STUDIES ON ACQUIRED IMMUNITY TO *TAENIA TAENIAEFORMIS* IN RODENTS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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ABSTRACT

This work is a study of both active and passively acquired immunity to Taenia taeniaeformis infection. The role of humoral components in serum as demonstrated by passive transfer of immunity and both active immunity following infection or immunisation were investigated. The antigens derived from larval T. taeniaeformis for immunisation studies were also used to raise immune sera, to monitor experimental infections, and to absorb out protective antibodies.

Metacestodes retrieved from mice were maintained in a defined medium for between ten and twenty-three days. The tegumental integrity of the metacestodes during maintenance was assessed by dye uptake and transmission electron microscopy (TEM). The medium in which the larvae had been held was used as a source of excretory-secretory antigen (ESA). Somatic antigen (SA) was derived from a saline extract of homogenised larval somata. Antisera raised against both ESA or SA preparations, were used to detect antigens in the used medium by the enzyme linked immunosorbent assay (ELISA). After verifying that the ELISA technique could quantitate antigens, and that the assay was not affected by the presence of serum in the medium, the effect of several factors on antigenic activity was investigated. The antigenic activity of the used medium did not appear to be unduly affected by the age of the larvae, by sealing the maintenance vessels, by variations in the integrity of the larval tegument or by the absence or presence of serum during maintenance. However, the beneficial effect of including serum in the maintenance was reflected in the dye uptake and TEM results.

The aim of studying these factors was to determine the optimal conditions under which the larvae would release maximal amounts of ESA.

ELISA was also used to monitor the humoral response of mice and rats experimentally infected with T. taeniaeformis against either ESA or SA as the detecting antigens in the assay. It was found that ESA was a more sensitive indicator of the infection than SA.

The ability of ESA and SA to protect rats against infection was investigated. Both these crude antigenic complexes seemed to possess similar immunising capacities. An association was found between the reactivity of these antigens in vitro by ELISA and their immunising or protective activity in vivo, which suggests that such antigens may provide a useful measure of the levels of protective antibody in the serum of the host.

Homologous and heterologous immune sera were obtained by immunising rats and sheep respectively with ESA or SA. The immune sera obtained in this manner was protective in passive transfer experiments. This appears to be the first time that serum from animals immunised with ESA has been demonstrated to confer protection against a helminth infection. Immune serum from experimental infections of six weeks duration conferred almost absolute protection, confirming previous published reports.

The highly protective immune globulins from active infections was absorbed with ESA, SA or live larvae. The absorption had a measure of success when assessed by in vitro estimation. However,

when used in passive transfer, the absorbed globulins retained their protective ability. Several possible reasons were adduced for this apparent failure. Thus there may have been insufficient concentration of antigen to remove all the protective antibodies from the highly active serum. Alternatively the protective antibodies produced in an active infection may not be induced exclusively by the soluble components in ESA or SA but possibly by both of these and even other antigens. Either of these suppositions would account for the partial efficacy of absorption found *in vitro*.

LIST OF ABBREVIATIONS AND SYMBOLS

Å	ångström (10^{-10} m)
B	Belgian strain (of Taenia taeniaeformis)
CO_2	carbon dioxide
°C	degree Celsius (temperature)
cm	centimetre
CNBr	cyanogen bromide
DEAE	Diethylaminoethyl
ELISA	Enzyme-linked immunosorbent assay
ESA	Excretory and secretory or metabolic antigens
FCA	Freund's complete adjuvant
FCS	Foetal calf serum
g	(acceleration due to) gravity
HEPES	4-2(2-Hydroxyethyl)-1-piperazine ethane
i.e.	that is
IgG	immunoglobulin class G
IgG_1	immunoglobulin G subclass one
IgG_2	immunoglobulin G subclass two
IgM	immunoglobulin class M
I.U.	International Units
l	litre
log	logarithm to the base 10
M	Malaysian strain (of Taenia taeniaeformis)
µg	microgram
µl	microlitre
µm	micrometre
mg	milligram

ml	millilitre
mm	millimetre
mmol	millimole
nm	nanometre
NS	In statistic: not significant
Pa	pascal (pressure)
PBS	phosphate buffered saline pH 7.3 (Dulbecco "A" Oxoid)
RPMI-1640	Roswell Park Memorial Institute cell culture medium - 1640 (Gibco)
SA	Somatic antigen
TRIS	Tris-(hydroxymethyl)-aminomethane
$U\ l^{-1}$	International units per litre
Us	Wilcoxon test statistic
$^{v}/_{v}$	volume per volume
$^{w}/_{v}$	weight per volume
7Sy1	immunoglobulin G subclass one with molecular weight around 150,000 (sedimentation coefficient 7 Svedberg unit)
7Sy2	immunoglobulin G subclass two with molecular weight around 150,000 (sedimentation coefficient 7 Svedberg unit)
-	In tables: Not done

GENERAL INTRODUCTION

The two parasites of major importance in taeniasis-cysticercosis are the tapeworms Taenia saginata (Goeze, 1782) and Taenia solium (Linnaeus, 1758). These two species are obligatory parasites of the intestinal tract of man. Taenia solium, the metacestode of which occurs mainly in pigs is the more serious pathogen because it not only causes porcine cysticercosis but also the often fatal cerebral cysticercosis in man. This parasite is however relatively limited in its distribution, although it still occurs widely in parts of Central and South America, Africa and South Asia. In contrast the metacestode of Taenia saginata which causes cysticercosis of cattle, is world-wide in distribution with an increasing incidence in many countries (Pawlowski and Schultz, 1972). It causes fiscal losses especially to the beef industry of East Africa, where there is a high prevalence of the disease. The losses are due to the condemnation of heavily infected carcasses, the cost of treating lightly infected carcasses (transport, freezing, boiling, or curing, etc.) and the reduction in value of these carcasses following treatment. Cysticercosis also hinders the meat export prospects of developing countries due to stringent quality control imposed by importing countries. The incidence of bovine cysticercosis is also increasing in many developed countries in Europe (Soulsby, 1975). In this case, the economic loss is to individual cattle-owners rather than on a national scale. The carcasses are rejected for human consumption on public health or aesthetic grounds. Some factors contributing to the increased incidence are: the overloading of sewer systems due to an increase in urban population,

the increasing use of sewage sludge as fertiliser for pastures, the intensive method of cattle rearing, and the role of birds, especially seagulls, in indirect transmission.

Man is the only definitive host and Bovidae the only intermediate hosts for Taenia saginata, while adult Taenia solium will only develop in man and some primates, with the metacestodes in pigs and to a lesser extent dogs. This restricts experimental work with these species, for obvious ethical and economic reasons. Hence, other species of the family Taeniidae have been employed as model systems. In this study, Taenia taeniaeformis (Batsch, 1786) which has a life cycle involving Felidae and rodents was used as the experimental model. This species was selected for several reasons. The hosts are easily and inexpensively maintained in the laboratory, and provide a ready supply of oncospheres or metacestodes. Accurate counts can be easily made of infections because the parasites localize in a single organ, namely the liver. Furthermore, the larval form exists aseptically within a capsule embedded in the livers of infected rodents and can be transferred easily to culture media without bacterial contamination.

Numerous studies have been undertaken aimed directly or indirectly at developing means of controlling cestode parasites. One method of controlling the larval population is by vaccinating the intermediate host. With the advent of improved culture systems, Urquhart (1980b) suggested that material derived from Taenia saginata stages maintained in vitro might be used as a vaccine against bovine cysticercosis. Alternatively, antigens released by

heterologous parasites in culture - the so-called excretory-secretory antigens - might offer a more practical source of material for such vaccines (Urquhart, 1980a).

The major problem associated with attempts to study helminth culture antigens is that of obtaining them in adequate quantities. Another problem is to establish the most favourable *in vitro* conditions to allow the metabolic processes to continue at an optimal rate for the longest possible period of time so that the antigens produced *in vitro* reflect those normally produced *in vivo*.

The objective of this study was to devise techniques for producing protection-inducing antigens, which might be applied to cysticercosis or other cestode systems such as coenuriasis or echinococcosis. The materials mainly used for the vaccination studies were excretory-secretory or somatic antigens derived from larval *Taenia taeniaeformis*. The soluble antigens in these preparations were also utilised to assess the value of serodiagnosis in infected rodents, and to produce immune sera for studies on passive transfer of resistance.

PART I

Studies on the antigenic products released by *Taenia taeniaeformis* metacestodes when maintained *in vitro*

CHAPTER 1

INTRODUCTION

In vitro culture of a parasite necessitates providing artificial conditions for its growth or differentiation. According to Smyth (1969) the term survival implies the maintenance of an organism *in vitro* at a metabolic level sufficient to keep its cells and tissues alive but not sufficient to allow growth or differentiation to occur. Hence, mere *in vitro* survival of the parasite cannot be termed "culture". In this work, no assessment was made of the processes which distinguish between growth and/or differentiation and survival, so it was considered appropriate to use the term "maintenance" rather than "culture" when referring to the *in vitro* medium and associated procedures.

The main aim of the maintenance system was to obtain metabolic products released from live, healthy metacestodes for studies on their immunogenic and antigenic properties. It was not intended to culture the larvae to obtain further development and therefore a maintenance medium that simply allowed for survival and metabolic processes to occur was adequate.

This series of experiments was primarily designed to optimise the production of metabolic antigens and reduce the amount of somatic antigens in the used maintenance fluids. This was sought because it was thought that the somatic components result from the decay of the parasite *in vitro*. It has been shown that some of these metabolic products result from active metabolism and are not merely somatic components released by slowly degenerating larvae (Brandt, 1980). Furthermore, metabolic products may be less

complex and so more suitable for use as a diagnostic antigen or as a protective immunogen. However, it was thought desirable to ascertain whether the parasites maintained their tegumental integrity *in vitro*.

Metacestodes of *Taenia taeniaeformis* were used in this *in vitro* work in preference to the more widely used oncospheres for several reasons. Thus, constraints in numbers of the feline definitive host limited the supply of eggs; the use of metacestodes dispensed with elaborate hatching and activation procedures, essential as a preculture requirement for oncospheres. Additionally, the relative large size of the developed metacestode offers an obvious handling advantage and may provide larger amounts of the desired product.

The maintenance periods were relatively short-term periods, no longer than 23 days. This was because preliminary observations showed that after that time some of the metacestodes lost their bladders and degeneration, though only slight, was visible.

REVIEW OF THE LITERATURE

Since *in vitro* culture did not constitute a major part of the work, an extensive review of the literature on *in vitro* culture of taeniid parasites has not been attempted. However, these studies which concern the cultivation of *Taenia taeniaeformis* at different stages of its life-cycle will be mentioned, with emphasis laid on relevant aspects.

The prime aim of *in vitro* culture in general was to study the biology of the parasites, that is, aspects of their nutrition, biochemistry and physiology, without the complexity provided by the host's environment. As work progressed in fulfilling the growth requirements for the parasite outside the host, other uses of *in vitro* culture were conceived. Smyth (1976) listed the uses of *in vitro* studies and one particular use he mentioned was to study the parasite's immunological behaviour. This should aid a better understanding of the mechanism of parasite survival within the host.

The work accomplished by other workers will be discussed under the headings of biochemical, physiological and immunogenic studies.

Biochemical studies

Haskins and Oliver (1958) studied the catabolic nitrogenous products released by mature *T. taeniaeformis* metacestodes incubated in aerobic, axenic conditions for 24-48 hours. Chemical analyses of the incubates revealed that less nitrogenous material was excreted compared to nematode larvae and that one-third of the total nitrogen was urea.

Von Brand (1973) speculated that worms maintained *in vitro* were probably abnormal to explain reported observations of carbohydrate leakage from larval and adult *T. taeniaeformis*. For this parasite, Von Brand and Bowman (1961) showed that both larval and adult forms mainly lost glucose and, in 1964, reported that the larvae required 100-200 mg per cent of glucose in the external medium before absorption could occur. As this leakage of carbohydrate was demonstrated only *in vitro*, the question remains as to whether it is the parasite's response to unfavourable external conditions or if it also occurs *in vivo* (Von Brand, 1973), as a normal physiological process.

The larval and adult *T. taeniaeformis* when maintained *in vitro* produced the same carbohydrate end-products by either aerobic or anaerobic fermentation. The products were lactate, pyruvate, acetate, succinate and ethanol. Lactate and acetate possibly originate by dismutation of pyruvate into carbon dioxide, lactate and acetate (Von Brand, Churchwell and Eckert, 1968). Von Brand and Alling (1962) studied the relationship between size and metabolic rate and found that the rate of respiration is neither weight nor surface proportional. Von Brand and Stites (1970) reported that *T. taeniaeformis* shows only slight stimulation of glyconeogenesis under the influence of carbon dioxide. The use of labelled glucose and galactose showed that the cestode can utilize both these since carbon atoms of both hexoses were incorporated into glycogen. Glucose is absorbed against a concentration gradient, the process requiring sodium, and glycerol is also absorbed readily (Von Brand, McMahon, Gibbs and Higgins, 1964; Von Brand and Gibbs, 1966).

Hustead and Williams (1977a) found that radio-iodinated proteins were taken up by larvae of both T. taeniaeformis and T. crassiceps in vitro. These proteins retained their structural and functional integrity. This readily accounts for the presence of host serum components within the bladder fluids.

For evaluating the maintenance of Hymenolepis diminuta, Fioravanti and MacInnis (1976) employed metabolic indices. These authors considered that wet weight, protein and glycogen levels served as useful indices for in vitro studies involving long-term incubation. However, Brandt (1980) found that the use of metabolic indices such as glucose uptake, ammonia and lactic acid production were not useful indicators of the adequacy of the culture system.

To screen the monogenean trematode Diclidophora merlangi for physiological experiments, Halton and Arme (1971) used the principle of dye exclusion to detect tegumental damage resulting from collection and handling procedures. The non-toxic dye used in the in vitro technique was 0.01% Evans blue dye in artificial seawater medium. They demonstrated that blue-staining areas revealed the presence of dye in the tegument. Furthermore, electron microscopic examination of the stained areas of the tegument showed that there was disruption of the surface membrane and associated cytoplasm. Murrell and Clay (1972) and Siebert and Good (1979) utilised this objective technique when working with Schistosoma mansoni and T. crassiceps respectively. These authors equated cytotoxic effects of immune serum on the worms with dye uptake.

Physiological studies

Heath and Elsdon-Dew (1972) hatched T. taeniaeformis eggs and cultured the oncospheres. Their basic medium NCTC 135 (Gibco) was supplemented with 20% rat serum (v/v) and rabbit blood cells. Young larvae with a cavity were observed in their cultures by ten days. In 1973(a), Heath developed a more satisfactory medium by substituting inactivated foetal calf serum for the natural host serum. Rabbit blood cells were again added to the medium. Air was used as the gas phase. The result was that immature larvae developed at a rate comparable to that in vivo. This latter medium was successfully utilised by Heath and Lawrence (1976) when culturing oncospheres of Echinococcus granulosus. Similar results were obtained if the basic medium was replaced by RPMI-1640 or McCoy's 5A.

Brandt (1980) however did not observe any post-oncospheral development when he employed Heath's medium to culture T. taeniaeformis. However he did obtain some development of the oncospheres when he used RMPI-1640 with L-glutamine and HEPES buffer (Gibco), modified with antibiotics and sodium pyruvate and supplemented with heat-inactivated foetal calf serum. The oncospheres after two weeks in culture showed signs of post-oncospheral reorganisation, in that they had doubled in size, formed a slit-like cavity and moved with gentle contractions at first and later with pseudopodia-like movements. Following this success with modified RPMI-1640, Brandt used this medium to culture mature metacestodes. He achieved better survival of the metacestodes when the medium was modified with sodium pyruvate. He also tried a diphasic medium, consisting

of the same liquid medium plus heat-coagulated Special Bobby Calf Serum as the solid substrate but no larval development or growth was reported.

Immunogenicity studies

In recent years, it has been shown that metabolic products of the cultured larvae of T. taeniaeformis contain "functional" antigens. Kwa and Liew (1977) and Ayuya and Williams (1979) maintained mature metacestodes in serum-free media for only 24 hours to obtain metabolic products for use as vaccines. For the same purpose, Rajasekariah, Rickard and Mitchell (1980b) cultured mature larvae for four to six days in serum-free media. Lloyd (1979) cultured oncospheres in RMPI-1640 with HEPES buffer and 10% v/v mouse serum for ten days; again the collection of metabolic products was the primary purpose but no details of development were given. All these workers recorded high protection levels in rodents against a challenge infection using vaccines consisting of the culture fluids in which the various larval stages of T. taeniaeformis had been incubated.

CHAPTER 2

MATERIALS AND METHODS

Experimental animals

All mammalian species used were specific-pathogen-free (SPF) animals obtained from the Centre for Laboratory Animals, University of Edinburgh. The mouse strain used was the random bred CF1. The rat strain was the random bred Sprague-Dawley. Cats used to maintain the life-cycle were obtained from the breeding colony of the above Centre. Following infection they were kept at the Wellcome Animal Research Unit, University of Edinburgh.

Establishment of Taenia taeniaeformis

Cats infected with the Belgian (mouse-adapted) or Malaysian (rat-adapted) strains of T. taeniaeformis (Brandt and Sewell, 1981a) were kindly provided by Dr. J. Brandt. The mouse-adapted strain will be referred to as T. taeniaeformis (B) and the rat-adapted strain as T. taeniaeformis (M). Later these cats were replaced by six month old female cats.

The proglottides expelled by the cats were collected daily by the staff of the Wellcome Unit, delivered to the laboratory and then washed and held at 4°C in normal saline plus antibiotics and fungizone (1 ml saline contained 100 I.U. penicillin, 100 μg streptomycin and 2 μg fungizone). The proglottides were then placed in a glass petri-dish and cut longitudinally with a scalpel. The contents of the petri-dish were washed into the top of a double-tiered sieve system. This consisted of two 100 mm diameter test sieves (Endecotts) with the upper sieve of pore diameter 53 μm and

the lower sieve with a microplate of 20 μm pore size. The cut proglottides were pressed manually and a spray of tap water was used to flush the top of the sieves followed by several washes with saline and antibiotic solution. The lower sieve retained the eggs and its contents were poured into a glass beaker. The sides of the sieve were rinsed and the residue added to the beaker. The suspension of eggs in 10 ml of saline was gently centrifuged in glass tubes at 250 g for ten minutes at room temperature. Eight millilitres of the supernatant was sucked off and the concentration of eggs estimated from the remaining 2 ml. The total number of viable eggs was estimated by counting the numbers in several 0.1 ml aliquots. These were taken with a 1.0 ml syringe while the beaker was on a magnetic stirrer, placed on a slide and counted under 100 x magnification. Viability was assessed from the morphology of the eggs i.e. those with thick brown shells having aligned hooks. Eggs were then stored in saline plus antibiotics at 4°C.

Batches of three week old CF1 mice were infected monthly, while three to four week old Sprague Dawley rats were infected periodically. The choice of ages for rats and mice was made on the basis of studies by Greenfield (1942) and Dow and Jarrett (1960) in which rodents of these ages were most susceptible to infection.

To infect these animals 200 viable eggs in 0.1 ml or 400 viable eggs in 0.2 ml normal saline were given to mice and rats respectively. Only eggs that had been stored for less than two weeks were used. Eggs were administered using cannulae made by removing the bevel from a 23 G 1" needle for mice and from a 20 G 1½" needle for rats

("Yale microlance" Becton-Dickinson). The tip was smoothed and covered by a 10 mm length of silicone rubber tubing, with half the length extending beyond the tip of the cannula.

Metacestodes from rats or mice with three to five month old infections were used to infect the cats. The intermediate hosts were killed by cervical dislocation and then their livers were removed and cysts dissected out. A few cysts from each liver were incised and the larvae removed to check for viability. This was done by placing the larvae in warm saline and examining it under a dissecting microscope. Viable larvae moved actively. The cats swallowed other intact cysts when these were placed at the back of the tongue. About 15-20 cysts were given to each cat, which were starved for 24 hours beforehand.

Proglottides were expelled by cats by one to two months after infection and collected daily. The cats were replaced annually.

Treatment of metacestodes prior to culture

Only the Belgian strain of T. taeniaeformis larvae or metacestodes were used for in vitro work. Mice infected for at least ten weeks were killed by cervical dislocation. Under a laminar sterile airflow cabinet (Microflow), the hair from the ventral region was shaved and the skin disinfected with "Hibitane" (ICI) followed by 70% alcohol. The abdomen was incised along the linea alba and the liver dissected out and placed in a petri-dish. Wearing sterile surgical gloves, each cyst capsule was incised with scissors and the larva extruded by gentle finger pressure on the capsule. The larvae were washed with copious amounts of physiological saline

in beakers, by refilling and decanting the fluid but retaining the larvae, which rapidly settles down, until host material was no longer visible. The larvae were then transferred into several disposable petri-dishes containing culture medium. A series of four to six washes was achieved by transferring the larvae (carried on the stem of an 18 G 1½" needle bent at 45°) from one petri-dish to the next before placing them in the final medium. Care was exercised to avoid damage to the larvae during this procedure. All materials used in this procedure were sterile and solutions were warmed to 37°C.

Maintenance procedure

The defined medium devised by Brandt and Sewell (1980) to support survival was used for maintaining the metacestodes. The following are the components of the medium:

RPMI-1640 with 25 mmol l^{-1} HEPES buffer and L-Glutamine (Gibco)

5% v/v 100 mmol (5 mmol l^{-1}) sodium pyruvate (Gibco)

100 I.U. ml^{-1} penicillin (sodium benzyl penicillin) (Gibco)

100 μg ml^{-1} streptomycin (streptomycin sulphate) (Glaxo)

As the activity of L-Glutamine diminishes fairly rapidly with storage, even at 4°C, according to the manufacturer's advice, this was routinely added just before use at 300 mg l^{-1}. This defined medium was the one routinely used in all experiments.

The metacestodes were kept in disposable sterile polystyrene petri-dishes (Sterilin) usually 90 mm x 15 mm but on one occasion 50 mm x 20 mm. The inner rim of the top lid was sealed using silicone stopcock grease (Dow-Corning), which was applied using a sterile 2 ml syringe.

Cultures were kept stationary in the dark at 37°C. The gas phase was air though the closed containers were airtight. The medium was changed after the first 24 hours and then routinely every 48 hours. During these changes the larva were handled in the same way as during the initial serial washes. The medium in which larvae had been maintained was now termed "used" medium. The used medium was centrifuged at 1000 G for 15 minutes to remove any tissue debris. The resultant supernatant was then decanted into sterile universal bottles and stored at -20°C.

Occasional samples of used medium were taken before storage for bacterial examination. The methods used were examination of Gram-stained smears of the debris after centrifuging medium samples, aerobic and anaerobic blood agar cultures and aerobic nutrient broth culture of medium and debris. These cultures were kept for two to four days and examined for bacterial growth daily. The batch of used medium was discarded if there was any evidence of bacteria in the samples. This, however, very rarely happened.

Protein estimation

Samples of used medium were assayed for protein concentration using a commercial protein reagent, "Bio-Rad Protein Assay" (Bio-Rad). This is a dye-binding assay based on the differential colour change of a dye in response to various concentrations of protein. The kit consists of a dye reagent concentrate and a lyophilised protein standard, which is bovine gammaglobulin. The protein standard was diluted with PBS to concentrations ranging from 10 μg - 100 $\mu g\ ml^{-1}$. The unknown medium sample was used neat

or diluted 1:2. The standard procedure adopted, according to the manufacturer's instructions, was that 0.8 ml of each sample, diluted standard and blank (PBS) was added to 0.2 ml of dye reagent concentrate and mixed well. After being allowed to stand for 15 minutes, the mixture was placed in a glass self-masking cuvette of 10 mm light path (Hellma) and measured against the blank in a SPG-200 spectrophotometer (Pye Unicam) at 595 nm. With the standard readings, a graph of absorbance versus micrograms of protein was plotted. A linear calibration graph was obtained. The protein concentration in the unknown medium sample was read from the standard curve. The protein standard was incorporated, each time protein estimation was performed, to control test variation.

Dye uptake

Metacestodes were examined for tegumental integrity by a dye exclusion technique according to Siebert and Good (1979). This was usually performed before an experiment to select for intact larvae. In the last experiment, dye uptake was performed before, during and after the period of *in vitro* maintenance. Larvae were given three washes in PBS in petri-dishes and then incubated for 30 minutes at room temperature in Hanks' balanced salt solution containing 0.05% Evans blue dye. After removal from the dye solution, the larvae were again washed three times in PBS and examined under a dissecting microscope. The entire procedure was carried out under sterile conditions. All larvae that stained blue were classified as being damaged and the damaged areas of the larvae were recorded. Larvae which did not take up any dye were regarded as being intact. The

dye uptake of each larva was graded according to degree of staining. In descending order of degree of staining (P = proportion of strobila which stained blue):

$$+++ \quad P > \tfrac{1}{2}$$

$$++ \quad \tfrac{1}{4} < P < \tfrac{1}{2}$$

$$+ \quad 0 < P < \tfrac{1}{4}$$

$$- \quad P = 0$$

Transmission electron microscopy

Representative proglottides of both damaged and undamaged areas as classified by dye uptake were processed for transmission electron microscopy. The procedure adopted for this purpose was that routinely employed by the Pathology Laboratory, Veterinary Field Station, University of Edinburgh.

Firstly, the tissues were fixed overnight at 4°C in 0.1 M sodium cacodylate buffer, pH 7.4, containing 2.5% glutaraldehyde. They were then rinsed in the buffer alone before being post-fixed in 1% osmium tetroxide for 1½ hours, then washed in buffer again. The specimens were then dehydrated in graded levels of ethanol, 20%, 50%, 90% and 100%, each dehydration step consisting of a 2 x 15 minute period. Following dehydration, the specimens were impregnated with epoxypropane (2 x 15 minutes) and then left for one hour in a mixture consisting of equal parts of epoxypropane and araldite. The specimens were then placed in pure araldite overnight at room temperature and finally embedded in fresh pure araldite and polymerised at 60°C for 48 hours. After polymerisation was completed, the araldite blocks were pared until the tissues were exposed. The

blocks were then cut on an ultramicrotome (Cambridge-Huxley Mark II). "Thick" sections of 1 μ were cut and stained with Toluidine blue. These sections were examined under a light microscope at 100 x magnification to select the desired area for ultrathin sectioning. The area selected was one which included the surface membrane and adjoining structures. Ultrathin sections at 800-1000 Å were then cut from the preselected area. Four to six sections from each specimen were picked up with a 200 mesh copper grid, 3 mm in diameter. The grids were stained with uranyl acetate (15 minutes) and then with Reynolds lead citrate (5 minutes).

The sections were finally viewed on a Philips 400 electron microscope at magnifications of up to 10,000. Electron micrographs however, were usually taken at lower magnifications because of the larger field.

Enzyme linked immunosorbent assay

All used media from these experiments were analysed for antigenic activity at the end of each experiment. For this purpose, the assay for detection of antigen was used. The method and materials used are described in detail in Part II.

Measurement of pH

In some cases the pH of a 25 ml aliquot of the used medium was measured at 20°C. For this, Pye Unicam Model 291 mark 2 pH meter was used, standardized against a reference solution (Standard buffer, BDH) pH 4.0, 7.0 or 9.0. The pH of the reference solution selected was the one which was estimated to be nearest to the pH of the unknown solution.

CHAPTER 3

EXPERIMENTS

The use of ELISA for quantitating antigens of T. taeniaeformis

Aim

In this first experiment of this part, an attempt was made to confirm that ELISA can be used to quantify ESA and SA. This was considered necessary before the ELISA was employed to assay antigen levels in samples of used medium in subsequent experiments. Also, the optimal dilution of used medium for the assay had to be determined.

