it may be advantageous for cestode larvae to limit if not entirely prevent the release of

their antigens if only to reduce the intensity of the inflammatory responses that they stimulate.

It is perhaps relevant to point out that spargana can migrate through the tissues of their hosts. Most cestode larvae are sedentary. It therefore may be important that these sedentary parasites do not stimulate marked inflammatory responses if such responses can damage or kill the larvae. If such marked responses are stimulated, the larvae cannot migrate away from the inflammed tissue. Spargana can migrate out of inflammed tissue. It may be, therefore, that spargana are able to survive in hosts to which they are highly antigenic because the spargana can migrate out of inflammed tissue and thereby avoid the effects of the responses produced by the hosts.

Finally, I wish to consider briefly the taxonomy of the spirometrid tapeworms. It is of some interest to note that immunological methods may help in the future to clarify the taxonomy of these tapeworms. I mentioned in section 1 that Wardle and McLeod (1952) included seventeen species of tapeworms in the genus Spirometra. Very little attention has been paid to most of them since they were first described. It is perhaps significant to note that when attention has been given to the taxonomy or general biology of any species of spirometrid tapeworm, doubts about the validity of the species seem to arise. This suggests that the validity of most species of spirometrid tapeworms

may be questionable but the validity of the less commonly used species has not been doubted because not enough is known about them.

According to Iwata (1933) the species Spirometra mansoni, S. ranarum, S. reptans, S. decipiens, S. okumuri and S. houghtoni are invalid and represent varieties of the species Spirometra erinacei Rudolphi, 1819. Yamaguti (1959) suggested that the species S. decipiens, S. houghtoni, S. okumuri and some other less-studied species are identical with the species Spirometra erinacei-europei Rudolphi, 1819 (a synonym of the species Spirometra erinacei Rudolphi, 1819). He (Yamaguti 1959) also considered the species Spirometra mansonoides to be invalid and identical with the species S. erinacei-europei Rudolphi, 1819. Mueller (1966) seemed sure of the validity of the species S. mansonoides whereas he expressed doubts (Mueller, 1966, 1967) about the validity of most other species of spirometrid tapeworms. Similar doubts about the validity of a number of species of spirometrids were expressed by Dubinina (1951). Thus there seemed to be ample justification for the opinion of Chandler and Read (1961, p.352) who stated that 'there is much confusion about the species of Spirometra'.

This confusion seems to have arisen because previous workers attempted to define species of spirometrid tapeworms by describing features of the morphology and anatomy that are much too variable.

For example, two of the characteristics which are used to identify species of spirometrids are the length and width of the proglottids and the fusion or otherwise of the lateral fields of testes and yolk-glands in the region of the proglottid just in front of the genital openings. Wardle and McLeod (1952) seemed to believe that these two criteria could be used to identify spirometrid tapeworms. However, the studies of Iwata (1933) and Joyeux and Houdemer (1928) clearly showed that these two features are far too variable and cannot be used to define the species of spirometrid tapeworms. The antigenic characteristics of these tapeworms may provide a better means of devising a new taxonomic system (see Kravtsov, 1967a). With this aim in mind, Kravtsov (1966, 1967b) has already started to study the antigenic characteristics of diphyllobothriid tapeworms but his studies are not yet complete.

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APPENDIX

The methods used to estimate the weight of materials and nitrogen per millilitre of the extracts of spargana

To estimate the amount of disolved materials in the extracts of spargana, 1ml samples of the extracts and 1ml samples of the saline solution from which the extracts were prepared were placed in weighed dishes and were heated to constant weight in an oven at 105°C. The results obtained are summarised in Appendix Table 1.

The total weight of nitrogen in the extracts was determined by means of the micro-Kjeldahl method (Kabat and Mayer, 1961, pp 476-477) followed by the estimation of ammonia by means of the micro-diffusion method described by Conway (1962, pp.7-13, 98-100). The results obtained from these estimations are summarised in Appendix Table 2.

APPENDIX TABLE 1

Estimates of the total weight of dry materials per millilitre of the extracts of spargana and of the saline solution used to prepare the extracts

Solution	Saline			Extract 1			Extract2			Extract 3		
Sample	1	2	3	1	2	3	1	2	3	1	2	3
Total weight of dry material (mg per ml)	8.5	8.5	8.5	26.0	26.0	26.0	19.9	19.4	19.6	19.1	19.8	19.7
Average total weight of dry material (mg par ml)	8.5			26.0			19.6			19.5		
Average total weight of dry material (mg) corrected for added NaCl.	-			17.5			11.1			11.1		

APPENDIX TABLE 2

The results used to estimate the weight of nitrogen per milliltre of the standard extracts of spargana

	Extract 1	Extract 2	Extract 3
Volume of diluted digested extract per Conway unit(ml)*	0.5	1.0	1.0
Titre of ammonium borate represented by equivalent volume of N/50 HCl† (three replicate titrations)	0.034 0.033 0.035	0.047 0.047 0.046	0.047 0.043 0.042
Average titre	0.034	0.047	0.044
Weight of nitrogen per millilitre of original extract(mg)	0.952	0.658	0.632

* 1ml of extract digestedwith 10ml of digest mixture and the resulting solution diluted with distilled water to a final volume of 50ml.

† Figures in this line represent the volumes of N/50 HCl used minus the volumes of N/50 HCl used to produce similar end points in samples of control solutions containing NaCl and digest mixture only(values for control solutions - Extracts 1 and 2:- 0.004ml N/50 HCl, extract 3:- 0.009ml N/50 HCl)

E R R A T U M

Page	Line	Correction
4	4	For "If" read "It"
23	10	For "abd" read "and"
36	16	For "filute" read "dilute"
38	2	For "Lilley" read "Lilly"
50	9	For "sensitised" read "sensitised and possibly unsensitised"
69	1	For "Legent" read "Legend"
73	2	For "wereas" read "whereas"
78	10	For "haemagglutin" read "haemagglutination"
80	7,12,23	For "Ostler" read "Osler"
82	5	For "Ostler" read "Osler"
86	21	For "infected" read "uninfected"
111	12	For "Which" read "which"
129	8	For "Urh" read "Uhr"
145	21	For "appeared in" read "appeared between"
171	13	For "Elsen-Dew" read "Elsdon-Dew"
176	21	For "Berezautsev" read "Berezantsev"
178	14	For "Mayaudon" read "Mayandon"

References

Jarrett et al (1966)	For "namatode" read "nematode"
Johnson (1911)	For "reptilion" read "reptilian"
Kajiwara (1960)	For "Kajiware" read "Kajiwara"
Osler (1963)	For "Ostler" read "Osler"