Experiment design

A neat sample of the used medium and dilutions of 1:5, 1:25, 1:125 and 1:625 of this sample were each assayed in the ELISA for detecting antigens using four replicates. The replicates of each dilution were arranged at random on the test plates by reference to a table of random digits (Rohlf and Sokal, 1981). This precaution of random arrangement was taken to allow for any variation between positions on the test plates.

Results

The mean corrected ELISA values for ESA and SA at the various dilutions of used medium are presented in Figure 1. It is evident from the graph that the neat sample and 1:5 dilution of used medium each produced similar values for both ESA and SA. However, the difference between the ESA and SA values increased with increasing dilutions, as the absorbance values fell off more rapidly using the SA system. At the dilution of 1:625, the SA system gave the same

reaction as the control while the ESA system retained considerable activity.

Discussion

The ELISA for detecting antigens was shown to be quantitative for the ESA and SA components of used medium. This used medium was a pool prepared from cultures over the first nine days of maintenance and may be assumed to be relatively rich in ESA. This supposition is supported by the fact that the results in the ESA system fell away less rapidly with dilution than those in the SA system.

In both cases however it seemed that a 1:10 dilution was a reasonable level to use routinely, since samples of other used media with lower concentrations of either ESA or SA might be expected to give lower results at this dilution. However this also implies that variations in SA content will have a greater pro-rata effect because of the steeper gradient with this system.

The effect of including serum in the ELISA for detecting antigens

Aim

In certain experiments, serum was added to the maintenance medium. This was done to compare both the survival of larvae and their release of antigens in serum-free and serum-added media. This experiment was therefore designed to investigate whether serum affected the ability of the ELISA to detect antigens.

Experiment design

The following solutions were prepared:

(i) Solution A — Serum-free used medium

(ii) Solution A_1 — Modified RPMI-1640:Solution A 1:10

(iii) Solution A_2 — Modified RPMI-1640:Solution A with 10% v/v SBCS 1:10

Control solutions:

(iv) Solution B_1 — Modified RPMI-1640:PBS-Tween 20 1:10

(v) Solution B_2 — Modified RPMI-1640:PBS-Tween 20 with 10% v/v SBCS 1:10

(vi) A range of dilutions in an approximately logarithmic scale of A_1 in B_1 and A_2 in B_2 were prepared, namely

1:1, 1:2, 1:6, 1:16, 1:40 and 1:100

These dilutions were then tested in the ELISA.

Note: SBCS (Special Bobby Calf Serum - Gibco)

PBS-Tween 20 (see Solutions used in ELISA page 74)

Results

Figure 2 shows that Solution A_1 in B_1 (serum-free) produced almost identical absorbance values to Solution A_2 in B_2 (with serum) at all dilutions used. This applied to the groups of components reacting with both anti-ESA and anti-SA gammaglobulins. Therefore, the presence of serum appears to have no effect on the detection by ELISA for ESA or SA levels in used medium. In this study, diluting the medium appeared to have less effect on the results with the ESA system but more with the SA system, thus in agreement with the results of the previous experiment.

Discussion

The evidence provided by this experiment allowed the addition of serum, for example as required in long-term maintenance of the

metacestodes, in the knowledge that this would not interfere with the ELISA detection of antigens.

The influence of age on the production of antigens

Aim

Previous workers have usually used three to six month old metacestodes to provide immunogenic *in vitro* culture products (Ayuya and Williams, 1979 and Rajasekariah *et al.*, 1980b). However the question arose as to whether the age of the metacestodes affects their production of antigenic metabolic products. An experiment was therefore conducted to study the relationship between age and release of such metabolic products.

Experiment design

Larvae aged three and ten months respectively were used for comparison, groups of 24 larvae of each age group being placed in sealed petri-dishes with 48 ml of medium. The medium was changed every 24 hours until day 11. The protein concentrations of the used medium samples were estimated at the end of the experiment in addition to the antigenic analysis.

The larvae in this experiment were not subjected to the dye test since at that time, this test had not yet been applied to these studies.

Results

Figure 3 shows that the ESA levels decreased with time for both three month old and ten month old larvae. However, for the three month old larval group where the maintenance period extended

beyond nine days, the ESA level appeared to increase after the ninth day. Similarly, the SA levels appeared to have the same trend as ESA levels. The fall in antigen levels seemed to be greater in the case of ESA relative to SA.

Table 1 shows the protein content of the used medium samples. Figure 4 suggests a positive correlation between protein concentration and antigen (ESA and SA) levels, as manifested by ELISA results for the used medium. This relationship was however significant ($P < 0.05$) only in the case of ESA levels produced by ten month old larvae.

Table 1 Protein content of used medium, expressed in $\mu g\ ml^{-1}$ after the maintenance of 3 month and 10 month old metacestodes

Age of metacestode (months)	Days in vitro										
	1	2	3	4	5	6	7	8	9	10	11
3	34	24	34	40	34	28	16	14	24	36	16
10	56	30	34	34	28	30	14	34	24	-	-

Discussion

These similar patterns of antigen release with time suggests that there is no quantitative difference in both ESA or SA levels in the used medium of three month old or ten month old larvae. Thus it appears that the age of metacestodes used in in vitro studies is not critical, within the three to ten month old range. This age range offers a practical handling advantage because of the size of the larvae (25-165 mm in length). Larvae less than eight weeks old are about 10 mm long and have a delicate thin coat, being

therefore more susceptible during recovery from the host. Larvae older than 12 months may be stained yellow or, in certain cases, covered with a white caseous substance. Hence, in all further experiments the metacestodes used for *in vitro* maintenance were obtained three to ten months after infection of the intermediate host.

The results of this experiment also suggested that the protein content of the used medium may to some extent be related to their antigenic load. Brandt (1980) found similar results in that the protein concentrations in culture medium did not fall with time. An analysis of his results shows the existence of a similar positive correlation between protein concentration and antigenic load, but this was not quite statistically significant.

A study of variation among singly maintained metacestodes

Aim

It was thought probable that there would be individual variations in the metabolic rate of the metacestodes maintained *in vitro*. This experiment was undertaken to check if the ELISA technique was able to detect these differences as they effect antigenic release.

Experiment design

All larvae used in this experiment were tested for tegumental integrity by the dye test at the beginning and at the end of the experiment. Only intact larvae were selected by this criterion of dye exclusion. All larvae were three months old.

Group I was five larvae placed individually in 50 mm x 20 mm petri-dishes with 10% v/v FCS (Foetal calf serum - Gibco) added to the medium. Group II was four larvae placed individually in the same size of vessel as Group I but were serum-free. Each larvae received an equal amount of medium, that is, 2 ml. The larvae were maintained until day 11 with the first change of medium at 24 hours and thereafter every 48 hours.

Results

The corrected ELISA results are in Table 2 which shows that between individual larvae not only are there different levels of both ESA and SA released at any one time but for each larva there were markedly varying levels of these antigens over the whole of the maintenance period.

There appeared to be no particular trend with regard to antigen production. There was no difference in ESA levels between Groups I and II. However, the SA levels for Group I were significantly higher than those for Group II on days 3 and 11 (two-sample t-test, $P < 0.05$).

To obtain an estimate of the variability, the coefficient of variation was calculated. The values were 49% (ESA Group I), 28% (ESA Group II), 47% (SA Group I) and 45% (SA Group II). The overall coefficient of variation (CV_1) was 45%.

Discussion

Individually maintained larvae appeared to release the same amount of ESA and SA whether the medium contained serum or not.

Table 2 Corrected ELISA values for serum-added (Group I) and serum-free (Group II) used medium in which single larva had been maintained, analysed using the ESA and SA antisera. Absorbance at 450 nm.

Antisera in ELISA	Group	Larva number	Days of maintenance				
			3	5	7	9	11
anti-ESA	I	1	0.357	0.307	0.372	0.317	0.386
		2	0.058	0.294	0.252	0.260	0.249
		3	0.255	0.144	0.650	0.547	0.261
		4	0.518	0.676	0.427	-	0.552
		5	0.576	0.592	-	-	0.253
	II	1	0.340	0.546	0.454	0.340	0.454
		2	0.416	0.403	0.375	0.183	0.207
		3	0.545	0.444	0.522	0.537	0.524
		4	0.365	0.521	0.449	0.554	-
anti-SA	I	1	0.861	0.466	0.519	0.810	0.651
		2	0.204	0.252	0.582	0.250	1.330
		3	0.502	0.622	0.392	0.448	0.701
		4	0.639	0.867	0.360	-	0.606
		5	0.558	0.290	-	-	0.393
	II	1	0.095	0.460	0.319	0.231	0.269
		2	0.230	0.358	0.825	0.241	0.074
		3	0.384	0.250	0.425	0.316	0.248
		4	0.252	0.416	0.293	0.258	-

The only exceptions to this were on days 3 and 11 in the case of SA, when the larvae in the serum-added medium released a much higher amount of SA. These higher SA readings may be related to the complex nature of antisera raised against SA, which will be discussed in detail in the general discussion.

The overall coefficient of variation (CV_1) is a useful but crude indicator of the variation which will be occurring in the usual maintenance procedure, that is, using groups of larvae. Assuming that each of the larvae acts as an independent unit, the coefficient of variation for a group of n larvae is $CV_1/\sqrt{n}$.

The influence of sealing the maintenance vessels on the production of antigens

Aim

This experiment was simply to assess if it was necessary to render the maintenance vessels airtight by sealing the lids of the petri-dishes. Serum-free and serum-added medium were other variables included for comparison.

Experiment design

Forty five-month old larvae, classified as intact by the dye test, were divided into four groups of ten. Each group was placed in 20 ml of the medium. The lids of the petri-dishes of Groups I and III were sealed using silicone stopcock grease as described earlier, but those of Groups II and IV were left unsealed. Only Groups III and IV contained 10% v/v Special Bobby Calf Serum (SBCS - Gibco). Medium was changed, processed and stored as usual, until day 19 when the experiment was terminated.

Results

Inspection of Figure 5 suggests that there were similar ESA levels and similar trends over time in both the sealed and unsealed vessels, whether they contained serum or not. There was no overall difference in ESA level between serum-free and serum-added media.

The somatic component produced contrasting results. In the serum-added medium (Figure 6) overall levels and trends were similar in sealed and unsealed vessels, but in serum-free medium the SA level was consistently higher in the sealed vessels over the first six readings; over this whole period this difference was significant (paired t-test, $P < 0.05$). Furthermore, from Figure 6, in the unsealed vessels only, the SA level was significantly higher in serum-added medium than in serum-free medium. Thus, overall, the SA level was lower in serum-free medium and unsealed vessels.

Another parameter considered was the colour change in the medium. Plate 1 shows samples of the four sets of used media arranged according to time of collection. It can be seen that the colour change was more intense in the sealed vessels than in the unsealed vessels containing the same medium. This is shown by the changes in colour from the original phenol-red through orange and finally to yellow. Group I has more orange and yellow fluid samples than Group II and so does Group III compared to Group IV.

Discussion

The ESA levels did not show any difference between sealed and unsealed vessels. The assumption was that the sealed vessels would prevent the loss of CO_2 produced by the larvae. The presence of CO_2

is thought to be conducive for metabolism of the metacestodes. Hence the results of this experiment should shed some "light" on the controversy between the relative importance of anaerobic and aerobic conditions for these helminths (Ward, 1982). Anaerobic conditions might tend to lessen the deleterious effects imposed by in vitro conditions. Therefore, if the somatic component levels are associated with degenerative processes, they should be lower in the sealed vessels. As this was only true for part of the experiment using serum-added medium (Figure 6) and, even then only to a level which can be accounted for by chance variation, the study does not support this hypothesis. There is even a suggestion of the opposite effect in serum-free medium, although because only one petri-dish of each type was used, external factors may easily have confounded the results.

The greater colour change from red to yellow in the sealed vessels may indicate greater acid production by these metacestodes in these vessels, resulting from a greater metabolic rate. More likely though, it resulted from a reduced loss of CO_2 compared to that from the unsealed dishes.

The effect of larval integrity on the production of antigens

Aim

The state of taeniid larvae used for in vitro studies are often not considered. There exists the possibility that larvae damaged during handling procedures may behave in an abnormal manner in vitro. This experiment was designed to compare the levels of antigenic metabolites released by intact or damaged larvae, as classified by the dye test.

Experiment design

The damaged larvae used were obtained by the usual handling procedures, and the damage was not intentionally inflicted. The larvae were from the same batch of five-month old larvae used in the previous experiment, the results in that experiment providing the intact larvae control for this study. All the damaged larvae used had only a little evidence of tegumental disruption as dye uptake was limited to the two ends, that is the scolex and bladder regions, with unstained strobila in between.

Three replicates, each of 12 damaged larvae in 24 ml of serum-free medium, were set up, Groups DT_1, DT_2 and DT_3. As this experiment was carried out in parallel with the previous experiment, the control group of intact larvae (I) was Group I from that experiment. This control contained ten intact larvae and an equivalent amount of medium, that is, 2 ml per larvae. The experiment ended on day 15.

Results

The ELISA results for ESA and SA levels present over the maintenance period are shown in Figures 7 and 8 respectively. No trends over time are apparent. Paired t-tests ($P < 0.05$) show the ESA levels in two groups of damaged larvae (Figure 7 - DT_2 and DT_3) to be significantly higher than the control, and SA levels in one group (Figure 8 - DT_1) to be significantly lower than the control. Otherwise the results between the other groups were not significantly different.

Discussion

The fact that the SA level in the damaged groups was not raised relative to the intact group was surprising, as it was expected that more somatic components would be released by the damaged larvae. A possible explanation is that the degree of damage incurred by the larvae was insufficient to release significantly greater somatic components than came from the control (intact) larvae. If more severely damaged larvae had been used with extensive stained strobilar areas, then the SA levels might have been elevated sufficiently to be detected.

It would also seem probable that the apparent differences between the damaged and intact larvae are merely a chance reflection of the large variation between larvae showing through in the groups.

The practical application of this finding is that results obtained in such *in vitro* studies using larvae which have been slightly damaged by the usual handling procedures are not invalidated. Nevertheless, if the numbers of larvae permits, it is obviously desirable to use totally intact larvae.

The question of whether the dye test is a reliable indicator of the state of larval tegument led to the next experiment.

Appraisal of the assessment parameters used in the maintenance of metacestodes

Aim

This experiment had three objectives. Firstly, the dye test and transmission electron microscopy (TEM) were employed to assess

the effectiveness of the former in appraising larval tegumental integrity. Secondly, the release of antigenic metabolites by the larvae into a serum-free medium and two media containing serum were compared. Thirdly, the pH values of the used media were monitored and the colour changes observed to assess the value of these parameters as indicators of the suitability of the culture media.

Experiment design

Only sealed maintenance vessels were used in this study. The media for Group I was serum-free but Groups II and III contained 10% v/v Special Bobby Calf serum (SBCS - Gibco) and 10% v/v Foetal Calf serum (FCS - Gibco) respectively. Group I had four replicates or maintenance vessels and Groups II and III had five replicates each.

One maintenance vessel or petri-dish in each group was marked. All petri-dishes had ten larvae each except the marked ones which contained 15 larvae each. The larvae were between $6\frac{1}{2}$ to $9\frac{1}{2}$ months old and were pooled before the larvae were randomly selected for each group.

The usual changes of medium were performed until day 23. At each change, one larva was taken at random from the marked petri-dish in each group and subjected to the dye test. The degree of staining was graded as described earlier. Then representative sections of stained and unstained areas were processed for TEM. The volume of medium in the petri-dishes was then adjusted so that there was a constant 2 ml of medium per larva. At the end of the experiment, all remaining larvae were exposed to the dye test.

For the second part of the experiment, the used media were analysed by ELISA for detection of ESA and SA. This was conducted at the end of the experiment when all the used media samples up to day 23 were available.

For the third part of the study, the pH of all the used media was measured and the colour change from the original unused media was noted.

Results

Dye test and TEM: The results with the larvae taken at each medium change and used for both dye test and TEM are in Table 3. As both unstained and/or stained sections of each larvae were processed for TEM, the state of the tegument for these sections were observed. Table 3 illustrates this association between dye uptake and tegumental integrity. Dye exclusion from any area of the strobila almost always corresponded to an intact tegument. However, dye uptake almost invariably coincided with areas of tegumental disruption. The tegument was considered intact if TEM showed the presence of normal tegumental structures. Plate 2 shows examples of the various states of larval tegument transpired during the course of the maintenance period.

Table 4 and Plate 3 show the results from the end of the first part of the experiment when all remaining larvae were exposed to the dye test. The larvae in the serum-free medium are relatively reduced in width. The proportion of larvae showing less than a quarter of the length stained is significantly reduced in the serum-added media as compared to the serum-free medium ($\chi^2_{(1)} = 45.47^{**}$). There was however no difference between the SBCS- and FCS-added media ($\chi^2_{(1)} = 0.24$, not significant).

Table 3 Results from dye uptake and electron micrographs of the single larva taken at each medium change

Days in vitro	0	3	5	7	9	11	13	15	17	19	21	23
Group I	-	+	+++	-	+++	-	++	+	+++	+++	+	+
(serum-free)	abc/-	abc/bc	abc/c	abc/-	-/nil	abc/-	abc/bc	abc/bc	abc/nil	0	abc/bc	abc/c
Group II	-	-	-	+++	-	-	-	+	+	-	-	++
(SBCS)	abc/-	abc/-	abc/-	-/c	bc/c	abc/-	abc/-	abc/c	abc/c	abc/-	abc/-	c/nil
Group III	-	-	-	-	-	+++	+++	-	-	-	-	-
(FCS)	abc/-	abc/-	abc/-	abc/-	abc/-		-/c	abc/-	abc/-	abc/-	abc/-	abc/-

KEY: P = proportion of strobila stained; - P = 0, + $0 < P < \frac{1}{4}$, ++ $\frac{1}{4} < P < \frac{1}{2}$, +++ $P \geq \frac{1}{2}$

a = microtriches, b = thick homogeneous layer, c = muscle bundles and "cells"

nil = no such structures observed in unstained/stained areas respectively

e.g. abc/- the unstained area has structures a, b, c; specimen had no stained area
abc/c unstained area has structures a, b, c and stained area has structure c only

Table 4 Dye uptake by larvae after 23 days in vitro in three maintenance media

P = Proportion of strobila stained

	$P = 0$	$0 < P < \frac{1}{4}$	$\frac{1}{4} < P < \frac{1}{2}$	$P > \frac{1}{2}$
Group I (serum-free)	3	10	21	0
Group II (SBCS)	12	30	0	2
Group III (FCS)	13	27	0	3

Analysis for antigens: For the second part of the experiment, the ELISA values obtained using the samples of used medium are presented in Appendix Tables 6 and 7. From the mean values, a time course for ESA and SA ELISA values were graphed in Figures 9 and 10 respectively. In Figure 9 it appeared that the ESA levels produced in all three media generally decreased steadily with time until days 13 to 17 when the decline ceased. In Figure 10, the SA levels fluctuated with time in the serum-free and SBCS-added media until day 19 when there seemed to be an increase in levels. This apparent initial decrease, followed by increasing levels of SA was more markedly manifested by the FCS-added medium, where levels fell until day 15 after which time increasing levels were detected.

pH measurement and colour change: Figure 11 is the pH values of the used media monitored every 48 hours throughout the experiment. The pH values of used media in all three groups appeared to be fairly steady with time. There was no significant dissimilarity between the trends produced by the three groups. The striking difference was that there was a bigger reduction in pH in

serum-added media than in the serum-free medium. In the serum-free medium (Group I) the maximum reduction in pH relative to the pH for unused serum-free medium (pH 7.70) was 0.55 which occurred on day 7. In SBCS-added medium (Group II), the maximum reduction in pH was 1.05 on day 21 relative to unused SBCS-added medium (pH 7.85), and for FCS-added medium (Group III) the maximum reduction was 0.85 on day 19, relative to unused FCS-added medium (pH 7.85).

Plate 4 shows vials containing samples of used media, arranged in chronological order from left to right according to the day of maintenance. The colour change was from red through orange to yellow in the serum-added media (Groups II and III) from day one onwards. But in the serum-free medium there was the same colour change only until day 7; thereafter the medium retained the original colour.

Discussion

Assessment of dye test: Table 3 shows that unstained strobila almost always produced electron micrographs which showed an intact tegument. The only exception was one specimen (Group II day 9) in which the microtriches were absent. The tegument was considered intact if electron micrographs revealed the presence of normal tegumental structures. According to Smyth (1969) a normal cestode tegument comprises of a distal and perinuclear cytoplasm. The distal cytoplasm is made up of spine-like processes called microtriches at the free surface, below which is a thick homogeneous layer containing vesicles and vacuoles, lying on a prominent basement membrane. The perinuclear cytoplasm consits mainly of layers

of smooth muscle beneath which are large tegumental "cells". These structures of a normal tegument have also been observed by Threadgold (1962), Siddiqui (1963) and Engelkirk, Williams and Signs (1981) working with adult Dipylidium caninum, larval Taenia species and larval T. taeniaeformis respectively.

Dye uptake however coincided with areas of disrupted tegument, lacking one or more tegumental structures. Hence electron microscopy confirmed that dye uptake is indicative of disruption to tegumental integrity.

Tables 3 and 4 show that more larvae were damaged sooner and to a greater degree in serum-free medium than in serum-added media. This is hardly surprising as the importance of using sera in in vitro cultures have been well established. The majority of larvae in the serum-added medium showed very little staining even after 23 days of maintenance. Even in the damaged specimens, the staining was limited to the ends of the strobila, namely the scolex and bladder regions. The fact that there was no apparent difference in larval integrity as between the FCS- and SBCS-added medium constitutes an advantage in using the latter as it is much less costly.

Comparison of antigenic release: The amount of ESA produced by the three types of media as analysed by ELISA decreased with time to much the same extent in all three media. However, in each case this decline appears to have ceased by about day 13, and is perhaps somewhat later in the serum-free media. It is possible that if the maintenance period had been extended beyond 23 days, the apparent increase in ESA level would have continued. The initial decrease

is probably due to slowing down of metabolic processes caused by larval adaptation to the in vitro conditions.

There is an even more marked tendency for a consistent rise in SA levels from about day 17 onwards. In view of the cross-reactions between the two antigens and the two sera it seems possible that the small apparent rise in ESA is merely a reflection of the rise in SA. This would be consistent with the hypothesis that there is a steady decrease in ESA and a corresponding tendency for SA to increase as the metacestodes degenerate. However, Brandt (1980) who cultured T. taeniaeformis for 18 days, found that SA appeared to be released at the same rate throughout this time. He deduced from this finding that the components shared between ESA and SA continued to be formed and released at a similar rate. This appears to be contraindicated by the present study.

Neither the ESA nor the SA levels reflect the dye uptake or TEM results. As there was more damage to larvae in serum-free medium the somatic level in those samples was expected to be high, assuming that SA might derive from material leaching out of the denuded tegument absent in electron micrographs of stained strobila.

Assessment of other parameters to monitor the suitability of the maintenance medium: The results shown in Figure 11 and Plate 4 suggested that there was more metabolic activity by the larvae in the serum-added media, leading to higher production of acids and thus to a greater reduction in pH and more drastic colour change, despite the additional buffering capacity of serum, than occurred in the serum-free medium.

The results of the dye test, TEM, pH and colour change were to a large extent in agreement in showing that serum was beneficial for the long-term well-being and metabolism of T. taeniaeformis metacestodes in vitro. However, the analysis of antigenic content in used medium by ELISA did not indicate that the absence or presence of serum had any effect on the release of either ESA or SA. It is however possible that the 23-day maintenance period was not long enough for the absence of serum to show an effect.

Brandt (1980) also cultured these metacestodes for a longer period of time (32 days) and derived a curvilinear relationship for ESA in media with time. The eventual increase in ESA concentration occurred earlier i.e. 14 days, in serum-free medium to which sodium pyruvate was not added. For both the serum-free medium and for serum-added medium to which sodium pyruvate was present the increase in ESA concentration occurred after 18 days. A similar pattern to this probably occurred in the present experiment with its effects just appearing about day 15. Brandt also speculated that the apparent increase in ESA might really have been due to an increasing concentration of SA in the media, together with the rather non-specific antisera used.

In concluding, it is evident that dye uptake, TEM, pH and colour change of the medium served as reliable assessment parameters to monitor the condition of metacestodes in vitro.

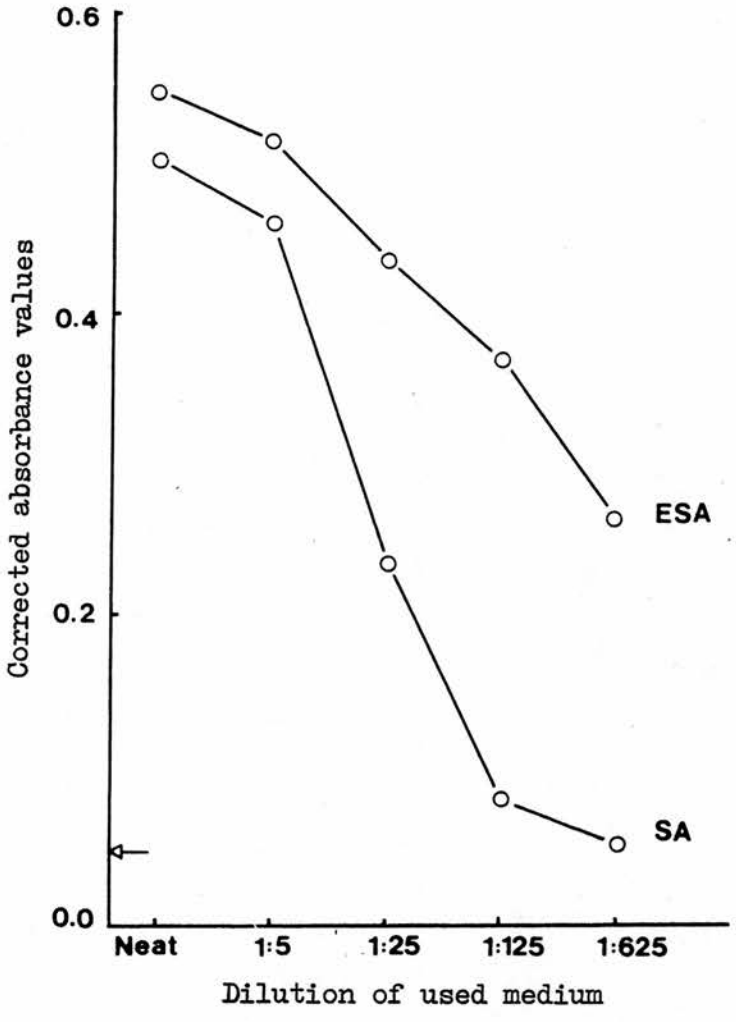

Figure 1 Detection of ESA and SA by ELISA with various dilutions of a pool of serum-free used medium. The arrow refers to the absorbance value obtained for unused medium. For the corrected absorbance values see Appendix Table 1.

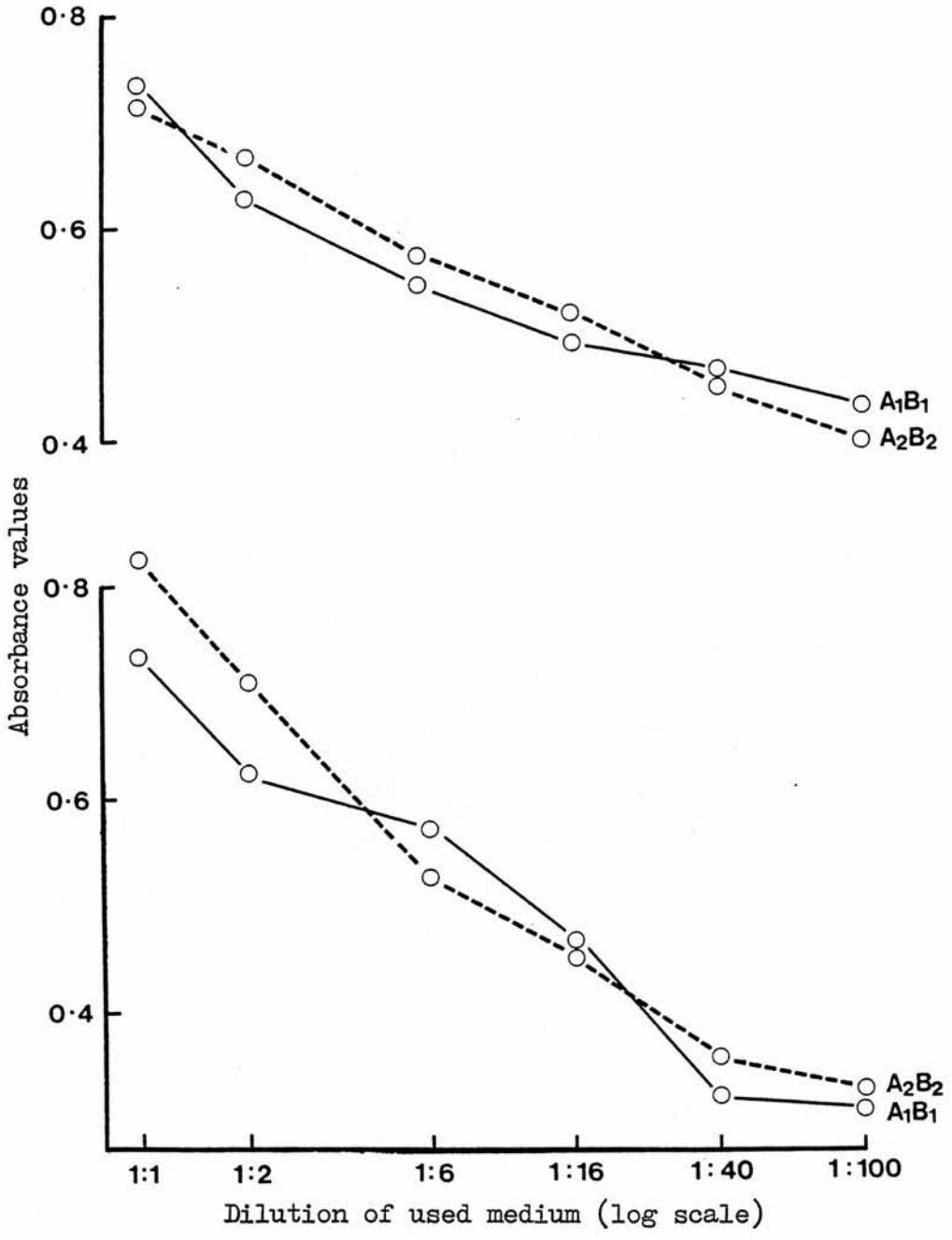

Figure 2 Detection of ESA (upper figure) and SA (lower figure) by ELISA in a pool of used medium, with and without the addition of serum during the assay (A_2B_2 and A_1B_1 respectively). Various dilutions of these two solutions were used.

For the mean absorbance values see Appendix Table 2.

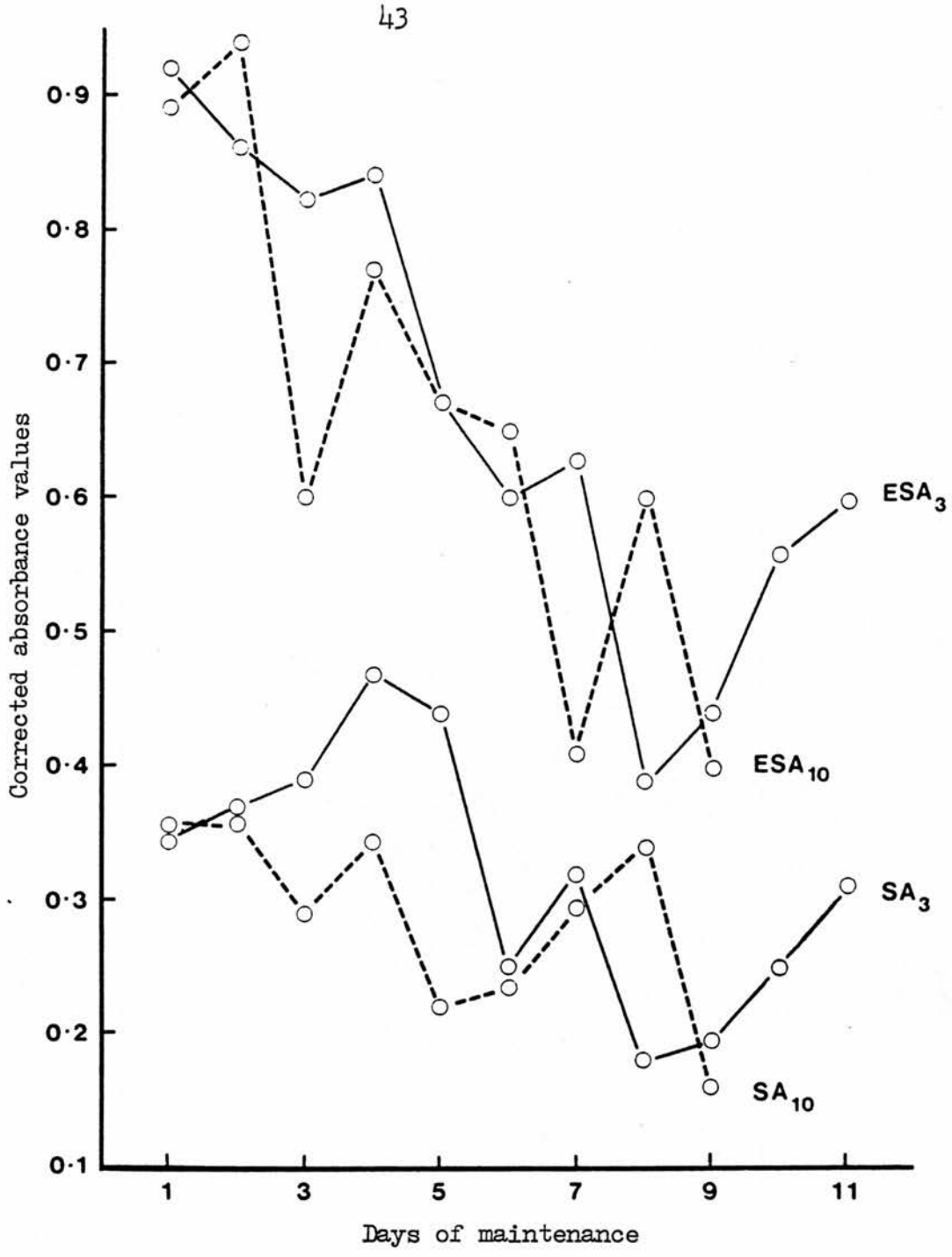

Figure 3 The pattern of ESA and SA release into the medium in which 3 month and 10 month old larvae had been maintained, as detected by ELISA.

For the corrected absorbance values see Appendix Table 3.

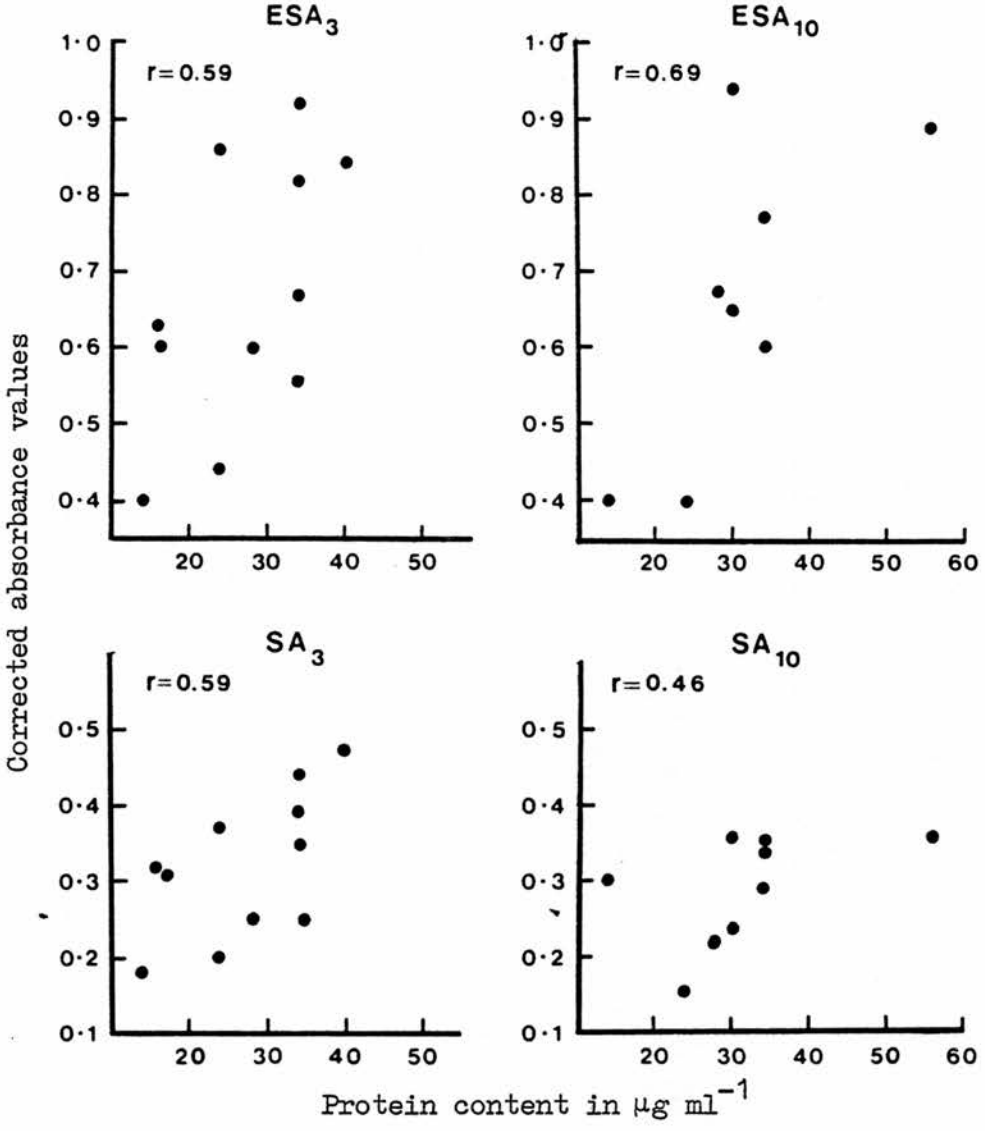

Figure 4 Scatter diagrams and correlations obtained by ELISA between protein content and the absorbance values when detecting ESA and SA in media in which 3 month and 10 month old larvae had been maintained.
(r = correlation coefficient)

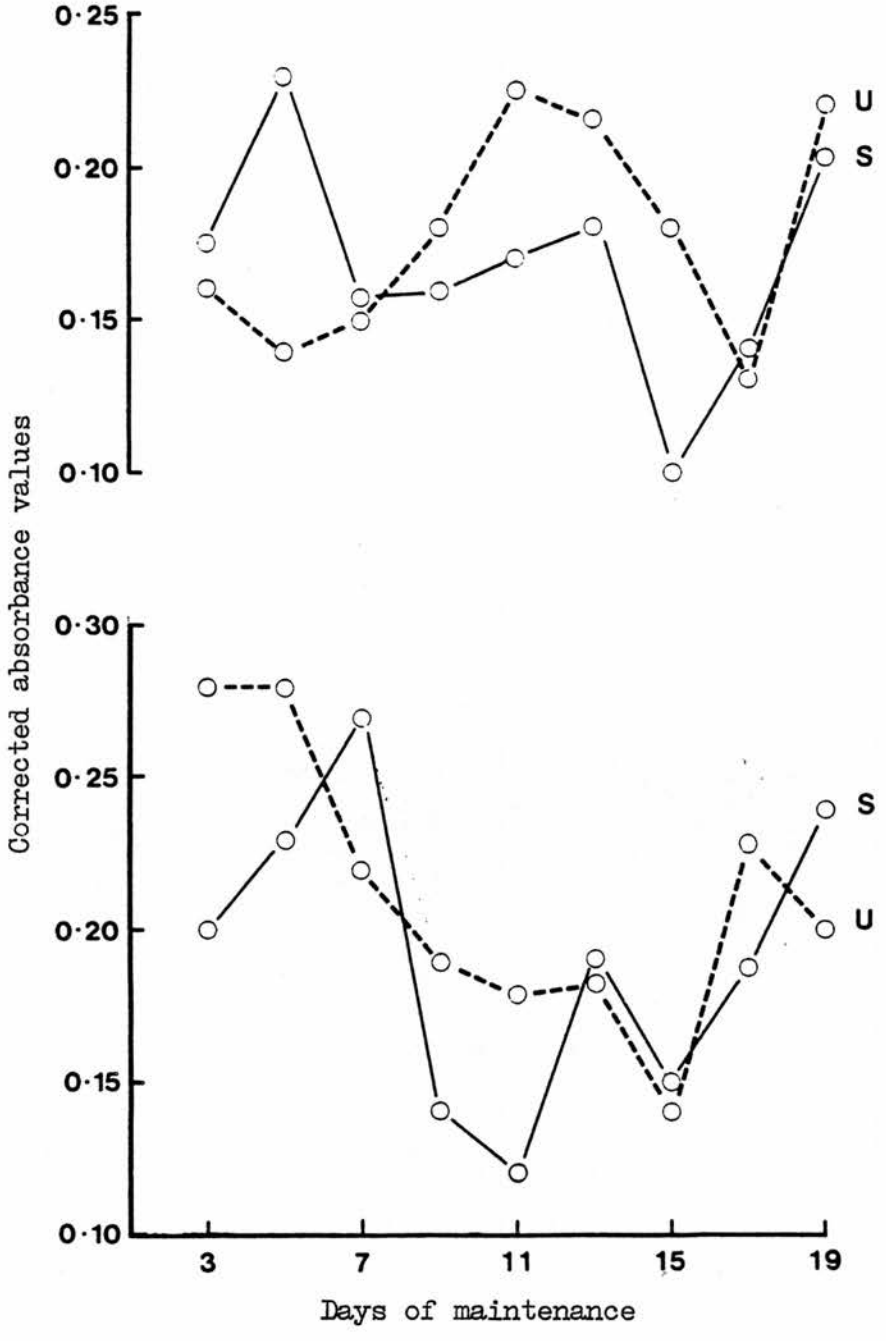

Figure 5 Absorbance values obtained by ELISA when detecting ESA in media in which larvae had been maintained in sealed (S) or unsealed (U) petri-dishes containing serum-free (upper figure) or serum-added (lower figure). For the corrected absorbance values see Appendix Table 4.

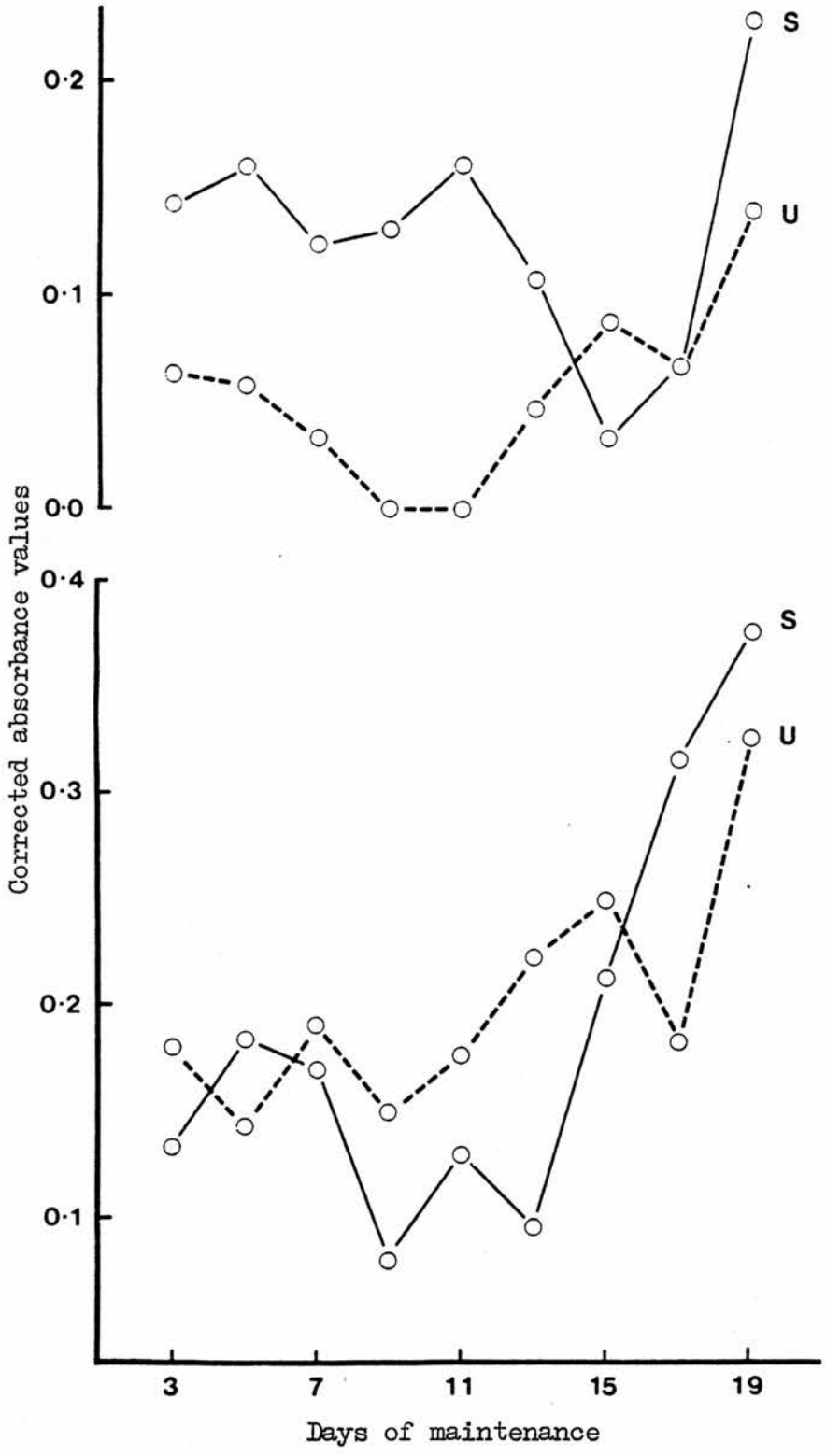

Figure 6 Absorbance values obtained by ELISA when detecting SA in media in which larvae had been maintained in sealed (S) or unsealed (U) petri-dishes containing serum-free (upper figure) or serum-added media (lower figure). For the corrected absorbance values see Appendix Table 4.

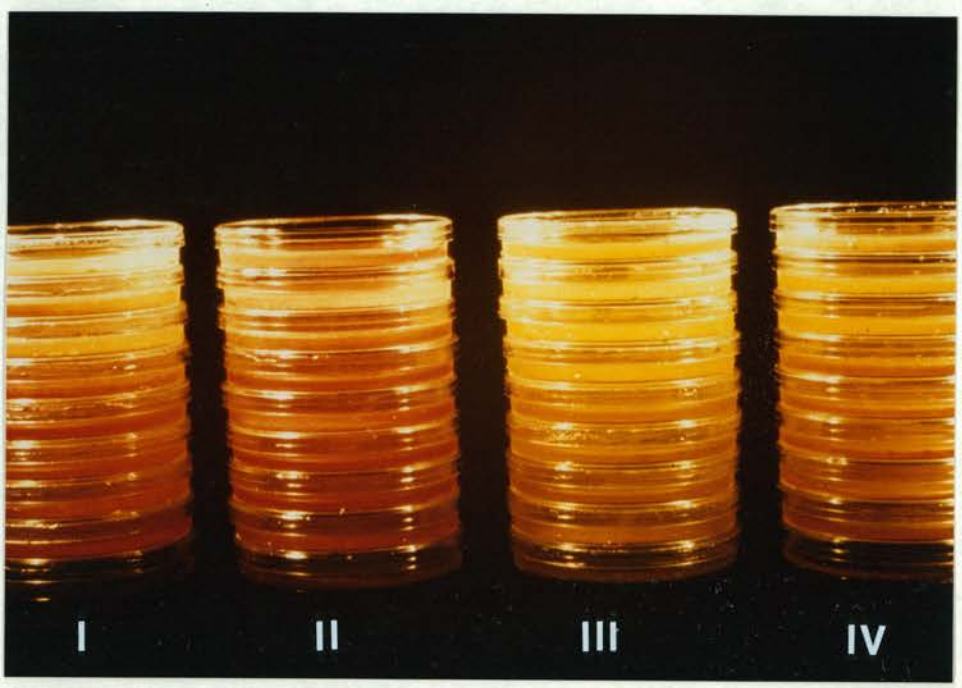

Plate 1 Colour changes in media in which groups of larvae had been maintained in the following manner:

I - sealed petri-dish; no serum in the medium

II - unsealed petri-dish; no serum in the medium

III - sealed petri-dish; 10% SBCS in the medium

IV - unsealed petri-dish; 10% SBCS in the medium

The petri-dishes in each group are arranged from top to bottom by increasing day of maintenance.
(SBCS - Special Bobby Calf Serum)

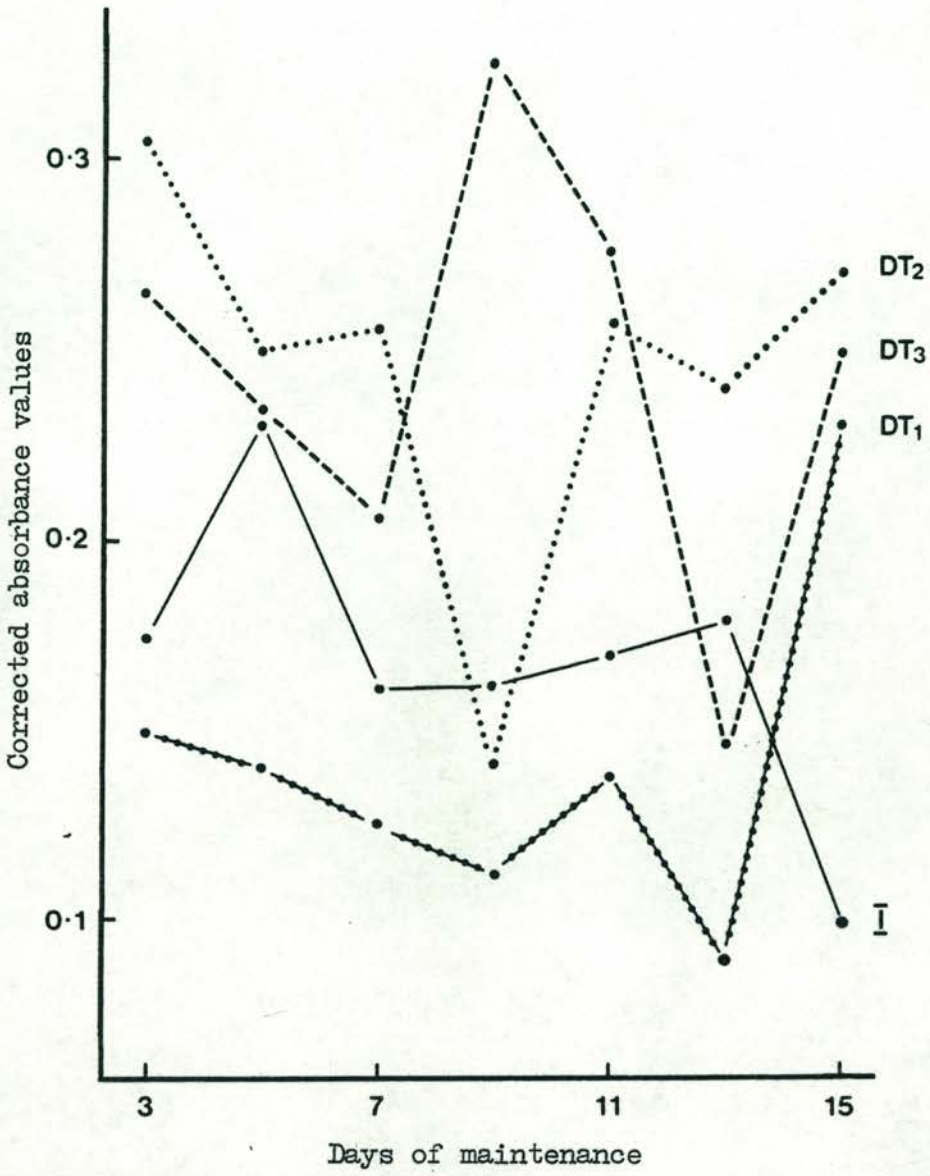

Figure 7 Absorbance values obtained by ELISA when detecting ESA in media in which three groups of damaged larvae (DT_1, DT_2 and DT_3) and one group of intact larvae (I) had been maintained.

For the corrected absorbance values see Appendix Table 5.

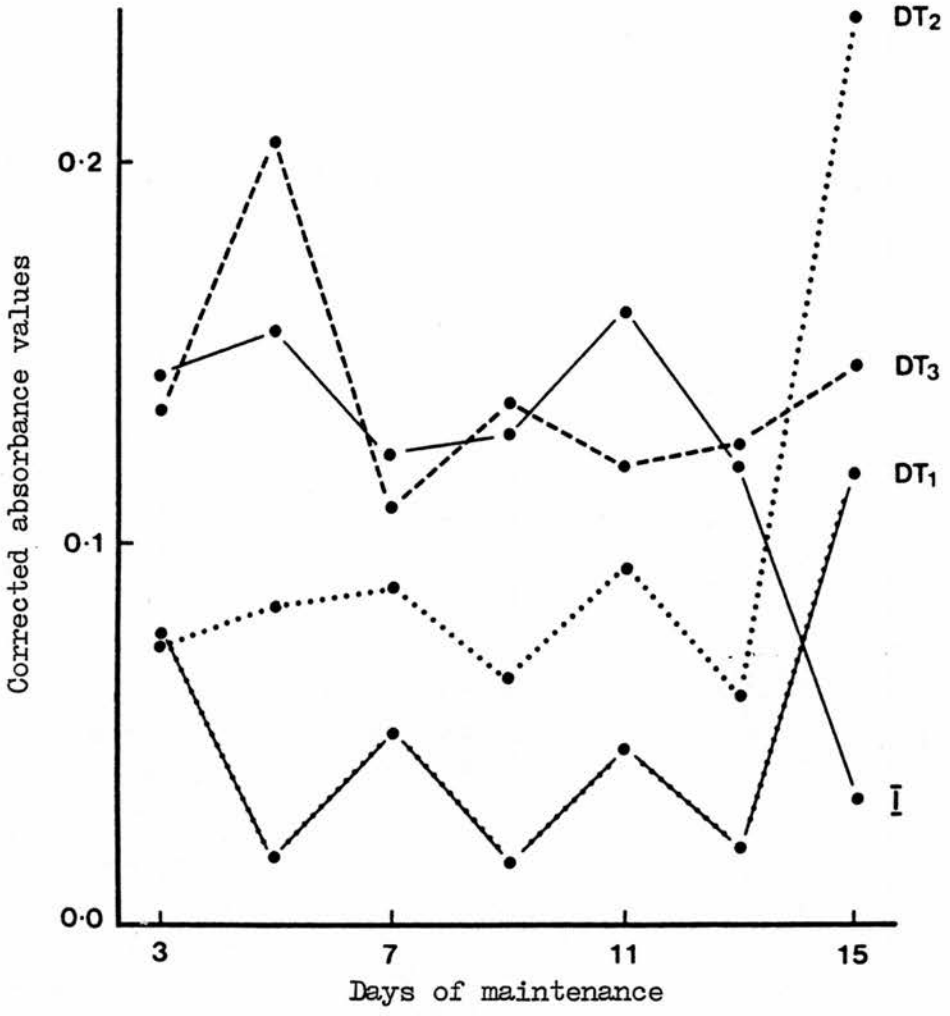

Figure 8 Absorbance values obtained by ELISA when detecting SA in media in which three groups of damaged larvae (DT_1, DT_2 and DT_3) and one group of intact larvae (I) had been maintained.

For the corrected absorbance values see Appendix Table 5.

Plates 2a-i

Transmission electron micrographs of transverse sections of *Taenia taeniaeformis* larvae, with examples of intact and disrupted teguments observed after *in vitro* maintenance

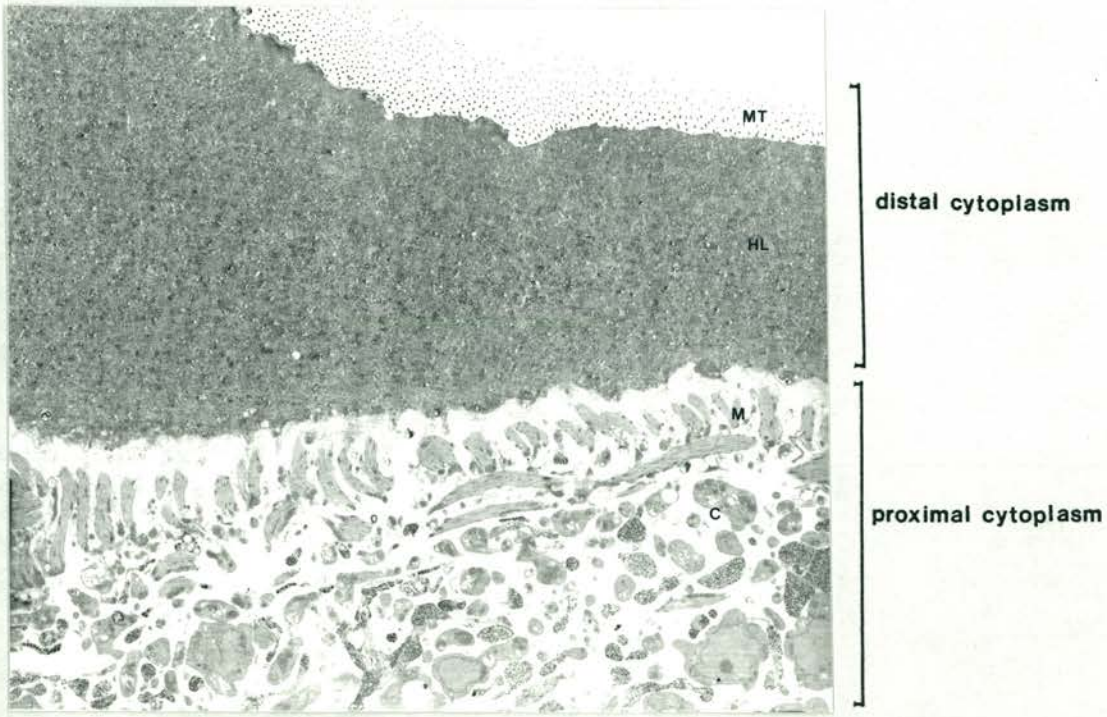

Plate 2a Normal tegument - the distal cytoplasm comprises microtriches (MT) and a thick homogeneous layer (HL); the perinuclear (≡ proximal) cytoplasm comprises muscle bundles (M) and tegumental cells (C). 2150 x

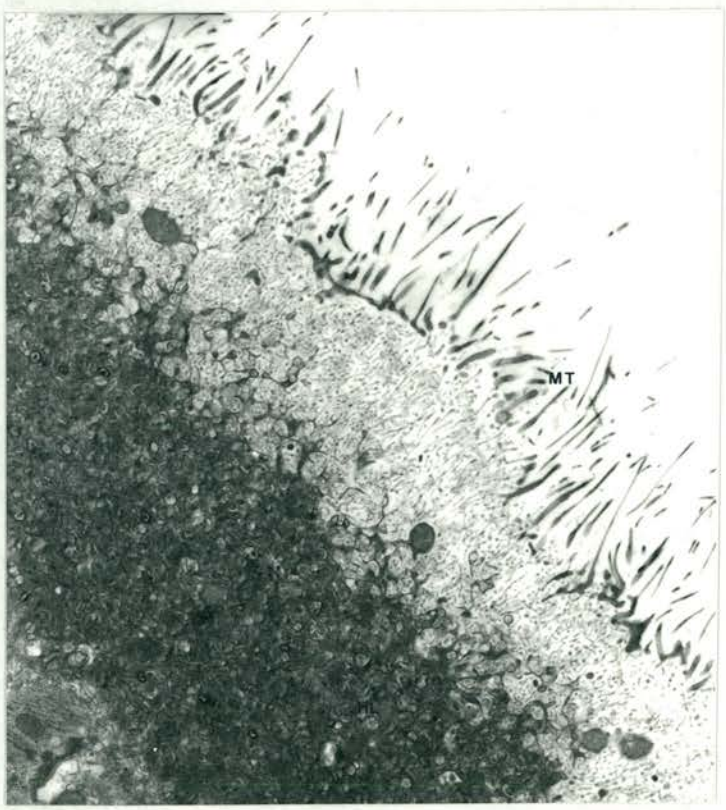

Plate 2b Normal tegument - the distal cytoplasm with microtriches (MT) and thick homogeneous layer (HL) and proximal cytoplasm with muscle bundles. 6000 x

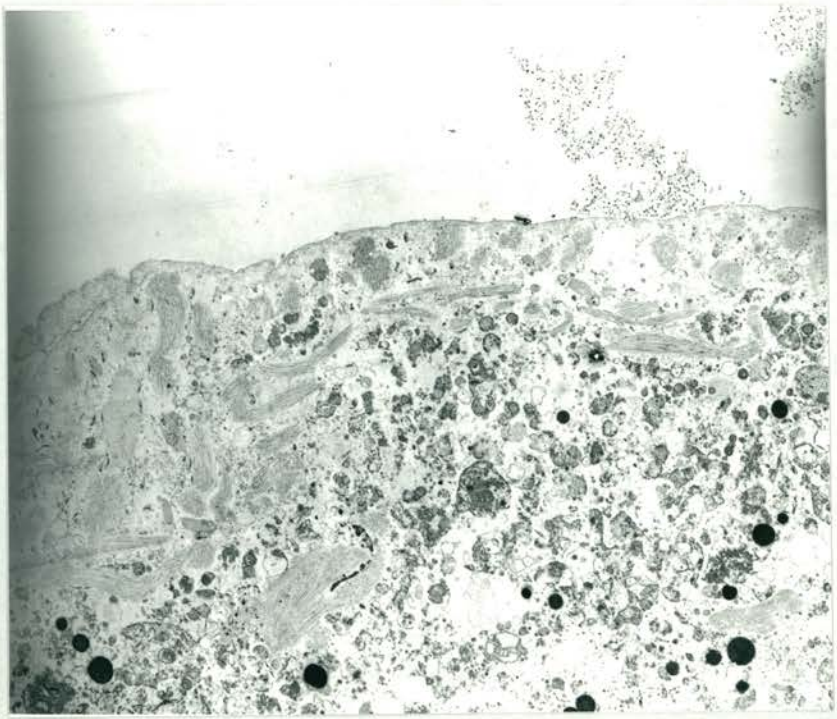

Plate 2c Group I - day 5. Section of area with dye uptake. Absence of both microtriches and thick homogeneous layer. 2150 x

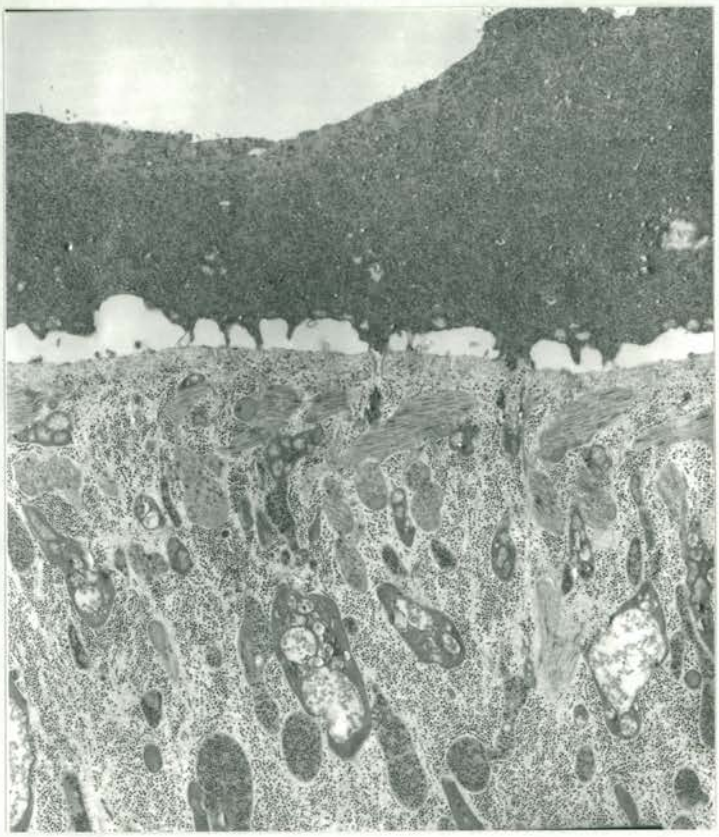

Plate 2d Group I - day 23. Section of area with dye uptake. Homogeneous layer is detaching from the larval body. 3550 x

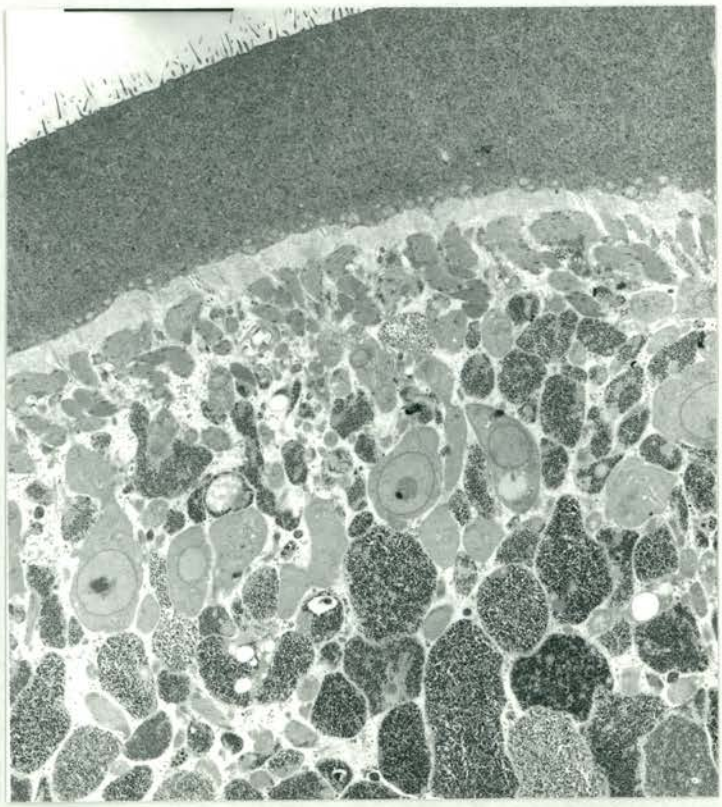

Plate 2e Group II - day 3. Section of area with dye exclusion. Presence of both distal and proximal cytoplasmic structures. 2150 x

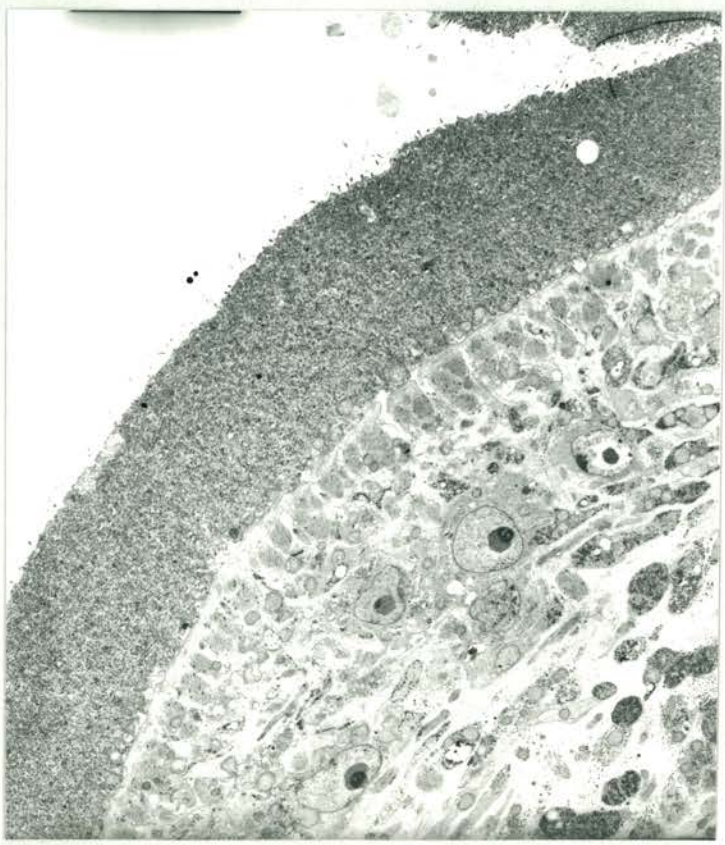

Plate 2f Group II - day 9. Section of area with dye exclusion. Presence of both distal and proximal cytoplasmic structures. 2150 x

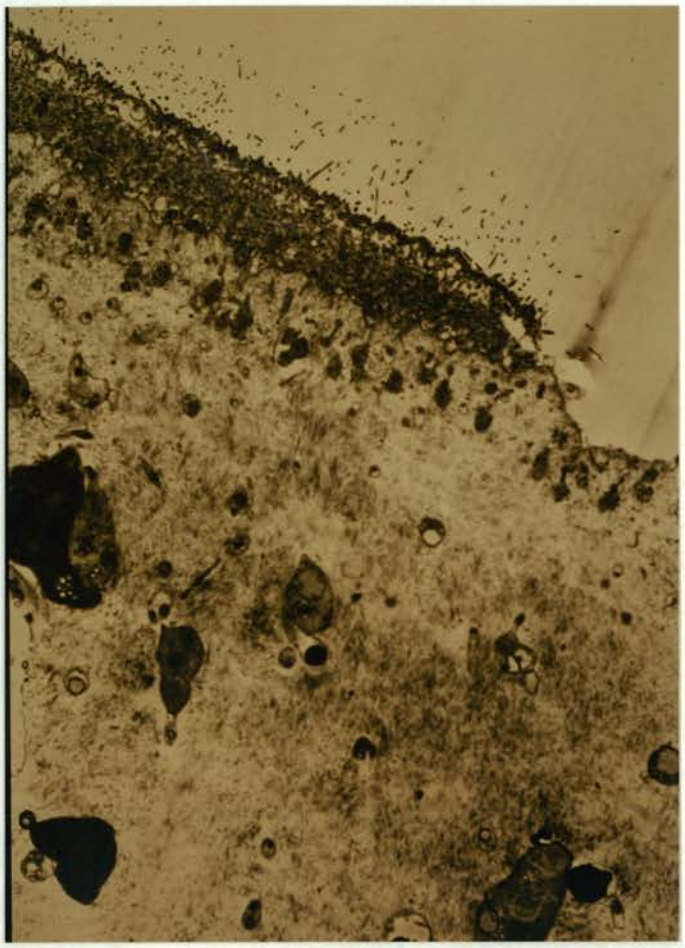

Plate 2g Group II - day 15. Section of area with dye uptake and exclusion. Intact tegument on the left and disrupted tegument on the right. 2150 x

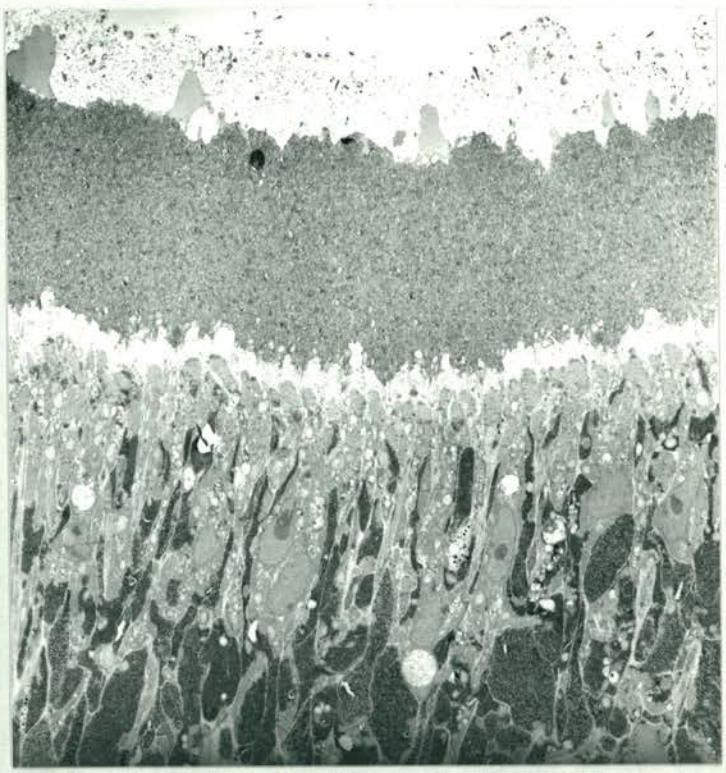

Plate 2h Group II - day 19. Section of area with dye exclusion. Presence of normal tegumental structures. 1650 x

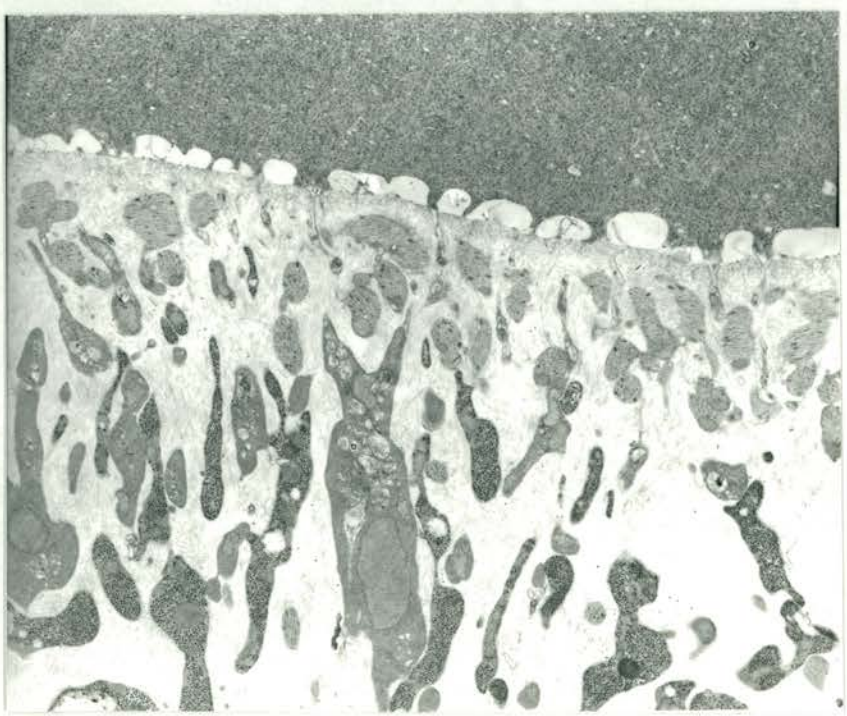

Plate 2i Group III - day 13. Section of area with dye uptake. Homogeneous layer is detaching from the larval body. 3550 x

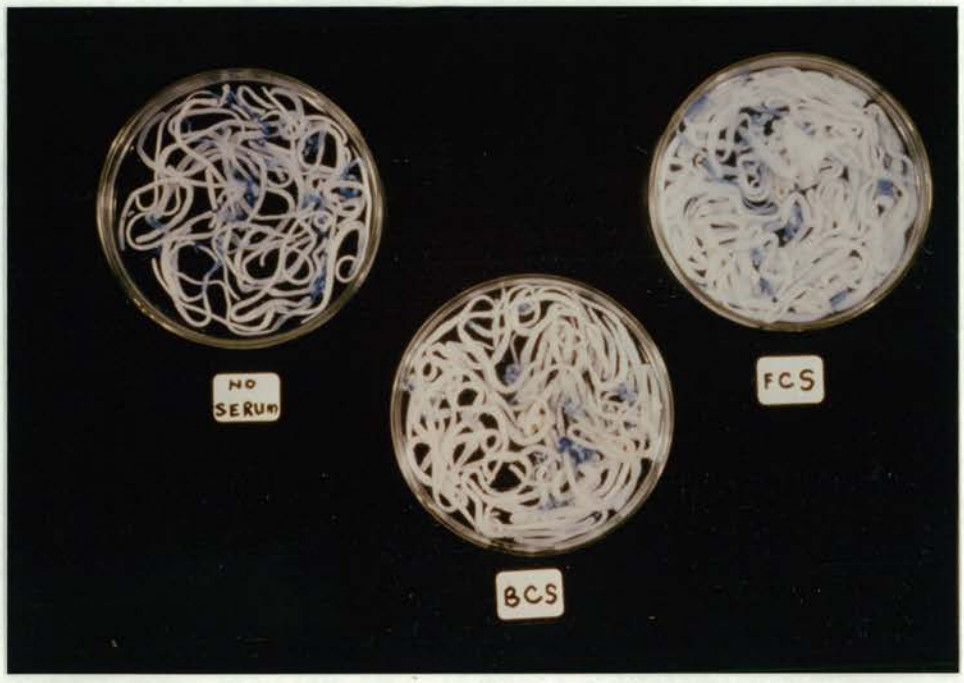

Plate 3 Dye uptake by larvae after maintenance for 23 days in serum-free medium or SBCS-added medium and for 21 days in FCS-added medium (SBCS - Special Bobby Calf serum; FCS - Foetal Calf serum).

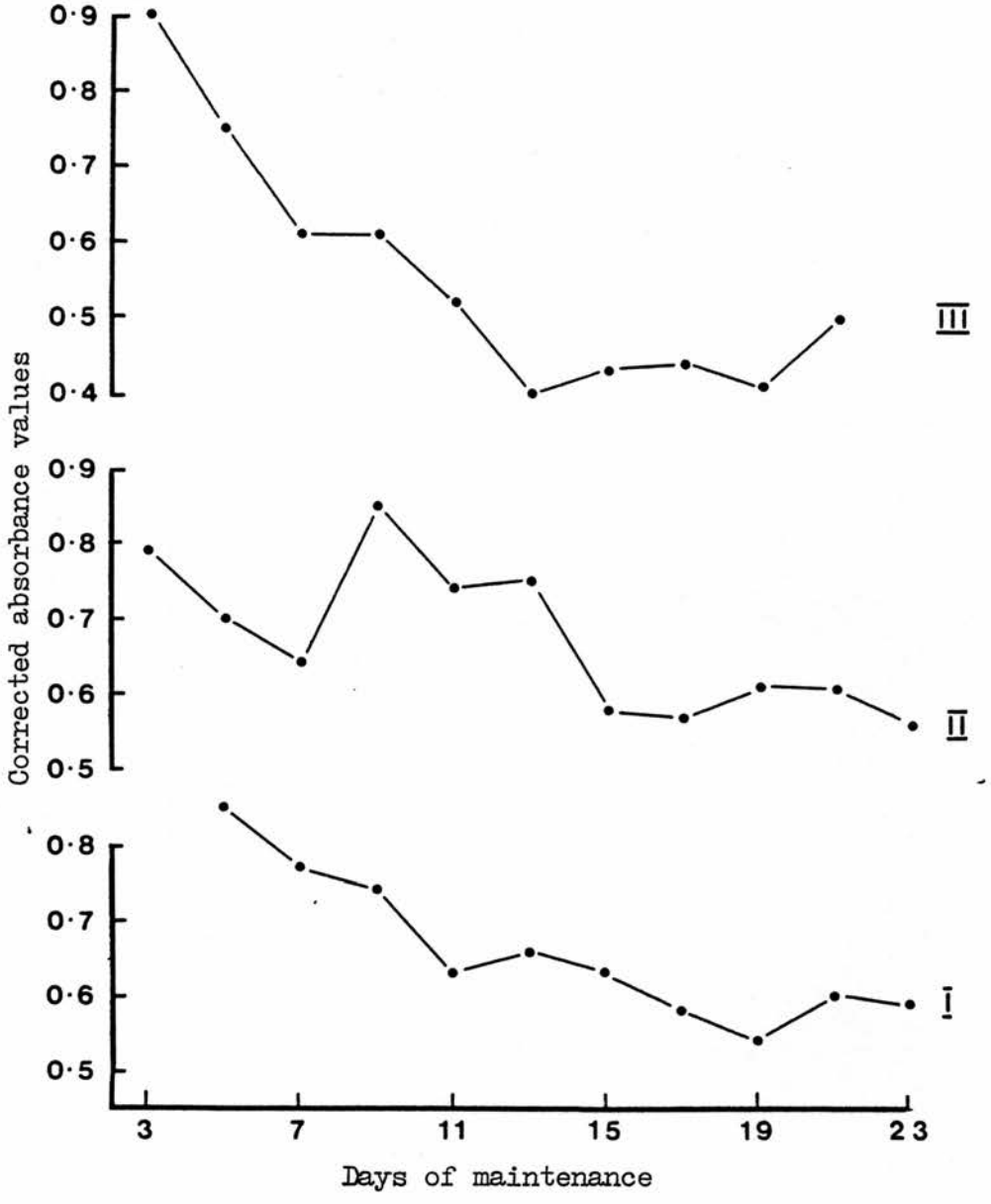

Figure 9 Absorbance values obtained by ELISA when detecting ESA in media in which larvae had been maintained. Group I was serum-free medium, Group II contained SBCS and Group III contained FCS in the media.
For the corrected absorbance values see Appendix Table 6.

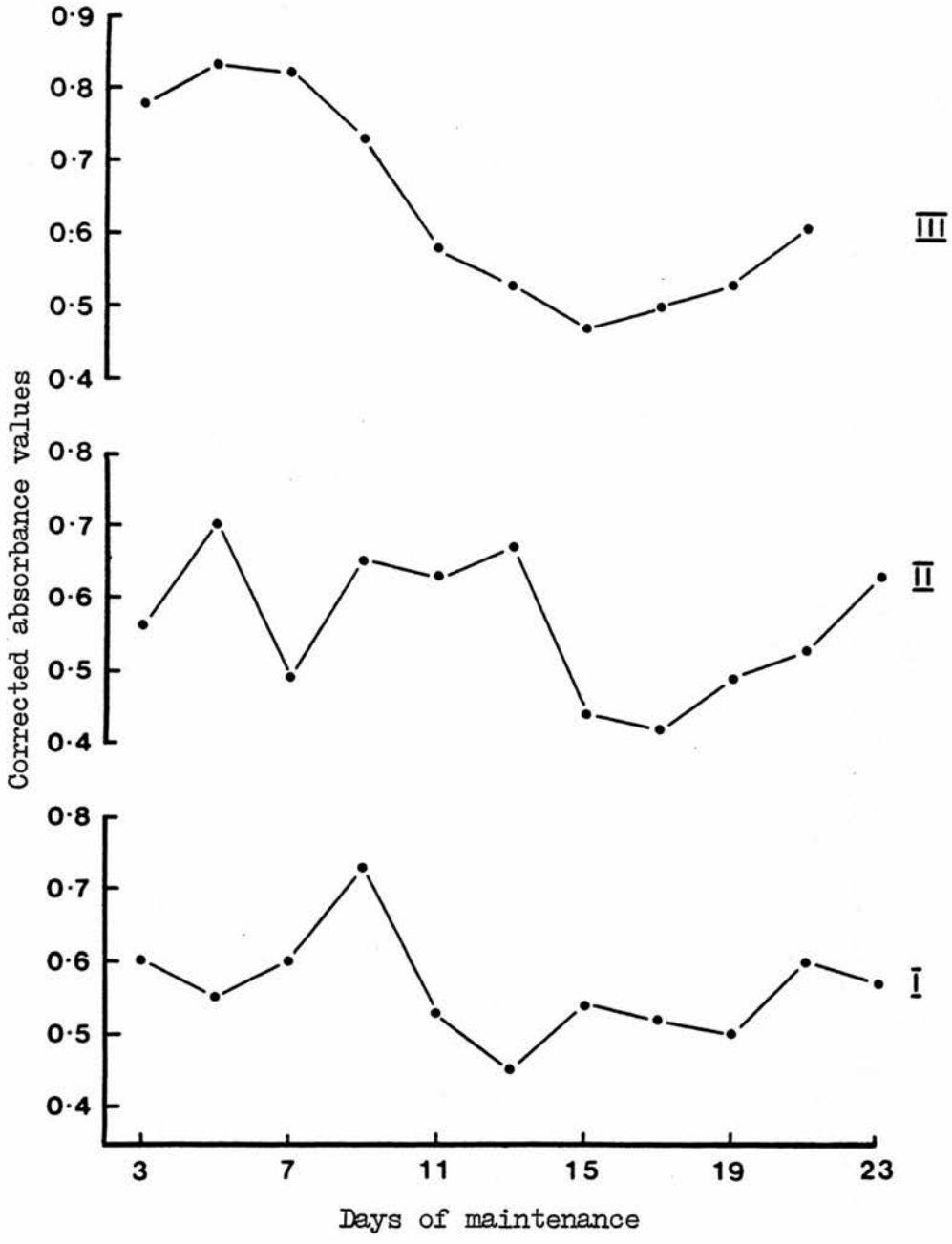

Figure 10 Absorbance values obtained by ELISA when detecting SA in media in which larvae had been maintained. Group I was serum-free medium, Group II contained SBCS and Group III contained FCS in the media.
For the corrected absorbance values see Appendix Table 7.

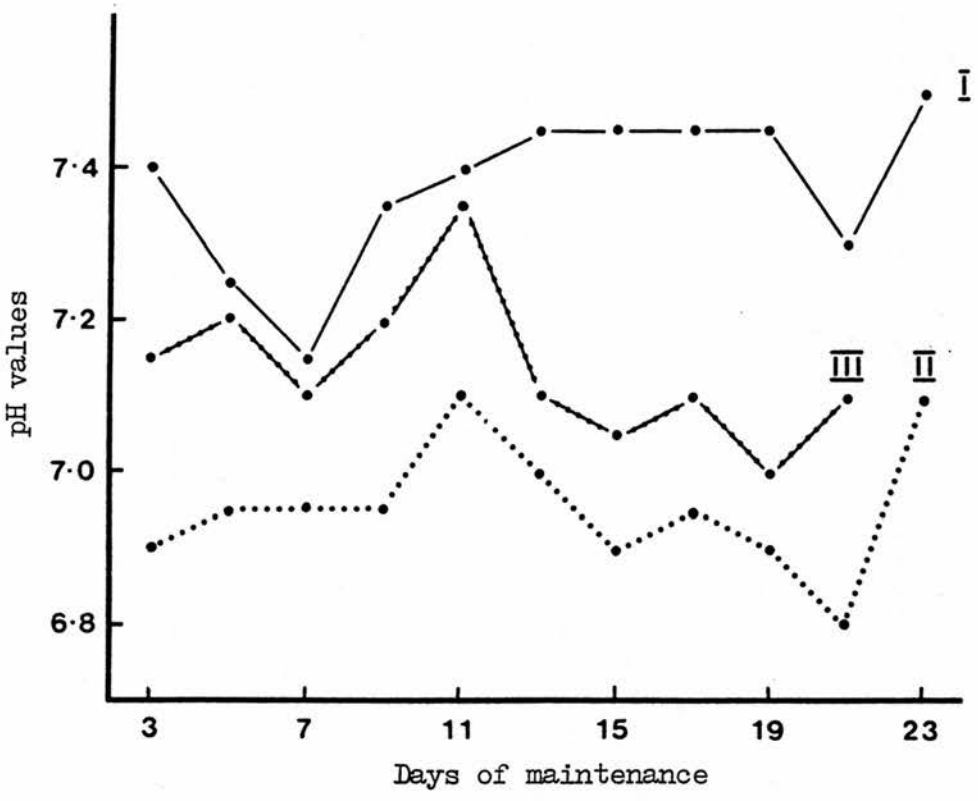

Figure 11 Variation in pH in media in which larvae had been maintained. Group I was serum-free medium, Group II contained SBCS and Group III contained FCS in the media.
For the pH values see Appendix Table 8.

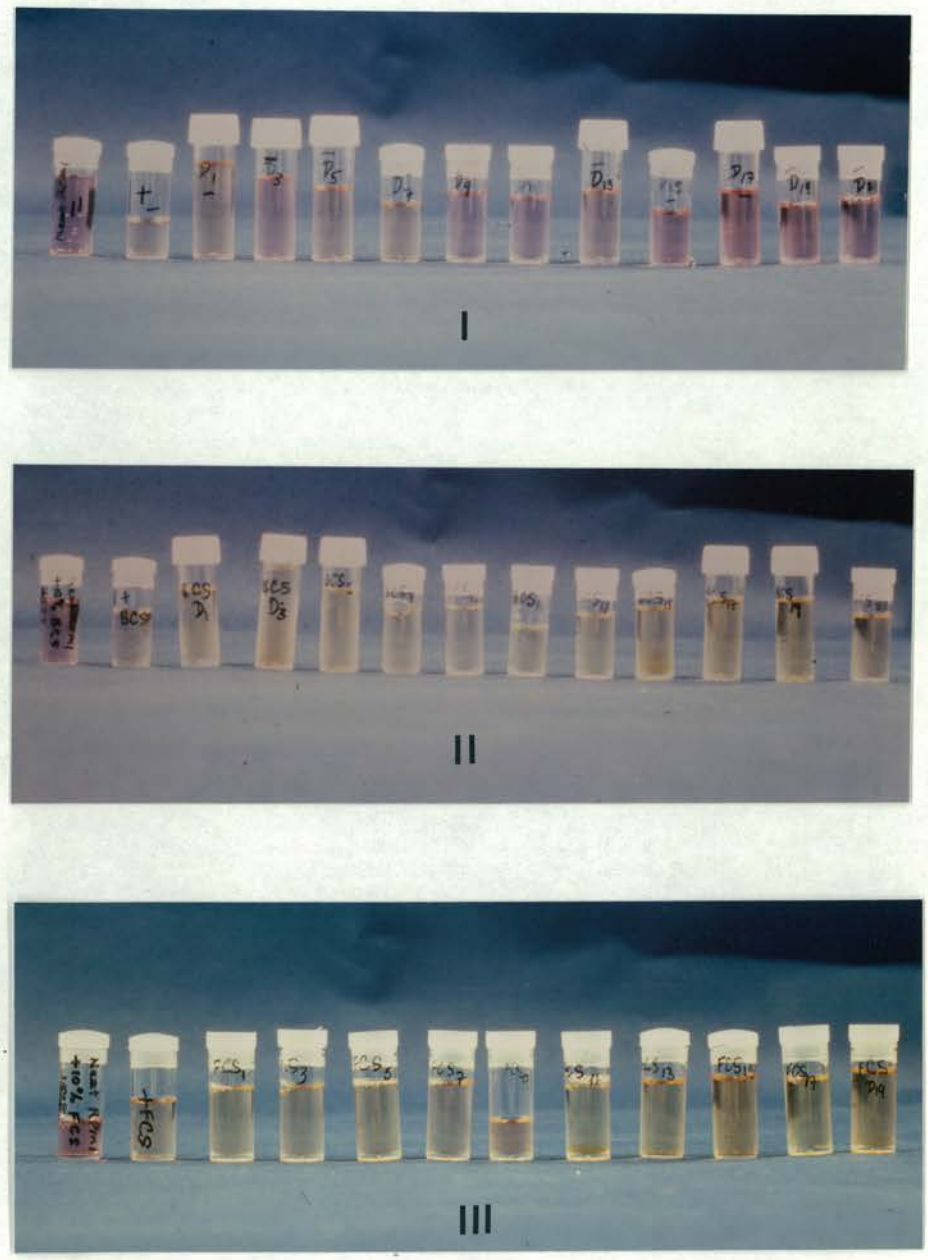

Plate 4 The colour of three types of media in which groups of larvae had been maintained; the medium in Group I was serum-free, the medium in Group II contained SBCS and in Group III contained FCS. The vials are arranged from left to right by increasing day of maintenance.

CHAPTER 4

GENERAL DISCUSSION

The results of the antigenic analysis by the ELISA have to be interpreted with caution. This is because the ESA and SA are far from pure and have shared components. Consequently so do the resultant antisera produced using these antigens. Hence when the assay is used to detect either ESA or SA, it is also to some extent detecting the other antigen. However in view of the different methods of antigen preparation and the different results obtained, these are clearly not entirely cross-reacting.

The electron micrographs show that there was eventual sloughing of the tegument when the parasite was maintained *in vitro* and that this usually involved both the microtriches and homogeneous layer beneath. The SA which produced the corresponding antisera used in the ELISA for detecting antigens was a saline soluble extract, and it is uncertain to what extent it is the same as sloughed tegument - though it will almost certainly contain common components. Therefore the amount of demonstrable SA might be expected to parallel the tegumental degeneration. A similar case can be made for the ESA since it has been shown that tritium-labelled proline is taken up by the tegument of *Hymenolepis diminuta* and served within the subtegumental cells for the synthesis of structural and enzyme proteins which are then incorporated into the tegument (Lumsden, 1966). Oaks and Lumsden (1971) found that a labelled carbohydrate (galactose) was taken up by adult *Hymenolepis diminuta* and incorporated into a protein fraction in the tegument. The labelled component was subsequently transported to the brush border

(≡ microtriches). The continual replenishment of this component in the tegument surface suggests that constant renewal of the surface coat appears to be a feature of the tapeworm tegument. The presence of numerous vacuoles, vesicles and metabolic enzymes in the tegument suggests that secretory (or excretory) processes are actively proceeding in this region (Smyth, 1972). Wilson and Barnes (1974b) working with Schistosoma mansoni confirmed that the vesicles contribute material to the surface of the tegument and suggested that they do so by fusing with the plasma membrane releasing their contents outside it. Thus the homogeneous layer of the tegument which is sloughed off in the damaged areas will also comprise the ESA. Kusel, MacKenzie and McLaren (1975) concluded that culture media in which Schistosoma mansoni had been held contained surface membrane antigens in both soluble and particulate form. They considered these to correspond to the materials released in vivo. Recently, Philipp, Parkhouse and Ogilvie (1980) showed that in vitro maintained Trichinella spiralis larvae and adults released radio-labelled surface proteins into the culture medium indicating a turnover of surface-labelled material in this species.

The observed in vitro damage to the tegument may be due to: (i) the parasite's response to inadequate in vitro conditions; (ii) the secretions of the parasite actively destroying its own tegument (Lawrence, Heath, Parmeter and Osborn, 1980). This would presuppose that in vivo, the secretions are removed by the host's circulation, so perhaps constituting "circulating antigen"; (iii) larval contact with the inanimate vessel surface having a deleterious effect on the tegument (Lawrence et al., 1980); (iv) a normal

"turnover" process analogous to that seen with Schistosoma species (Wilson and Barnes, 1974a) and (v) a combination of any or all of the above possibilities.

Finally, although using a defined medium where all the components are known and avoiding the use of serum, which contains unknown components, is desirable for analytical work, the dye test augmented by transmission electron microscopy showed that serum has a beneficial effect on the maintenance of the metacestodes.

PART II

Humoral response in mice and rats experimentally infected with *Taenia taeniaeformis*

CHAPTER 5

INTRODUCTION

The detection of specific antibodies or antigenic materials in the serum or body fluids of the host constitutes serodiagnosis of parasitic diseases (Kagan, 1979).

In this part, excretory-secretory and somatic antigens derived from metacestodes of T. taeniaeformis are used to investigate the antibody response in rats and mice experimentally infected with this parasite. These antigens were also used to produce antisera for detection of circulating antigens.

Glutamate dehydrogenase activity in the serum of the mice was also used to monitor the infection.

REVIEW OF THE LITERATURE

Detection of circulating antigen

Parasitic antigens have been shown to circulate in the serum and body fluids of hosts with a number of parasitic infections. Hence, Kagan (1974) suggested that the scope of parasite serology should not only cover the detection of antibodies but also that of antigens.

Information on detection of circulating antigen in helminths has stemmed from work with various species of Schistosoma. In this system, Berggren and Weller (1967) demonstrated specific circulating antigen in plasma of infected mice by immunoelectrophoresis. They also correlated the appearance of antigen with the worm burden and duration of infection. Gold, Rosen and Weller (1969) also detected a specific Schistosoma antigen by immunodiffusion and suggested that there was a direct linear relationship between the serum antigen concentration and worm burden. Bawden and Weller (1974) used a complement fixation test to confirm this relationship.

Von Lichtenberg, Bawden and Shealey (1974) and Nash (1974) both stated that circulating schistosome antigen (CSA) originated in the gut and not from the parenchyma or tegument. However, Kusel, MacKenzie and McLaren (1975) suggested the tegument as a further source of antigenic material released into the circulation.

As with the previous authors, Nash, Prescott and Neva (1974) perceived CSA to be detectable only in experimental hosts heavily infected with schistosomes. These authors and Nash, Nasir-Ud-din and Jeanloz (1977) confirmed that CSA was present in Schistosoma

worm material as well as in the sera of infected mice. Hence they concluded that CSA was derived from the worm and is either secreted or excreted into the circulation.

Santoro, Vandemeulebroucke and Capron (1979) not only detected CSA but also circulating immune complexes (CIC) six to seven weeks after infecting mice. They related the appearance of CIC to the nephropathy which occurs seven to eight weeks after infection in mice.

Van Knapen and Panggabean (1977) detected circulating antigen by ELISA in human and mouse sera during the acute stage of Toxoplasma gondii infection. They related this to the onset of parasitaemia in mice and clinical symptoms in human patients. They considered it to be advantageous to test for both antibody and circulating antigen levels in diagnosing acute toxoplasmosis as only one serum sample is then needed, instead of paired sera samples.

Ruitenberg and Van Knapen (1977) investigated serum antibody and antigen levels in Trichinella spiralis, Toxocara canis, Trypanosoma species and Toxoplasma gondii infections. They compared the ELISA and immunofluorescence techniques and found the former to be more sensitive in helminth infections. They suggested that detection of free circulating antigen especially was better at discriminating between the early phases and past experience of an infection.

Harrison (1978) detected antigen levels one to three weeks post-infection in sera from cattle experimentally infected with Taenia saginata, after which the levels remained high for about ten to twenty weeks.

Detection of antibodies

The serologic diagnosis of parasitic infections constitutes a field which has become so vast that extensive coverage would not fall within the scope of this dissertation. However the overall findings relating to the immunodiagnosis of cestode infection are presented.

The diagnosis of cestode infections has been reviewed by Williams (1979). He noted that the purification of a specific "antigen 5" had rendered the serological tests for echinococcosis as the most successful immunodiagnostic procedure for human helminthiasis. However the serodiagnosis of cysticercosis in domesticated livestock is much less successful. The reason for this lies partly in the lack of specific and characterised antigens. It is also because under field conditions, light infections with taeniid species are more common. Low antibody titres resulting from such infections require very sensitive diagnostic methods and the use of such methods increases the probability of false positive results occurring. Reviews by Fife (1971), Kagan (1974), Buck (1975) and WHO (1976a) concluded that the specificity and sensitivity of an immunodiagnostic procedure depends largely on the quality of antigen employed. According to Buck, the main requirement is that the serodiagnostic antigens should be able to detect and differentiate antibodies induced by specific parasitic infections.

Flisser, Perez-Montfort and Larralde (1979) reviewed immunodiagnostic tests for cysticercosis. They concluded that all common immunological methods were useful in diagnosis. The choice of a method depended more on the cost, ease of execution and the

purpose of the study than on sensitivity. However, Geerts, Kumar and Vercruysse (1977) disagreed that the serological techniques in current use were completely satisfactory. The indirect hemagglutination test (IHA) has been considered to be the test of choice for the diagnosis of Taenia saginata cysticercosis (Dewhirst, Cramer and Sheldon, 1967 and Gallie and Sewell, 1974). However one disadvantage of IHA is that it gives a high frequency of false positive reactions. Geerts, Kumar, Aerts and Ceulemans (1981) in comparing several procedures, found that whereas immunoelectrophoresis was the most specific it lacked the desired sensitivity. However ELISA was found to combine both sensitivity and specificity.

Enzyme linked immunosorbent assay (ELISA)

This technique was introduced by Engvall and Perlmann (1971, 1972). They introduced the idea of using enzymes conjugated to antibody or antigen to detect and measure antigen or antibody respectively.

Ljungstrom, Engvall and Ruitenberg (1974) and Ruitenberg, Steerenberg, Brosi, Buys, Ljungstrom and Engvall (1974) introduced the ELISA method into parasitology when it was used for the serodiagnosis of Trichinella spiralis infections. One of the first workers to apply the method to cestode infections were Farag, Bout and Capron (1975) who used it to detect antibody in hydatid disease. They found the test to be both very sensitive and highly specific when a purified antigen was used. ELISA has since proved to be a very sensitive and practicable test for the diagnosis of many parasitic diseases (WHO, 1976b and Voller, Bidwell and Bartlett, 1976).

One of the advantages of ELISA is that it may be used for the detection of antigens in body fluids (WHO, 1976b). This seems potentially useful as a means of discriminating between the presence of the living parasite and past experience of an infection, as established by Van Knapen and Panggabean (1977) and Ruitenberg and Van Knapen (1977).

CHAPTER 6

MATERIALS AND METHODS

Glutamate dehydrogenase

Principle

Glutamate dehydrogenase (GDH) was measured by spectrophotometric assay according to Ford and Boyd (1962). The enzyme activity in serum was determined by following the rate of oxidation of NADH in the presence of 2-ketoglutarate and ammonium ions. The mechanism of the reaction is as follows:

$$2 \text{ ketoglutarate} + \text{NADH} + NH_4^+ \rightleftharpoons \text{L(+)-Glutamate} + NAD^+ + H_2O$$

The equilibrium of the reaction lies far in favour of glutamate formation. The measurements are therefore made with 2-ketoglutarate and ammonium as substrates and NADH as coenzyme. The rate of oxidation of NADH is directly proportional to the reaction of the substrate and serves as a measure of the enzyme activity.

Method

The following reaction mixture was placed in an 8 ml test tube held in a waterbath of 25°C.

0.2 ml of NADH (Sigma) 1 mg ml^{-1}

2.2 ml of 0.1M phosphate buffer pH 7.5

0.2 ml of 33% $^w/_v$ ammonium sulphate

0.2 ml of serum (diluted to 1:4)

After 15 minutes preincubation, the mixture was placed in a 3 ml spectrophotometer cuvette (Sarstedt) with a 10 mm light path. The cuvette was then placed in a Pye Unicam spectrophotometer SP 1800

fitted with a constant temperature cell-housing kept at 25°C. Following the addition of 0.2 ml of 0.1M α-ketoglutaric acid (Sigma) pH 7.5, the first reading of extinction (E_1) at 340 nm was immediately taken. The final reading (E_2) was taken 40 minutes after the first reading. The enzyme concentration was calculated from the formula:

$$\frac{\Delta E\ min^{-1}}{6.3} \times \frac{\text{volume of cuvette}}{\text{volume of sample}} \times 1000 \times \text{dilution factor}$$

where $E\ min^{-1} = \frac{E_1 - E_2}{40}$

volume of cuvette	3 ml
volume of serum	0.2 ml
dilution factor of serum	4

The value obtained from the formula was expressed in moles of enzyme min^{-1} $litre^{-1}$ of serum at 25°C which is equivalent to U l^{-1}.

Preparation of antigens

All antigens used originated from the Belgian strain of T. taeniaeformis. Two preparations were used. One was a pool of defined medium in which metacestodes had been maintained in vitro for ten days. This was designated "ESA" for "excretory and secretory antigen", a common term for such predominantly metabolic antigens in helminthology. ESA is sometimes referred to as exogenous or exoantigens (Smyth, 1976). The details of the maintenance and processes for use were as described in Part I.

The other antigen used was a somatic extract of the metacestode. This was prepared according to the method of Gallie and

Sewell (1974) with certain extensions. Metacestodes (3-10 months old) were recovered from mice and washed thoroughly as for *in vitro* maintenance (Part I). Excess fluid from these worms was removed by blotting with filter paper. The worms were cut finely with a pair of scissors and this tissue mass weighed. In Bijou bottles 1 g of tissue sample was mixed with 2 ml of PBS and homogenised on an MSE Homogeniser for 3 x 1 minute periods. In between homogenisations the bottles were cooled in an ice-bath. After the suspension was well broken up, a further 2 ml of PBS was added to each bottle. The suspension was mixed overnight on a blood-mixer (Eschmann) at 4°C. The suspension was then centrifuged at 2500 g for 30 minutes at 4°C. The supernatant was withdrawn and ultracentrifuged on an L2-65B Ultracentrifuge (Beckman) at 100,000 g for 30 minutes at 4°C.

The precipitate resulting from the ultracentrifugation was stored at -20°C, to be used as a vaccine in one of the experiments in Part III.

The protein content of the final supernatant was estimated before being stored in aliquots of 300 µl at -20°C. This preparation was then designated "SA" or "somatic antigen". Similar antigens may also be known as endogenous or endoantigen, structural, bound or internal antigens (Smyth, 1976).

Blood sampling

The samples were taken by means of heparinized capillary tubes No. 442 (Sarstedt). Rats were anaesthetised with ether, a small cut with a scalpel blade was made in the tail and the blood taken

up into the capillary tubes. The tubes were centrifuged at 1500 g for 30 minutes at 4°C and the resulting plasma was stored at -20°C in labelled 0.5 ml microcapped centrifuge tubes (Hughes and Hughes).

Enzyme-linked immunosorbent assay (ELISA)

Micro-ELISA was generally conducted according to the methods and principles described by Harrison (1977) as detailed in Table 5. The assays were performed in polystyrene plates ("Mikrotiterplatte 96K F-Form aus Immulon" Greiner labor technik).

The optimal dilutions for the antigen and antiserum in the assay for detecting antibody were obtained from the results of a preliminary experiment. The ELISA procedure for detecting antigen (Table 6) was adopted from Brandt (1980). Dr. Brandt also provided the rabbit and guinea-pig normal and hyperimmune gammaglobulins raised against ESA and SA.

Solutions used in ELISA

Coating buffer	0.1M sodium carbonate buffer pH 9.6 with 0.02% w/v sodium azide (BDH)
PBS-Tween	PBS pH 7.3 (Dulbecco "A") with 0.05% w/v Tween 20 (Polyoxyethylene sorbitan monolaurate - Sigma)
Conjugates	Commercial horse radish peroxidase conjugated to IgG against heterologous species IgG; namely: Rabbit anti-mouse IgG (Miles) Rabbit anti-rat IgG (Miles) Rabbit anti-sheep IgG (Nordic) Goat anti-guinea-pig IgG (Nordic)
Substrate	Ortho-phenylene diamine (OPD) (Sigma) 35 mg OPD in 100 ml 0.1M citric acid/phosphate buffer, pH 6.0, containing 0.05% H_2O_2

Sulphuric acid solution	1 part of 1M sulphuric acid in 11 parts of distilled water
Washing solution	0.9% $^{w}/_{v}$ sodium chloride solution to which was added 0.05% $^{w}/_{v}$ Tween 20

Table 5 The stages in the ELISA for detecting antibody

Stage 1	Antigen (ESA or SA) diluted in coating buffer to the optimum concentration and adsorbed to plate.
Stage 2	Wash
Stage 3	Serum or plasma diluted in PBS-Tween to the optimum dilution and added to plate.
Stage 4	Wash
Stage 5	Conjugate dilution in PBS-Tween to 1:1000 and added to plate.
Stage 6	Wash
Stage 7	Substrate added to plate
Stage 8	The degree of colour produced by the substrate-enzyme reaction is measured on a spectrophotometer

Table 6 The stages in the ELISA for detecting antigen

Stage 1	Rabbit normal or immune gammaglobulins diluted in coating buffer to 30 $\mu g\ ml^{-1}$ soluble protein, and adsorbed to plate.
Stage 2	Wash
Stage 3	Serum, plasma or used medium diluted in PBS-Tween and added to plate.
Stage 4	Wash
Stage 5	Guinea-pig normal or immune gammaglobulins diluted in PBS-Tween to 50 $\mu g\ ml^{-1}$ soluble protein and added to plate.
Stage 6	Wash
Stage 7	Conjugate diluted in PBS-Tween to 1:1000 and added to plate.
Stage 8	Wash
Stage 9	Substrate added to plate
Stage 10	The degree of colour produced by the substrate-enzyme reaction is measured on a spectrophotometer.

General procedure

At every stage, 150 µl aliquots of the relevant solution were added to the wells using an adjustable pipette ("Pipetman P" Gilson). The plates were covered with a lid during incubation. The initial sensitization of the wall took place over three hours at 37°C followed by storage at 4°C for periods not exceeding 48 hours. The duration of incubation for each subsequent step was one hour at 37°C. In between each step the plates were washed at room temperature by emptying the wells and refilling them all with washing solution. This solution was left in the wells for five minutes and the washing procedure repeated twice more. After the final wash, the plates were shaken dry and any remaining liquid was removed by gently tapping the inverted plates on tissues. The next stage was begun at once.

The substrate was prepared immediately before use. The plates were left in a dark cupboard for the substrate reaction to proceed. After 30-60 minutes, during which time the colour changes were regularly checked, the enzyme reaction was stopped by adding an equal volume of sulphuric acid solution. The reaction was stopped when the visual checks revealed that the strongest reactions were well developed but still readable. At the same time, an equivalent amount of the acid solution was added to the remaining substrate which had also been left in the dark. The relative absorbance of the reaction product was read in a filterphotometer ("Vitatron" Fisons MSE) provided with a 450 nm filter. Zero absorbance was set against the substrate-acid mixture (blank). At regular intervals while reading the samples the zero setting was checked with the blank.

Controls and interpretation

Every sample tested in the ELISA was carried out at least in duplicate and the average result calculated. A control for false positive reactions in the ELISA for detecting antibody involved using saline-buffer instead of serum.

Controls for false positive reactions in the ELISA for detecting antigen consisted in replacing gammaglobulins from the hyperimmunised animal with gammaglobulins at the same concentrations from corresponding normal (i.e. not infected or not hyperimmunised) animal species.

The corrected ELISA values were obtained by subtracting the absorbance values for the controls from the absorbance values for the test, as in the following formulae: (In (i) + indicates component present and - indicates component absent. In (ii) + indicates gammaglobulin from hyperimmunised animals and - indicates gammaglobulin from corresponding normal animals.)

(i) For ELISA to detect antibodies:

$$\begin{matrix}\text{Antigen:} \\ \text{Antibody:}\end{matrix} \begin{bmatrix}+ \\ +\end{bmatrix} - \begin{bmatrix}+ \\ -\end{bmatrix}$$

(ii) For ELISA to detect antigens:

$$\begin{matrix}\text{Rabbit gammaglobulins} \\ \text{Antigen} \\ \text{Guinea-pig gammaglobulins}\end{matrix} \begin{bmatrix}+ \\ + \\ +\end{bmatrix} - \begin{bmatrix}+ \\ + \\ -\end{bmatrix} - \begin{bmatrix}- \\ + \\ +\end{bmatrix} + \begin{bmatrix}- \\ + \\ -\end{bmatrix}$$

A different approach was adopted in the ELISA for detecting antibodies, when samples from an experiment had to be assayed on different days. A formula was designed which takes into account

day-to-day variation, thus enabling values obtained from different days to be standardized. This formula also gives the advantage of allowing comparisons to be made between the results from one experiment and those from another. Samples consisting of sera from infected and normal rats with high and low ELISA values respectively were separately pooled. Aliquots from these two pools of positive and negative sera were preserved at -20°C. Dilutions of these reference sera were made at the same level as for the test sera and included in each group of tests. The ELISA values for these positive and negative sera assayed on a certain day 0 were taken as the constant or reference values and used in the following formula to standardize ELISA values from day-to-day:

Corrected absorbance

$$= \left[Un - Nn\right] \times \left[\frac{Po - No}{Pn - Nn}\right]$$

where Un test serum assayed on day n

Nn negative serum assayed on day n

Pn positive serum assayed on day n

No negative serum assayed on day 0

Po positive serum assayed on day 0

CHAPTER 7

EXPERIMENTS

Preliminary experiments with ELISA

The effective antigen-antibody dilution range was studied for the ELISA for detecting antibodies. Chequer-board titrations of various protein concentrations in antigens (ESA and SA) against positive (infection) and negative (normal) sera were carried out. Five soluble protein concentrations of 1, 5, 10, 15 and 20 $\mu g\ ml^{-1}$ were used against $^1/50$, $^1/100$, $^1/200$, $^1/400$ and $^1/800$ dilutions of serum.

Results

Figures 12 and 13, 14 and 15 show the ELISA values of the chequer-board titrations for sera from mice and rats respectively. Using these figures, the optimum antigen concentration and serum dilution were selected for all subsequent ELISA tests. These were concentrations and dilutions which would give maximal economy of the material in use, consistent with good discrimination between positive and negative sera, minimal results with negative sera and a satisfactory ability to quantify the activity of the sera. Hence for both ESA and SA, 10 $\mu g\ ml^{-1}$ soluble protein was selected and serum dilutions were set at 1:100.

The use of ESA and SA to measure the serological response of mice experimentally infected with T. taeniaeformis (B)

Aim

In two groups of mice experimentally infected with the Belgian strain of Taenia taeniaeformis, the measurement of antibodies in

the serum was performed using ESA and SA as the detecting antigens in ELISA. These same antigens had previously been used to raise heterologous antisera in rabbits and guinea-pigs (Brandt, 1980) which were then employed as the detecting gammaglobulins in ELISA to locate circulating antigens in the serum of these mice. Additionally, the activity of an enzyme, glutamate dehydrogenase (GDH), was monitored for the duration of the infection.

Experiment design

Two breeds of mice were used in this experiment, forming the two groups. One group was 30 eight week old BKW female mice and the other group was 33 three week old CF1 female mice. Each mouse was infected with 200 eggs. The collection for serum started before infection (Day 0) then on 1, 3, 5 and 7 days after infection of the mice. Thereafter blood was collected weekly until day 49. At each blood collection, three mice were lightly anaesthetized with ether, then the blood vessels of the neck were severed with a pair of scissors and the blood allowed to collect in a universal bottle. The serum of the three mice were pooled. The livers of the killed mice were dissected out. The state of the hepatic cysts was recorded and counts of the cysts were made. A cyst was considered viable if it contained a living larva and non-viable if the larvae was dead, that is, invaded by host cells.

At the end of the experiment, all the serum samples were analysed for antigen and antibody levels and GDH activity. Antigen levels were looked for in sera samples of days 0, 1, 3, 5 and 7. The serum was diluted 1:50 for this purpose. Antibody levels were

looked for in samples of days 0, 14, 21, 28, 35, 42 and 49. The enzyme activity was monitored until day 28 of the infection.

Results

The counts and appearance of the hepatic cysts from the livers of mice are given in Table 7. The cysts were visible by day 5 (BKW mice) and day 7 (CF1 mice) after infection. The CF1 mice seemed to have a higher level of infection than the BKW mice, as there were more cysts per mouse in the former group.

Table 7 The state and numbers of cysts from the livers of mice taken at fixed times; three mice each time, after an oral infection with 200 eggs each of T. taeniaeformis (B)

Day after infection	BKW	CF1
1	No larvae visible	No larvae visible
3	No larvae visible	No larvae visible
5	Numerous viable cysts	No larvae visible
7	Numerous viable cysts	15, 22, 50 viable cysts
14	9, 25, 30 viable cysts	Numerous viable cysts
21	Numerous cysts mainly viable but a few non-viable	Numerous viable cysts
28	5 non-viable cysts, 1 viable cyst and 4 viable cysts	Numerous viable cysts
35	9 non-viable cysts, numerous viable cysts, 2 non-viable cysts	Numerous viable cysts
42	-	Numerous viable cysts
49	-	Numerous viable cysts

The enzyme assay results for GDH presented in Figure 16, show that the enzyme activity increased sharply to a maximum peak around day 5 (CF1 mice) and day 7 (BKW mice), after which the enzyme activity in sera fell steadily to regain pre-infection values.

In Figure 17, the circulating antigen level in the sera of CF1 mice increased rapidly to peak at day 5, then decreased abruptly on day 7. This was detected by antisera raised against ESA but with the SA system the antigen level was only detectable on day 5 and had decreased to a low level on day 7.

Both groups of mice gave similar antibody responses over the course of the infection period (Figure 18). The levels steadily increased from day 14 until about day 35. This occurred with both types of antigen (ESA and SA), although there appeared to be generally more antibodies reactive against SA than against ESA in the sera from the BKW mice but the reverse occurred in the sera from the CF1 group. On the whole the antibody levels were lower in the sera from the BKW mice.

The use of ESA and SA to measure the serological response of rats experimentally infected with T. taeniaeformis (M)

Aim

As in the previous experiment, the antigen and antibody levels in the serum of rats given a single oral infection of the Malaysian strain of Taenia taeniaeformis were measured.

Experiment design

Thirty three-week old female Sprague Dawley rats were divided randomly into three groups of ten. Rats in Group I were each fed with 400 eggs, those in Group II with 600 eggs and those in Group III with 800 eggs. Plasma samples were collected as described, before infection at 1, 3, 5 and 7 days after infection and thereafter at weekly intervals until week 12. The plasma samples from each rat were not pooled but were labelled and stored separately. The rats were necropsied at the end of 12 weeks and the number of living larvae in the liver of each rat was counted.

The detection of antigens and antibodies were by the ELISA, carried out after all the plasma samples collected during the 12 week period were available. For the antigen detection sera samples were diluted to 1:200.

Results

The counts of living larvae harboured in the livers of the experimental rats are given in Table 8. There seemed to be no association between the magnitude of egg dose and the number of larvae that was established.

Table 8 Number of living larvae in the livers of rats 12 weeks after oral infection with T. taeniaeformis (M) eggs at three levels

Number of eggs given	Number of live larvae per rat	Mean Number	% of dose
400	12, 14, 28, 50, 67, 124, 135, 225	82	20.5
600	3, 4, 7, 13, 14, 30, 57, 98, 326	61	10.2
800	10, 22, 25, 28, 51, 54, 179, 279, 600	138	17.2

In Figure 19, the mean levels of the antigens reacting with the ESA antisera appeared to peak around days 3 and 5, then decreased to levels below that of the pre-infection plasma (day 0) but increased again on day 42. Days 3 and 5 were the only times at which the levels were significantly above that on day 0 (two sample t-test, $P < 0.05$).

In Figure 19, the antigens reacting with the SA antisera were detected at mean levels above that of the pre-infection plasma only on days 21 and 28. However only the level on day 28 was significantly above the level on day 0 ($P < 0.05$). The pre-infection plasma (day 0) gave values which were significantly zero when assayed for the presence of circulating antigens with either ESA or SA antisera (paired t-test, $P < 0.05$).

In Figure 20, antibodies detected by ELISA using ESA and SA showed an increasing trend from week 2. The level of antibodies detected by ESA and SA were significantly higher than at day 0 beginning from weeks 3 and 4 respectively (two-sample t-test, $P < 0.05$). The level of antibodies peaked around weeks 6 and 7, decreased slightly and thereafter remained fairly steady. The sera tended to give a stronger reaction with ESA than with SA, after week 3.

The data shown in Figures 21 and 22 which were obtained using ESA and SA in ELISA respectively, were selected on the basis of dividing the rats into two groups, one in which the rats had less than 100 larvae and the other of rats which had more than 100 larvae. This was done to see if the two groups had dissimilar

humoral responses. Again, the sera seemed more reactive to ESA than SA in both groups. The results from these groups were similar in that they produced peak levels of antibodies around weeks 6 and 7. However, it was obvious that rats having more than 100 cysts produced a stronger antibody response. This was true for both types of antibodies, detected by ESA or SA. To test the strength of this relationship between number of larvae and antibody response, correlation coefficients were calculated. These coefficients were obtained by computing log (number of larvae) and ELISA values of sera at week 6. The correlation coefficients for antibodies detected by ESA and SA versus log (number of larvae) were 0.72 and 0.73 respectively. These positive correlations are highly significant ($P < 0.01$).

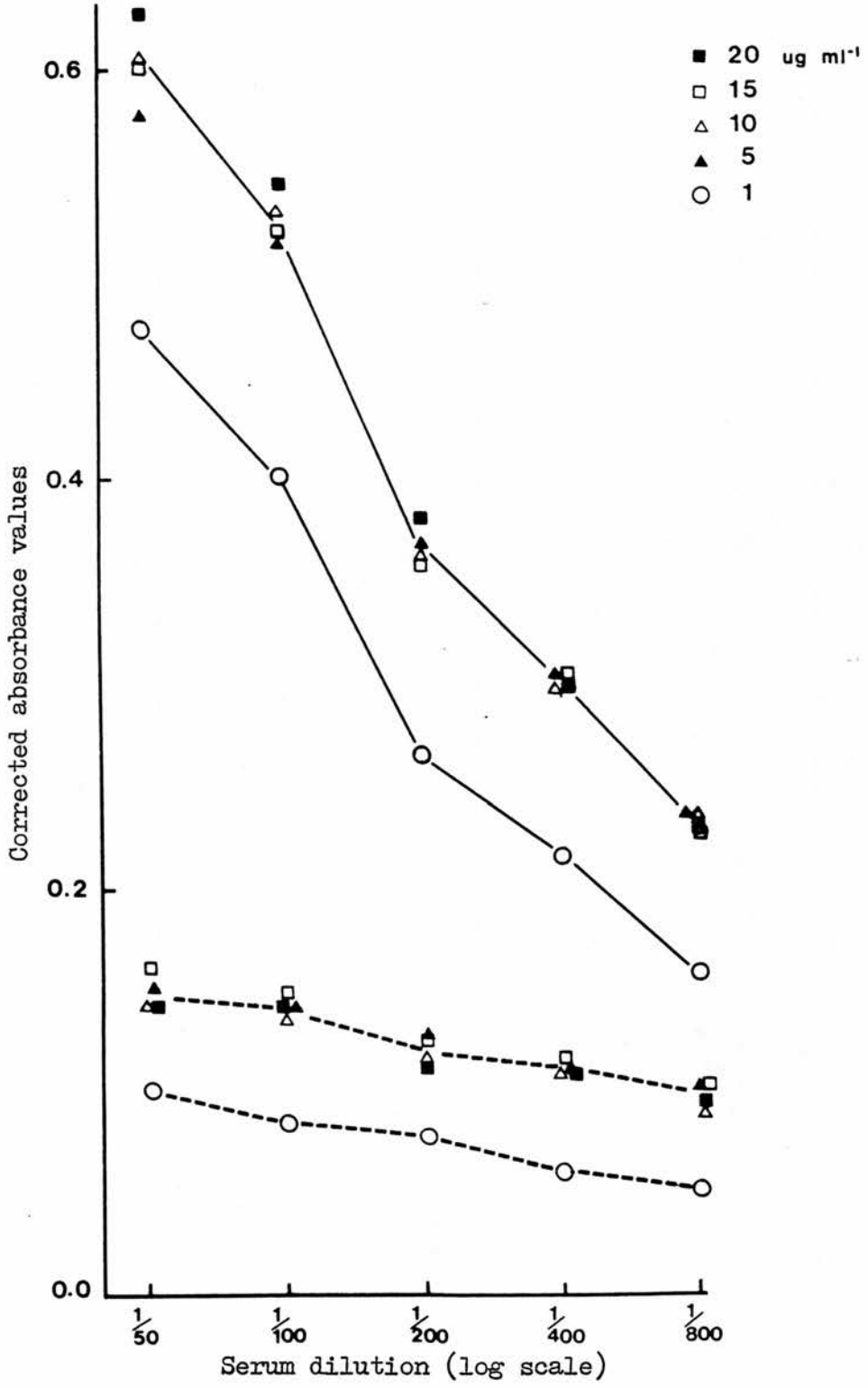

Figure 12 Titration of mouse sera against five concentrations of ESA soluble protein by ELISA. The unbroken lines represent positive serum and the broken lines represent negative serum.

For the corrected absorbance values see Appendix Table 9.

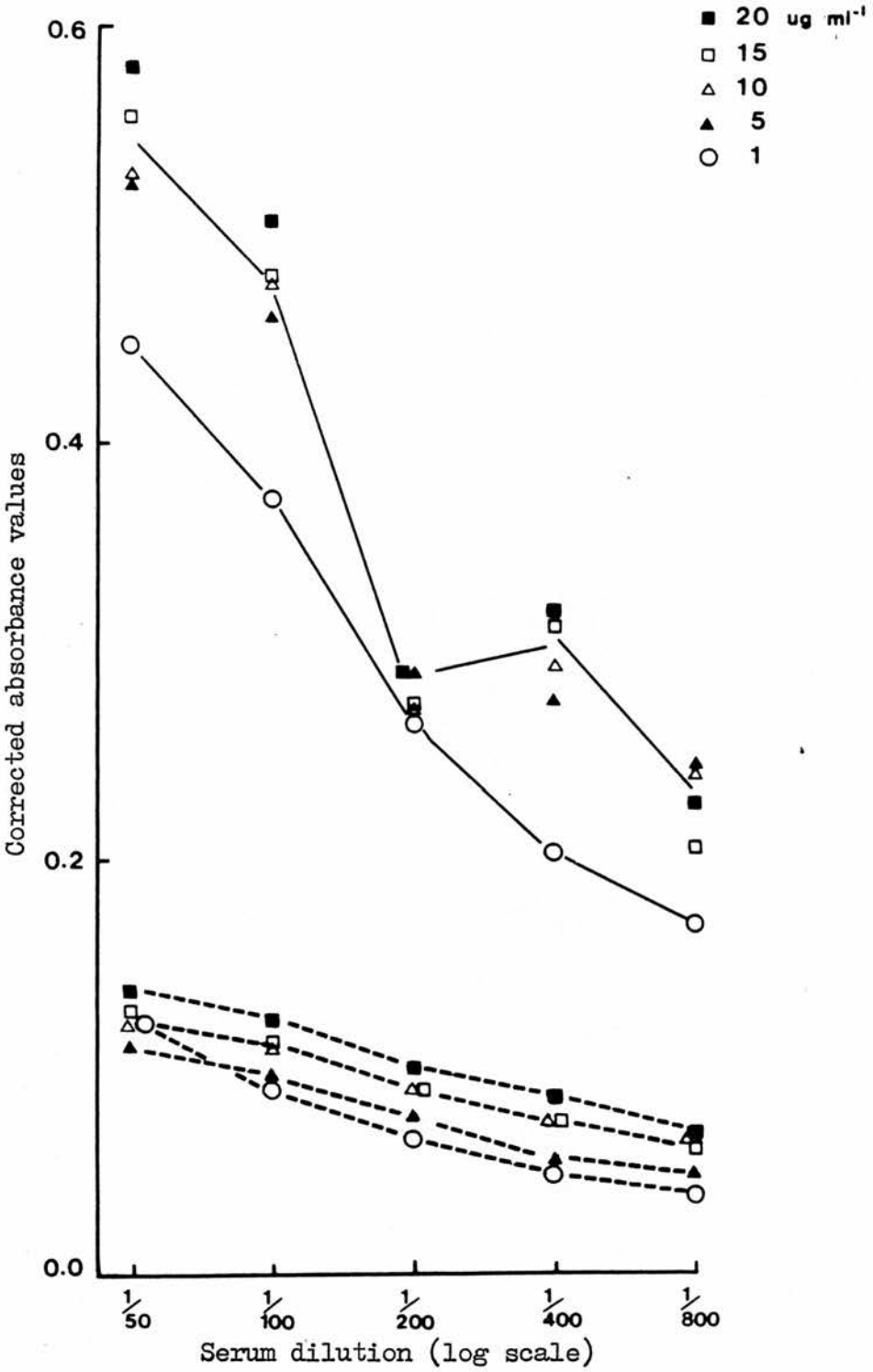

Figure 13 Titration of mouse sera against five concentrations of SA soluble protein by ELISA. The unbroken lines represent positive serum and the broken lines represent negative serum. For the corrected absorbance values see Appendix Table 9.

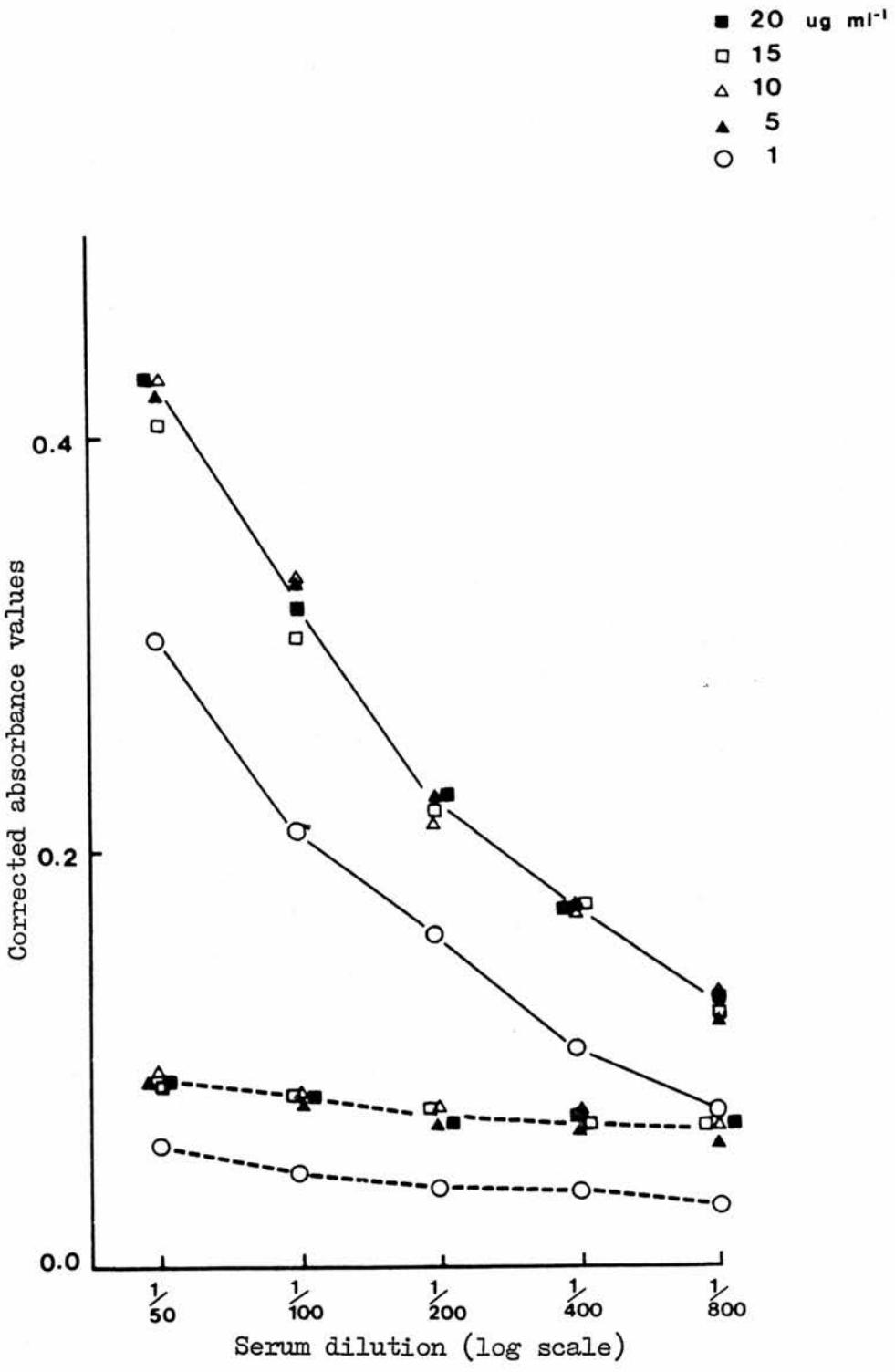

Figure 14 Titration of rat sera against five concentrations of ESA soluble protein by ELISA. The unbroken lines represent positive serum and the broken lines represent negative serum.

For the corrected absorbance values see Appendix Table 10.

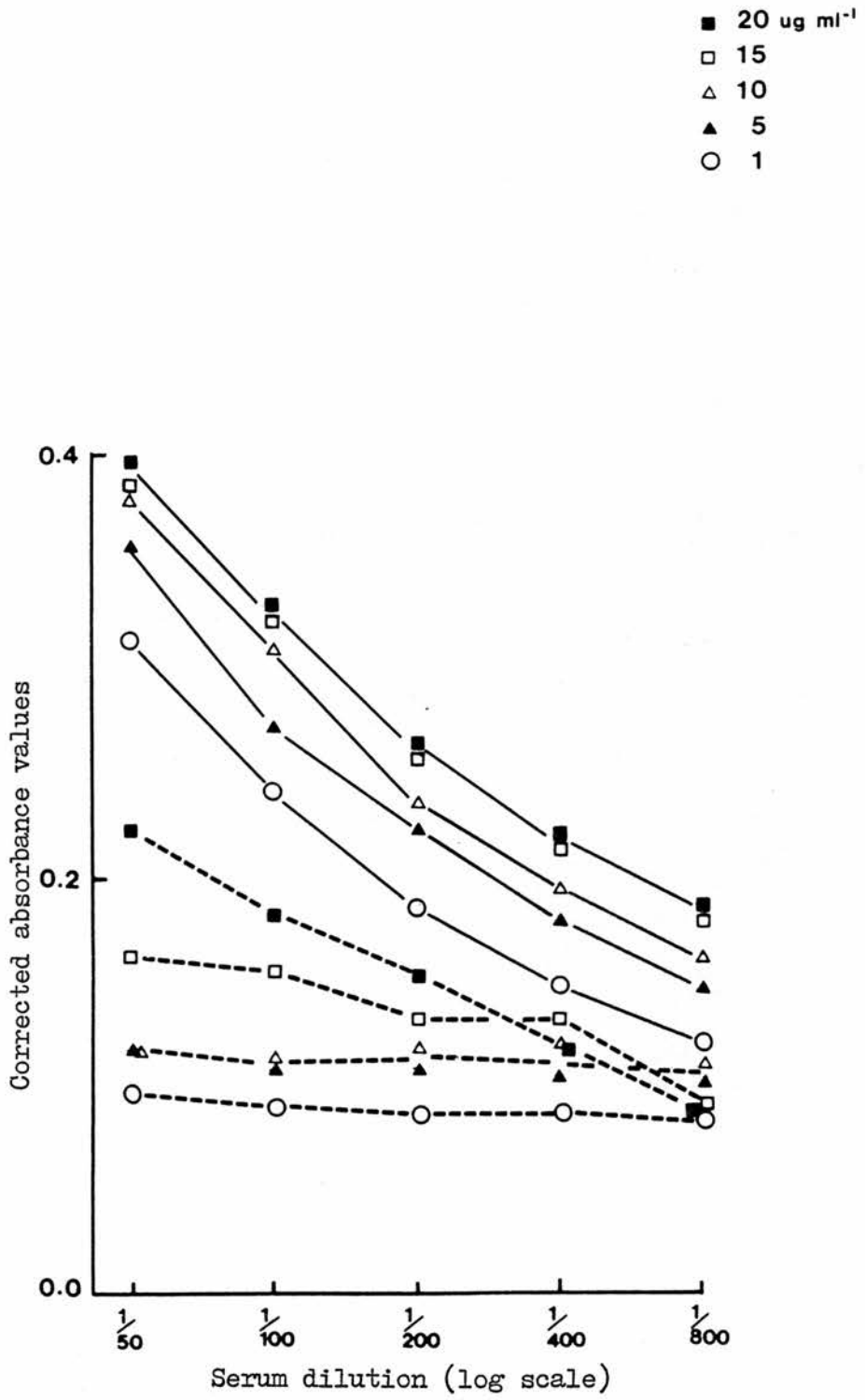

Figure 15 Titration of rat sera against five concentrations of SA soluble protein by ELISA. The unbroken lines represent positive serum and the broken lines represent negative serum.

For the corrected absorbance values see Appendix Table 10.

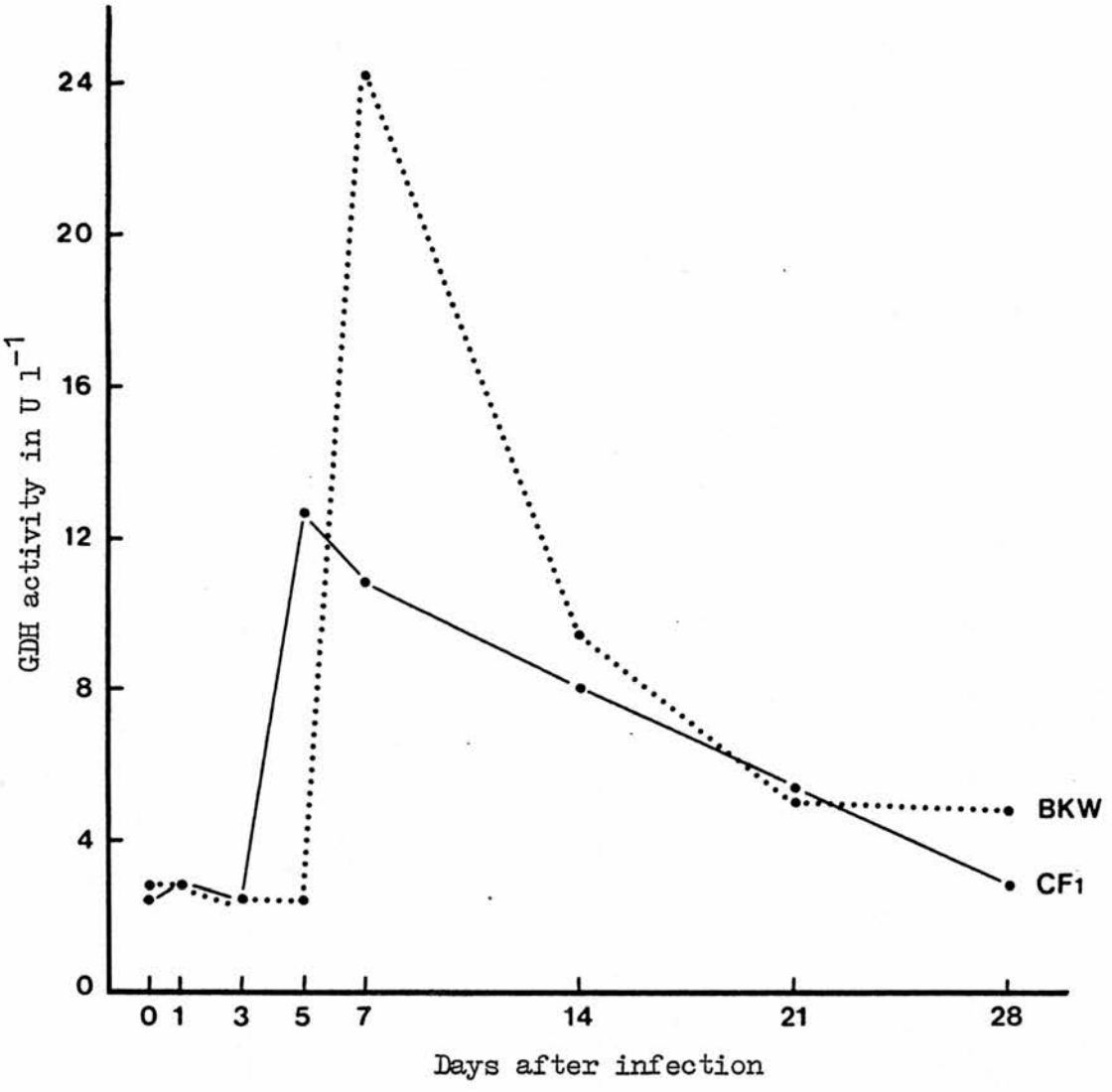

Figure 16 Serum glutamate dehydrogenase levels in two groups of mice (BKW and CF1) infected with T. taeniaeformis (B). For the GDH values see Appendix Table 11.

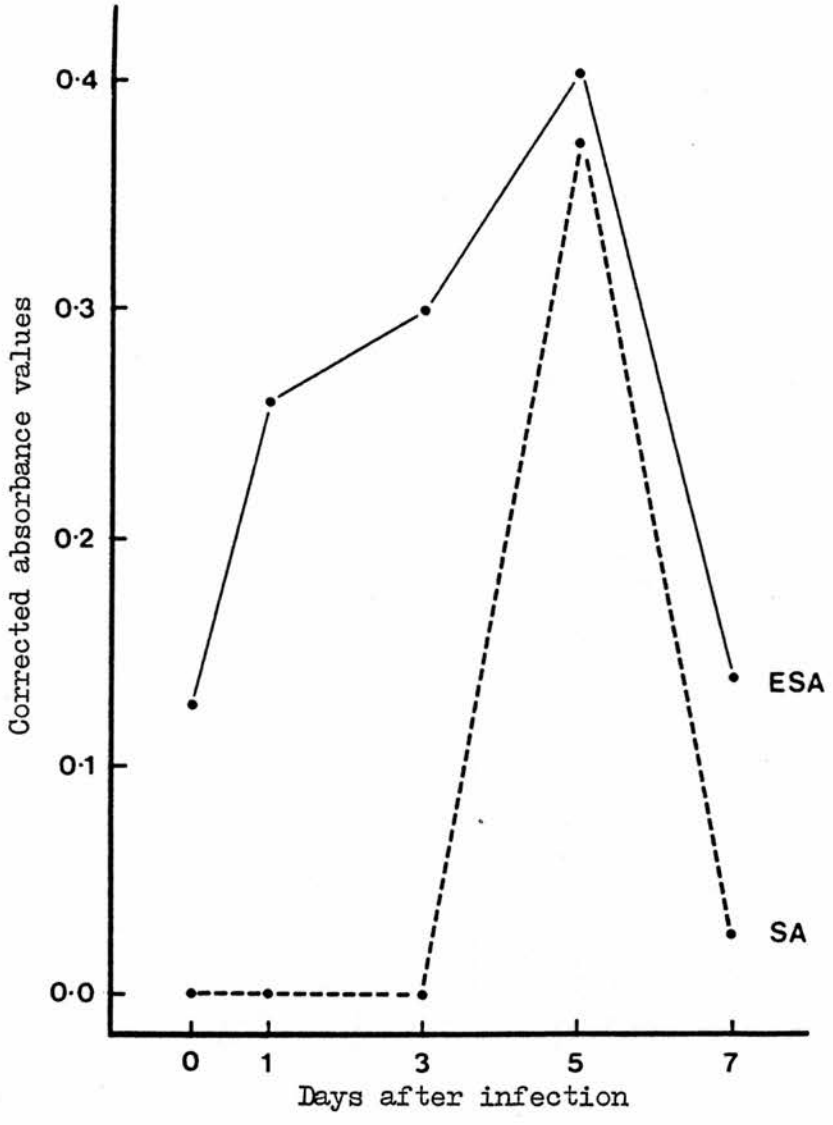

Figure 17 Absorbance values obtained by ELISA when detecting antigens in the sera of CF1 mice infected with T. taeniaeformis (B), using antisera raised against ESA or SA.

For the corrected absorbance values see Appendix Table 12.

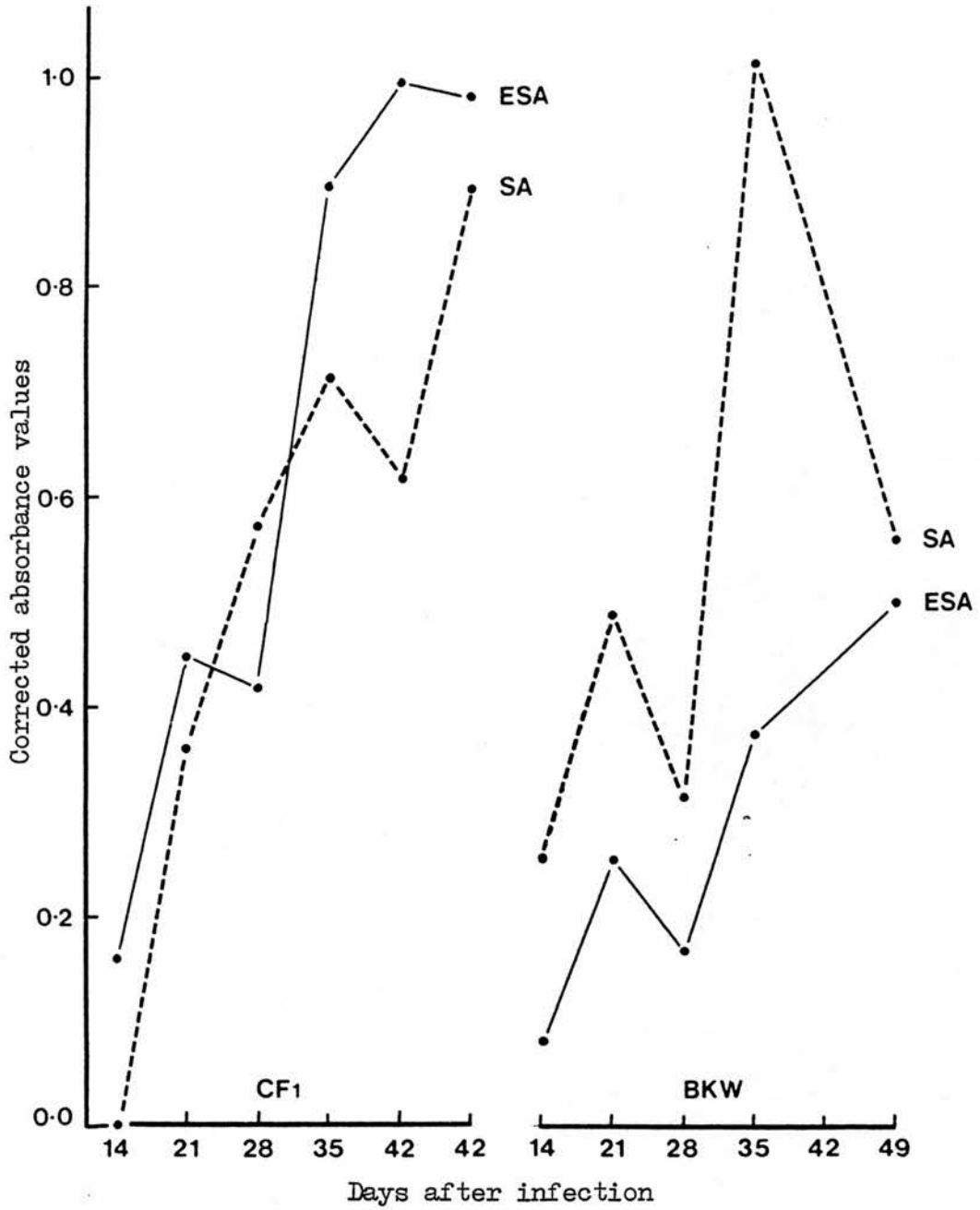

Figure 18 Absorbance values obtained by ELISA when detecting antibodies in the sera of two groups of mice (BKW and CF1) infected with T. taeniaeformis (B) using ESA and SA.

For the corrected absorbance values see Appendix Table 13.

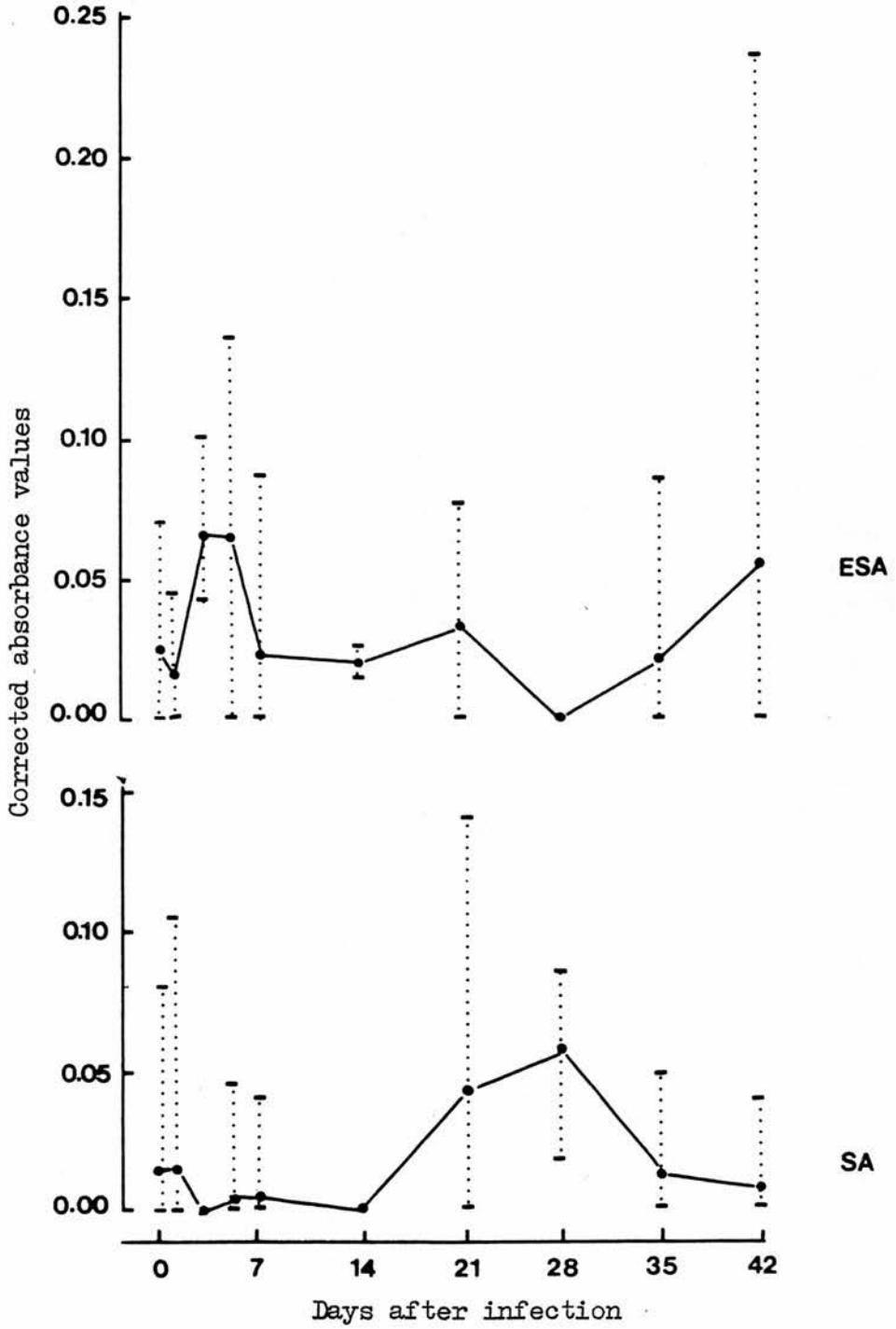

Figure 19 Mean absorbance values obtained by ELISA when detecting antigens in the plasma of rats infected with T. taeniaeformis (M), using antisera raised against ESA or SA. The bars represent the maximum and minimum absorbance values. For the mean and range of corrected absorbance values see Appendix Table 14.

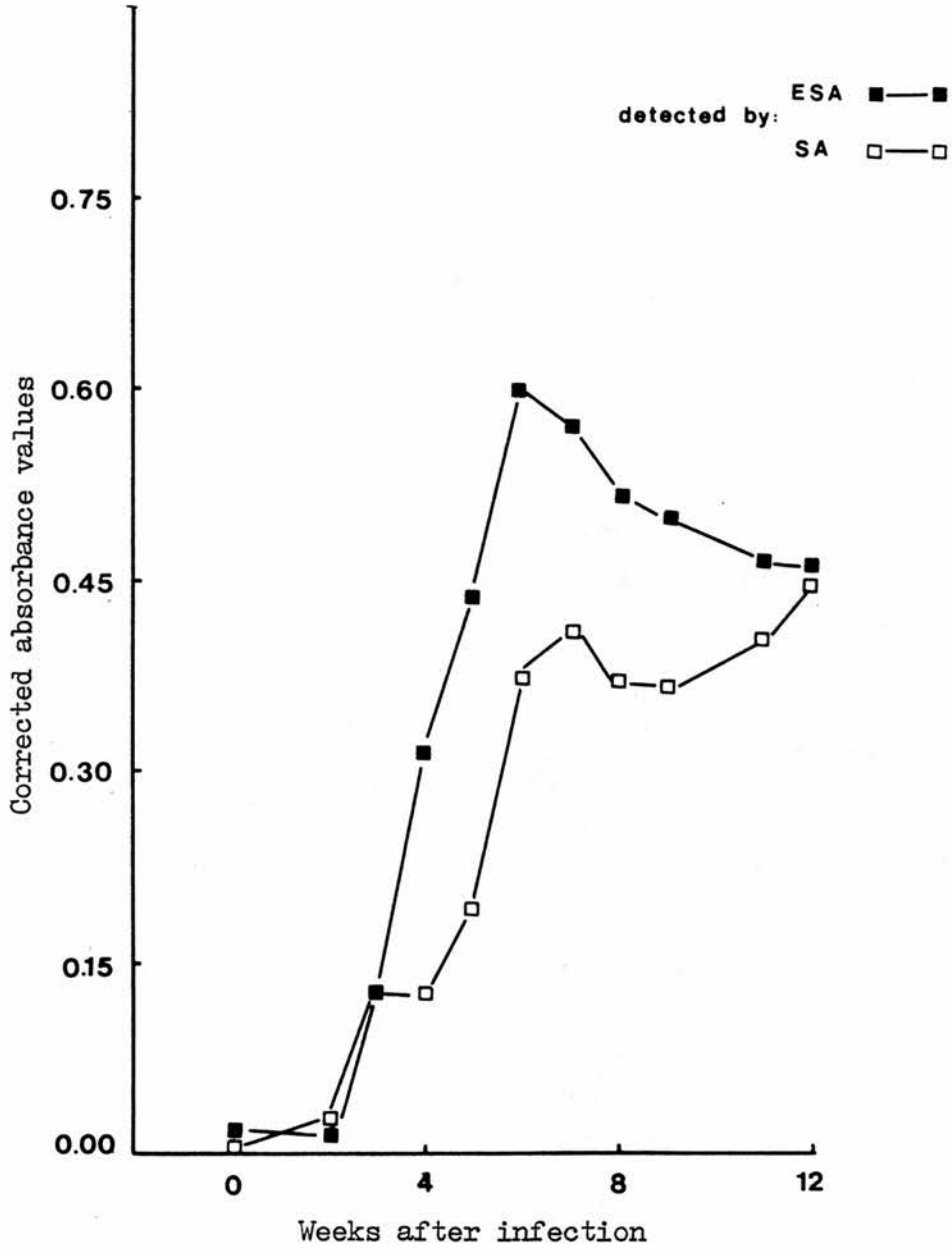

Figure 20 The mean antibody response of rats infected with T. taeniaeformis (M), detected by ELISA using ESA and SA.

For the corrected absorbance values of individual rats see Appendix Tables 15 and 16.

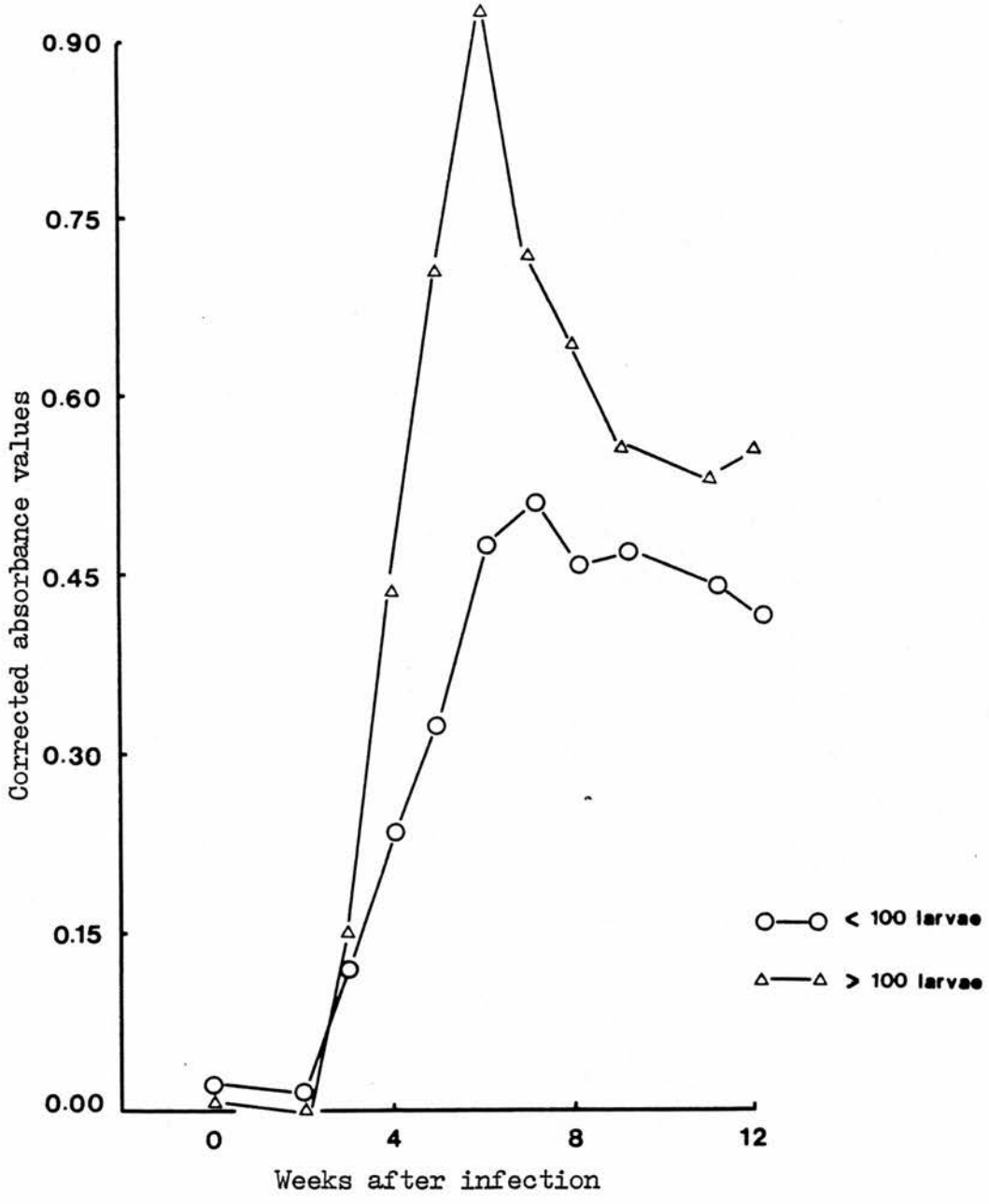

Figure 21 The mean antibody response of rats with light (< 100 larvae) and heavy (> 100 larvae) infections, detected by ELISA using ESA.

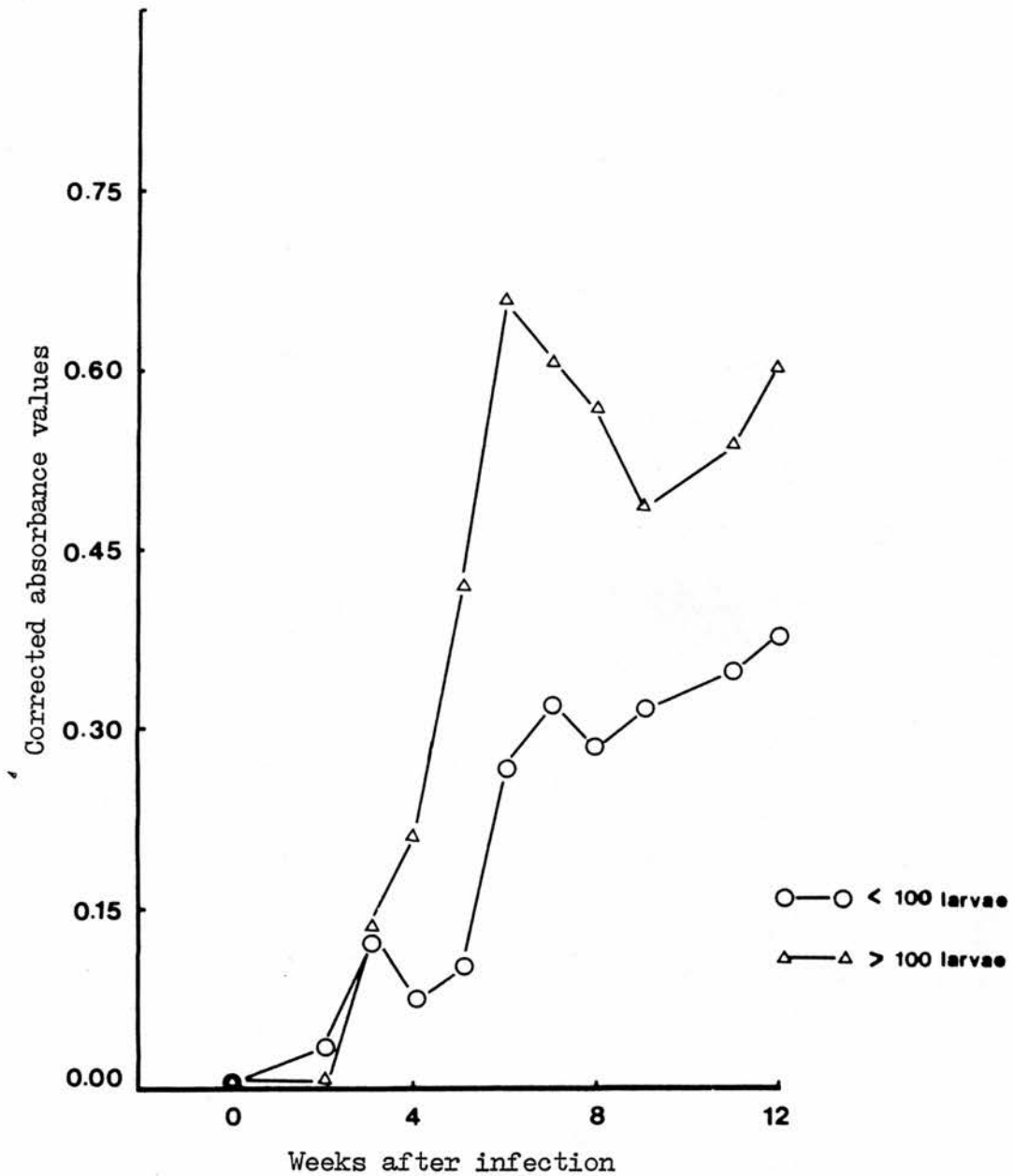

Figure 22 The mean antibody response of rats with light (< 100 larvae) and heavy (> 100 larvae) infections, detected by ELISA using SA.

CHAPTER 8

DISCUSSION

Glutamate dehydrogenase

Bullock and Curtis (1924) studied the cellular reaction about developing T. taeniaeformis larvae. They observed inflammatory and necrotic processes in the first week and maximum activity of host cell proliferation eight to ten days post-infection, after which the active processes gradually subsided. Lewert and Lee (1955), Banerjee and Singh (1969) and Banerjee (1972) also observed a similar sequence of events surrounding the cysts in rat liver. Lewert and Lee found that during the early period of development, the parasite produces a collagenase-like enzyme which softens and disrupts surrounding liver cells to facilitate absorption of nutrients. Older larvae in turn showed a decreased production of this enzyme. The GDH activity observed with the mouse serum in this study is consistent with these authors' observations, for this enzyme is known to be elevated in serum when there is leakage from damaged liver cells.

Boyd (1962) obtained a GDH value of 9.2 ± 3.2 U l^{-1} for normal rat serum. He also found that though GPT (glutamate pyruvate transaminase) and GDH were active in the liver, the former was the more sensitive indicator of liver necrosis in the rat. Thus it might have been better to have monitored this enzyme.

Egg dose and infection

In this study, there seemed to be no correlation between the numbers of eggs administered and the number of cysts that developed.

Hinz (1962), Weinmann (1970) and Mitchell, Rajasekariah and Rickard (1980) working with T. taeniaeformis infection in rats and mice found an inverse relationship existed between these two parameters. They suggested that high egg doses lead to an early accelerated immune response which is prejudicial to the establishment or subsequent survival of the larvae in the liver. Flisser, Perez-Montfort and Larralde (1979) suggested a tendency towards a linear relationship between the number of eggs administered and the number of established cysticerci, though a very low average percentage efficiency of establishment of 1% was quoted.

The range of egg doses and the interval between egg doses used to produce the infection probably determines, to a large extent, the relationship to be found between egg dose and infection. If the interval between the egg doses is too small, the effects of dose size on establishment cannot be seen clearly. Perhaps this was the case in the experiment with rats. The rat host in this study exhibited a wide range of susceptibility to infection, probably due to it being an outbred strain.

Detection of antigens

In the mouse model, the apparent transient increase in ESA and SA around days three and five after infection may indicate larval material released into the circulation by dead or dying larvae.

In the rat model, where the period of antigen detection was extended, ESA was again prominent very early during the infection after which time diminishing levels were detected. However, the

ESA levels appeared to increase again by day 42. This may mean that these later antigens are those being released by established developing larvae. The SA levels were only elevated around days 21 and 28. Attempts to correlate worm burden with circulating ESA or SA proved futile in both mice and rats.

Harrison (1978) was able to detect circulating antigen in cattle experimentally infected with Taenia saginata for a much longer period of time. The high dilution of sera (1:200) used in the present work must have decreased the amount of antigen to such an extent as to be undetected. This high dilution was unavoidable because of the small amount of sera available, caused by the following problems. Firstly, the difficulty of collecting blood early in the infection (days 1-7) due to the frequent 48 hourly bleedings, which was compounded by the small size of the rats at this stage. Secondly, the sera was required for both antigen and antibody analysis and so had to be sub-divided into small quantities. Thirdly, the ELISA for detecting antigens was unsuccessful at the first attempt which wasted the allocated amount of sera. Thus a second attempt had to be on minute amounts of the remaining sera.

Detection of antibodies

Antibody levels in both rats and mice, as detected by either antigen, gave a similar pattern. Both antigens were able to detect rising antibody levels from week three (ESA) and week four (SA) onwards. Kwa and Liew (1978) using a passive haemagglutination technique detected antibody to a somatic extract of T. taeniaeformis by four to five weeks after an experimental infection. The sharp

rise in antibody levels after this time observed by these authors, was similar to findings in this work. This sharp increase in antibody levels after week two is probably in response to the increased release of antigens from migrating oncospheres and early stages of developing larvae which had succumbed to the host's immune mechanism, as well as antigens released by the surviving larvae. The fact that ESA appeared to be more sensitive than SA in detecting the humoral response after week three, is probably because the antibodies being produced are increasingly the result of the persistent release of metabolic antigens by living established larvae, whereas the antibodies reacting to SA are mainly stimulated by antigens present over a short period appearing from the non-surviving early forms.

Other workers who have noticed that ESA or similar metabolic products are more sensitive than SA in the serodiagnosis of other infections include Sadun and Norman (1957) and Slanga and Ivey (1962) with Trichinella spiralis; Sadun, Schoenbechler and Bentz (1965) with Schistosoma mansoni; and Harrison (1982) with Taenia saginata. Additionally, Kagan and Oliver-Gonzalez (1958) and Anderson, Sadun, Rosen, Weinstein and Sawyer (1962) observed fewer cross-reactions with an ESA than when using SA to diagnose Schistosoma infection and human oesinophilic meningitis (Angiostrongylus cantonensis) respectively.

There was no correlation between the numbers of eggs administered and humoral response, which agrees with the findings of Kwa and Liew (1978). However the highly significant correlation

between the antibody level and number of established larvae suggests that concentration of antibodies reacting to ESA and antibodies reacting to SA may be related to the amount of antigen released. Morris, Proctor and Elsdon-Dew (1968) failed to relate parasite burden with either precipitin or hemagglutination titres, when working on Taenia solium infection. Their view was that the complexity of antigens used negated any trend. However, Rickard and Outteridge (1974) found that in active infections of Taenia pisiformis, the number of cysts present in rabbits at post-mortem was directly related to lymphocyte reactivity in the blood.

To relate the findings of Hinz, Weinman and Mitchell et al. with this work, it appears possible that the higher number of cysts survived because the low egg dose caused only a weak early immune response, not sufficiently adverse to the survival of the majority of larvae. However, the large number of surviving larvae per host produced a persistent later high titre immune response. This high titre must either consist of antibodies which do not harm the parasite or more likely came about after the parasite had developed its evasive immune mechanism. Campbell (1938a, b) called this phenomenon "early" and "late" immunity. Presumably, the opposite sequence of events was occurring in the hosts harbouring the lower level infections, after heavier infective doses.

PART III

Immunisation of rats with antigens derived from metacestodes of *Taenia taeniaeformis*

CHAPTER 9

INTRODUCTION

The world-wide prevalence, public health hazards and socio-economic burden on the livestock industry arising from taeniid disease makes control very desirable. These aspects of taeniid infections have been lately reviewed by Arundel (1972), Pawlowski and Schultz (1972), Soulbsy (1975) and Grindle (1978).

Gemmell (1978) discussed the various options for controlling hydatidosis and cysticercosis. One of the options for controlling the larval population is to prevent infection by immunisation. For active immunisation of the intermediate hosts, the types of vaccines used may be living - normal or irradiated infective larvae, or non-living - including somatic antigens or excretory-secretory antigens. In each case these may be derived from the homologous or heterologous species. It is these non-living vaccines which have been studied in the following immunisation experiments.

There are numerous publications regarding the protective value of these two types of antigen. These have reported variable results in the success of their vaccines. Since this variation may be due to different techniques in vaccine production, an attempt was made to clarify the immunogenicity of the antigens produced for the study.

REVIEW OF THE LITERATURE

Somatic antigens

Miller (1931, 1932) and Campbell (1936) showed that extracts and homogenates of adult and larval T. taeniaeformis may confer substantial immunity in rats to a challenge infection. Interest in this type of antigen however waned afterwards as there was little success in other model systems (Clegg and Smith, 1978). Gemmell's experiments (1964, 1965, 1966, 1969a, 1969b) appeared to demonstrate the superiority of viable parasite material over killed material for the stimulation of resistance to metacestodes in intermediate hosts. Forthcoming evidence however proved that non-living parasite antigens would confer a high degree of protection against parasite infections (Subrahmanyam, 1981).

In the 1970s, work carried on with somatic extracts of T. taeniaeformis also proved successful (Kwa and Liew, 1977; Ayuya and Williams, 1979; Lloyd, 1979 and Rajasekariah, Rickard and Mitchell, 1980b).

A high level of immunity was achieved by vaccination with sonicated oncospheres from both T. taeniaeformis (Rajasekariah, Mitchell and Rickard, 1980a and Rajasekariah et al., 1980b) and T. saginata (Rickard and Brumley, 1981) against a subsequent challenge infection in mice and calves respectively. The oncospheral products found to stimulate the highest level of immunity were the ultracentrifuged pellet and uncentrifuged, sonicated oncospheres. Hence, these workers suggested that the antigen stimulating a protective immunity may be membrane associated or particulate in nature since the centrifugal force seemed pertinent. They suggested

that sonication caused only partial solubilisation. Rajasekariah et al. (1980b) and Rickard and Brumley (1981) discussed the particulate nature of the antigens in relation to these also being secretory. They referred to other workers (Lethbridge and Gijsbers, 1974) who had shown that the secretions from the penetration glands of oncospheres of Hymenolepis diminuta are contained in a membrane.

Rajasekariah, Rickard, Mitchell and Anders (1982) proceeded to solubilize oncospheres with sodium deoxycholate prior to, and during sonication. The 100,000 g supernatant fluids of the sonicated, solubilized product stimulated a high degree of resistance against infection to T. taeniaeformis in mice to a degree approaching that in mice vaccinated with the crude sonicate. The corresponding supernatant of the non-solubilized sonicated product when used to vaccinate mice, resulted in five times more the mean number of larvae compared to the solubilized supernatant, though remained significantly lower compared to the controls. The results of this study supports the hypothesis of a particulate protection-inducing antigen in the larvae of T. taeniaeformis.

Excretory-secretory antigens

Weinman (1970) stated that observations with living material generally indicated that the antigens most functional in eliciting protective immunity are associated with the more actively metabolizing stages, including the active oncosphere. This is the basis for workers introducing or implanting live parasites into the host animals to stimulate immunity (Rickard and Bell, 1971a and Musoke and Williams, 1976). The speculation was that the live

parasites would release excretions and secretions which play a role in protective immunity. With the advent of improved *in vitro* culture techniques for oncospheres, workers have explored the possibility of collecting metabolic or ES antigens, using these products as vaccines instead of the live organisms directly.

Rickard and Bell (1971b) demonstrated the immunising capacity of metabolic substances when ES antigens collected from *in vitro* culture of oncospheres were effective in immunizing sheep against infection with *T. ovis*. High levels of immunity induced with oncospheral ESA vaccines against *T. saginata* and *T. ovis* infections were reported by Rickard and Adolph (1976 and 1977 respectively), by Lloyd (1979) and Rickard and Brumley (1981) against *T. saginata* infection and Heath (1976) against *T. pisiformis*.

There were however also reports regarding the failure of oncospheral ESA to protect against *T. saginata* infection (Wikerhauser, Brglez, Dzakula, Asaj and Matic-Piantanida, 1978 and Mitchell and Armour, 1980).

Lloyd (1979) used oncospheral ESA while Kwa and Liew (1977) and Ayuya and Williams (1979) used ESA of mature metacestodes to successfully protect mice and rats against a challenge infection with *T. taeniaeformis*.

Rajasekariah *et al.* (1980a, 1980b) utilised ESA from oncospheres, immature and mature metacestodes of *T. taeniaeformis* from mice and found that all stages would confer protection to mice against infection. Here, as with the SA, the centrifugal force

used in preparing the vaccines played an influencing role, as the supernatants of used media from the oncospheres and metacestodes centrifuged at 3500 g and 4500 g respectively were either only marginally host-protective or failed to induce immunity. These findings suggested that functional antigens in both oncospheral or metacestode ESA are membrane bound or particulate, a situation similar to that in SA.

As a contribution to their evasive or survival mechanism, parasite products have been shown to interfere non-immunologically with the host defence system. Hammerberg and Williams (1978) demonstrated that factors present in the cyst fluid of metacestodes of T. taeniaeformis and in their in vitro products were able to deplete complement levels in the serum of normal rats. Hammerberg, Dangler and Williams (1980) extracted materials from the surface of these metacestodes and found that these materials were not only capable of consuming complement but also inhibited coagulation. These authors identified the surface materials as a highly sulphated polysaccharide or proteoglycan. The observations suggest that the products from T. taeniaeformis if released in vivo may constitute an important evasive mechanism for parasite survival.

CHAPTER 10

MATERIALS AND METHODS

Experimental animals

Three to four week old Sprague-Dawley rats were used. Five to ten of these rats were usually allocated for each treatment, referred to as a group.

Antigens

The non-living antigens, ESA and SA, were derived from the mature metacestodes of T. taeniaeformis strain B, and produced as described in Parts I and II respectively. In brief, the ESA was the supernatant of serum-free used medium centrifuged at 1000 g for 15 minutes and the SA was the supernatant of larval homogenate ultracentrifuged at 100,000 g for 30 minutes.

The SA had a protein content of 2-8 mg soluble protein per ml, depending on the number of larvae used during the extraction procedure. Because of this high protein content, SA was usually used neat and was even occasionally diluted for use as a vaccine.

ESA, however, had a very low protein content of about 50-100 µg per ml. Therefore ESA was concentrated before use as a vaccine. Two methods of concentration were employed, namely lyophilisation and dialysis.

Lyophilisation was carried out on a freeze-dryer ("10-140 BA MDC Macro dry ice Mobile", Virtis). For this 50 ml or 25 ml samples were evenly shell-frozen on the wall of round-bottomed 500 ml or 250 ml flasks respectively. Shell-freezing was done by rapidly

rotating the flasks in a mixture of isopropylene alcohol (BDH) and solid CO_2. Sublimation was carried out at about 0.5 PA over about six hours. The lyophilised powder was redissolved in sterile PBS to a tenth of its original volume and stored at -20°C.

Dialysis was performed for three to four hours with the ESA in Visking tubing cellulose membrane (Scientific Suppliers), against a solution of polyethylene glycol ("Carbowax 6000" BDH). The whole procedure was kept at 4°C. The tubing retains molecules in excess of about 20,000 molecular weight.

Protein estimation by measuring the extinction at 260 nm and 280 nm

This method was developed by Warburg and Christian in 1941 and is described in "Data for Biochemical Research" by Dawson, Elliott, Elliott and Jones. By this method the phenylalanine, tryptophane and tyrosine content of the sample is measured but not total protein. The technique, however, is rapid, simple to perform and non-destructive to the sample.

The extinction of an appropriately diluted protein solution was measured at both 260 nm and 280 nm on a Pye Unicam spectrophotometer SP1800. The ratio E280/E260 was measured. Using this ratio, a factor for calculating the protein concentration could be read off from a standard table. The protein concentration was estimated using the following formulae:

$$\text{Protein concentration (mg/ml)} = E280\ \text{nm} \times F \times {}^{1}/_{d} \times D$$

where F = the factor mentioned above
d = length of the light path in cm (0.2)
D = dilution used

Immunisation

The antigens were administered intraperitoneally in three divided doses given every 48 hours. The amount injected per rat was assessed on the basis of the soluble protein content. For the first two experiments, the protein content of ESA was measured by the Bio-Rad protein assay and that of SA by the Warburg and Christian method. In subsequent experiments, the protein content of both antigens was usually measured by the Bio-Rad assay. This was done so as to equalize, as far as possible, the amount of protein for each antigen.

In experiments 1 and 2, the amount of ESA and SA given per rat was 1500 μg and 4200 μg soluble protein respectively. These amounts were chosen because they had produced the best results in previous experiments (Brandt, 1980). In subsequent experiments in an attempt to standardize conditions, 1500 μg of protein was administered for all antigens.

Challenge infection

The challenge infection was given by mouth as described in Part I. In all except experiment 1, a fixed number of "viable" eggs were given. The viability was assessed from the proportion which hatched and activated *in vitro* by the technique derived from that of Brandt and Sewell (1981b). The modifications to this technique were the use of "Chloros"(ICI) as a source of hypochlorite and the use of 30 seconds agitation on a Whirlimixer (Fisons) in place of agitation with a Pasteur pipette. The total number of eggs given was then the number of viable eggs desired divided by

the proportionate viability.

In all experiments, except experiment 2, the time interval between the start of vaccination and challenge was three weeks.

Necropsy and interpretation of results

The rats were killed with ether eight weeks after challenge. The liver was dissected out and the number of cysts containing living larvae were counted.

Either the students' t-test or a Wilcoxon two-sample test whichever was appropriate, was used for statistical analysis of the number of living larvae. The non-parametric Wilcoxon test was used where there were many null counts in the data. The t-test was not applicable in these cases because the normality assumption of the test was not satisfied, even after transformation of the data.

CHAPTER 11

EXPERIMENTS

Aim of experiments

The initial studies were designed to investigate the immunogenicity of ESA and SA. The ESA employed was a serum-free used medium in each case. The variables studied were the dose for the challenge infection and the time interval between immunisation and challenge. Because of the results published by other workers during the period of study, attention was later also given to the insoluble fractions which formed the "residues" of the method used to obtained soluble SA from larval material.

All experiments included sham vaccinated (with unused medium or saline) and challenged controls.

Immunisation of rats with ESA or SA followed by different levels of challenge infection

Experiment design

Forty-five three-week old female rats were randomly divided into three groups of 15. Rats in one group were each vaccinated with 1500 µg soluble protein of ESA, in another group with 4200 µg soluble protein of SA and in the remaining group with unused medium.

Within each group, the rats were then further divided into three sub-groups of five. Three weeks after vaccination, the sub-groups for each treatment were challenged with different doses of eggs. Rats in the three sub-groups were each given 30, 90 or 270 viable eggs respectively.

The ESA was concentrated by lyophilisation and the unused medium (for the control group) was lyophilised to the same extent.

Results

The counts of living larvae in the livers of the rats at necropsy are presented in Table 9. Transforming the data by log (y + 1) where y represents the larval counts, Figure 23 was plotted showing the mean transformed larval count of each group against log (egg dose). The SA sub-group challenged with the 90 egg dose was excluded from the statistical analysis because it appeared to give an anomalous result - zero counts.

Table 9 The numbers of living larvae in rats immunised with ESA or SA and from control rats, challenged 3 weeks later with 30, 90 or 270 viable eggs

	Number of viable eggs in challenge infection	Number of living larvae per rat
Sham	30	1, 2, 4, 5, 6
	90	2, 9, 10, 10, 20
	270	5, 17, 28, 54, 135
ESA	30	0, 0, 1, 1, 2
	90	0, 0, 3, 11, 19
	270	0, 2, 5, 29, 30
SA	30	0, 0, 1, 2, 3
	90	0, 0, 0, 0, 0
	270	1, 1, 2, 14, 132

The regression coefficients of the mean transformed larval counts on log (egg dose) for ESA, SA and Sham treatments were 0.66, 0.63 and 0.90 respectively (standard error of difference, SED = 0.458). These regression coefficients are not significantly

different and three parallel lines were fitted using the average slope of 0.73 (SED = 0.187). This indicates a relationship of the form

$$(\text{number of larvae}) = k\ (\text{egg dose})^{0.7}$$

suggesting that percentage infection defined as the ratio of number of larvae to egg dose, decreases with egg dose, i.e.

$$\text{percent infection} = k\ (\text{egg dose})^{-0.3}$$

The efficacy of the vaccines is demonstrated by the vertical distance between the line for sham and the other two lines, representing a reduction in the constant of proportionality k of the above relationship for the two vaccines relative to the control. The estimated vertical distance between the SA line and the sham line is 0.44 (SED = 0.200) and between the ESA line and the sham line is 0.47 (SED = 0.200). In both cases a one-tailed t-test shows significance at $P < 0.01$. This size of difference on the logarithmic scale corresponds to a reduction of the constant k by a factor of nearly 3, i.e. at any given egg dose the effect of either vaccine is to reduce the number of resulting larvae by a factor of about 3. Clearly there is no significant difference between the effects of SA and ESA.

For subsequent experiments, 100 viable eggs per rat was considered an optimal number for challenge infections. With this number of eggs, the number of cysts to be counted in the control group was kept to manageable levels and there were fewer failures to infect the controls.

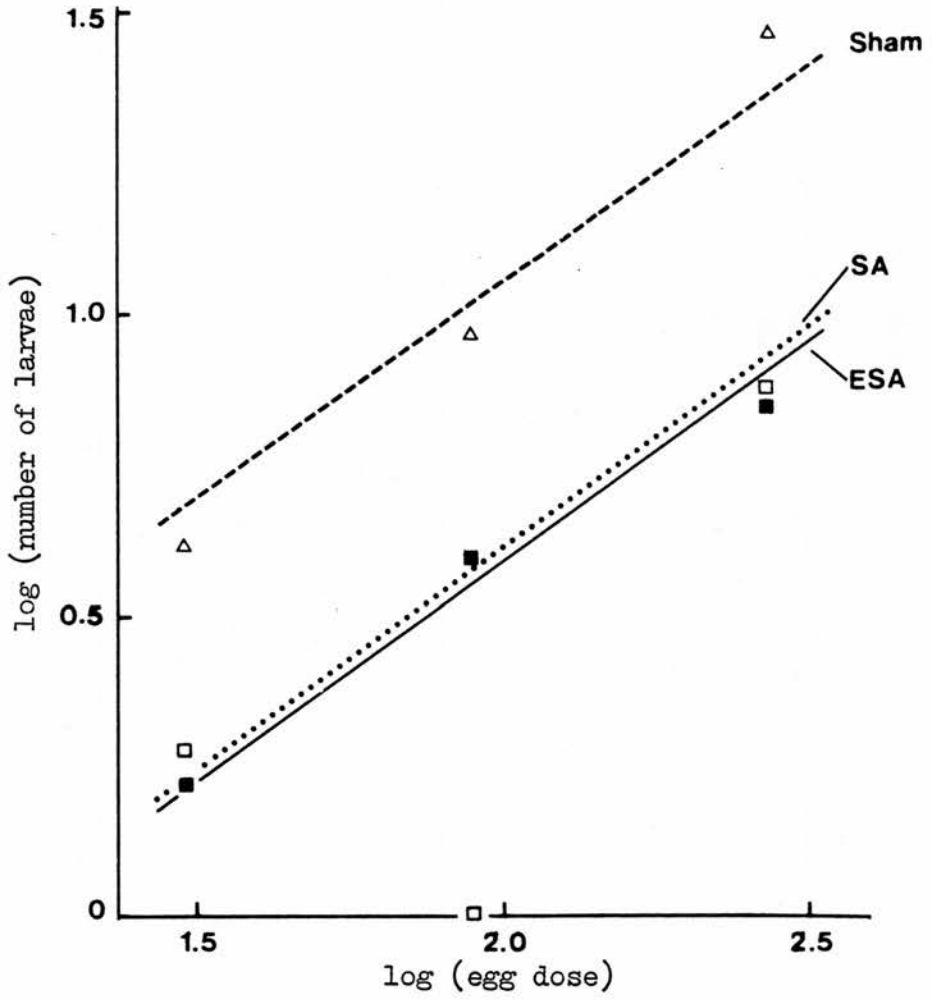

Figure 23 The relationship between the number of eggs in the infecting dose and the number of metacestodes developing in control and vaccinated rats. The slopes of the regressions on this data, excluding the anomalous zero for the group vaccinated with SA and given 90 eggs, did not differ significantly. The lines shown are those giving the best fit for each group with the pooled slope.

The lyophilised ESA presented difficulties, in that the vaccine appeared to be painful for the rats probably because it was very hypertonic. Six rats which died within a few hours of vaccination were immediately replaced. On necropsy, death was found to be associated with marked ascites. It appeared that the high concentration of salts in the concentrated vaccine caused this rapid movement of fluid from the surrounding tissues into the peritoneum. This led to the later use of dialysis for concentration of this type of vaccine in most cases.

Immunisation of rats with ESA or SA prior to challenge at different time intervals

Experiment design

The protocol of this experiment was similar to the previous experiment. Rats in the sub-groups were however challenged with a fixed number of viable eggs (100) given at different time intervals between vaccination and challenge. These intervals were 3, 5 and 7 weeks post-vaccination.

The ESA and unused medium for the sham-vaccinated group were both concentrated by dialysis to the same extent.

Results

Table 10 shows the number of living larvae counted from the livers of rats at necropsy. Adopting the same transformation as for the previous experiment, the transformed means for time intervals within each treatment (i.e. ESA, SA or sham) were compared but none of the differences were significant at the 5% level.

Table 10 The numbers of living larvae in rats immunised with ESA or SA and from control rats, challenged 3, 5, or 7 weeks later with 100 viable eggs

	Number of weeks between immunisation and challenge	Number of living larvae per rat
Sham	3	0, 1, 5, 8, 34
	5	1, 4, 5, 9, 14
	7	0, 3, 5, 5, 10
ESA	3	0, 2, 2, 57
	5	0, 0, 0, 1, 6
	7	0, 0, 1, 3, 5
SA	3	0, 0, 1, 4, 9
	5	0, 0, 1, 3, 5
	7	0, 3, 5, 4, 10

Comparing pooled means for the Sham, ESA and SA groups; there were significant reductions in the larval counts in the SA ($P < 0.10$) and ESA ($P < 0.05$) groups as compared with the sham-vaccinated group. There was however, no significant difference between the two vaccines.

On further pooling the data for ESA and SA, thus handling it as one vaccinated group, the overall transformed mean showed a significant ($P < 0.05$) reduction in larval counts as compared to the sham-treated group.

Immunisation of rats with lyophilised or dialysed ESA

: Aim

The aim of this experiment was to ascertain whether the method of concentrating ESA exerted an effect on its immunogenicity. This is in view of the apparent difference in the efficacy of the ESA

vaccines in the previous two experiments. Two methods of concentration were tried, lyophilisation and dialysis.

Experiment design

Rats were vaccinated with 1500 µg soluble protein of lyophilised ESA (ESA_L) or dialysed ESA (ESA_D) or sham-vaccinated with saline. All rats were challenged three weeks later with 100 viable eggs.

Results

The data in Table 11, handled by the two-sample Wilcoxon test show that ESA, concentrated by either lyophilisation or dialysis is capable of stimulating protective immunity in rats to a challenge infection. The difference between the two preparations of ESA was significant ($Us = 77$, $P < 0.05$). Nevertheless, subsequent batches of ESA to be used as vaccines or hyperimmunisations were dialysed. This method of preparation was preferred partly because for logistic reasons regarding lyophilisation, since the equipment was temporarily unavailable. However, other factors involved in this decision were that the dialysed vaccine gave a level of protection considered adequate; that other workers have employed dialysis successfully to concentrate ESA (Ayuya and Williams, 1979) and that the lyophilised ESA appeared to be harmful to the rats.

Table 11 The numbers of living larvae in rats immunised with lyophilised ESA or dialysed ESA and from control rats challenged 3 weeks later with 100 viable eggs

	Number of living larvae per rat									
Sham	0,	7,	14,	22,	28,	39,	52,	57,	62,	79
$ESA_{(L)}$	0,	0,	0,	0,	0,	0,	0,	0,	1,	9
$ESA_{(D)}$	0,	0,	0,	2,	4,	4,	5,	5,	12,	17

Immunisation of rats with insoluble somatic antigens or cyst fluid

Aim

This experiment was designed to compare the immunogenic capacities of SA (100,000 g supernatant), insoluble somatic antigen (ultracentrifuged pellet of 100,000 g precipitate) and cyst fluid. The method of extracting these fractions were as previously described (in Part II).

Cyst fluid was obtained by tearing intact bladders of T. taeniaeformis metacestodes with a 23 G needle. The fluid was separated from larval tissue by sedimentation, carefully pipetted off and centrifuged at 2500 g at 4°C. The resulting supernatant was labelled cyst fluid and stored at -20°C until used.

Experiment design

Rats were vaccinated with 1500 µg soluble protein of SA insoluble somatic antigen (ultracentrifuged precipitate) or cyst fluid, or sham-vaccinated with saline. All rats were challenged three weeks later with 100 viable eggs.

Results

The numbers of living larvae in the livers of the rats are shown in Table 12. All the types of antigens employed produced highly significant resistance ($P < 0.01$) against the challenge infection. Cyst fluid, used for the first time in these studies gave a high level of protection.

Table 12 The numbers of living larvae in rats immunised with SA, insoluble somatic antigen or cyst fluid and from control rats, challenged 3 weeks later with 100 viable eggs

	Number of living larvae per rat									
Sham	1,	3,	11,	14,	16,	17,	24,	26,	39,	71
SA	0,	0,	0,	0,	0,	1,	3,	13,	20,	22
Insoluble somatic antigen	0,	0,	0,	0,	0,	1,	1,	1,	3,	6
Cyst fluid	0,	0,	0,	0,	0,	0,	1,	2,	5,	11

CHAPTER 12

DISCUSSION

Resistance against T. taeniaeformis is manifested by significantly fewer living larvae in the livers of vaccinated rats after challenge, than in control rats. This finding confirms that protective activity is present in ESA and SA. The overall results obtained with ESA, however, were rather lower than those previously reported in similar experiments (Table 13) but were similar to those of Brandt (1980).

The lower results obtained in this study may partly be attributed to the use of mature metacestodes as a source of antigen, the oncosphere having been more frequently used. Heath (1973b) found that the capacity to stimulate protective immunity in T. pisiformis infection was associated with the immature larval stages. Larvae up to 15 days of in vitro development, when implanted subcutaneously or intramuscularly, were able to confer an absolute resistance to an oral challenge infection, whereas implanted, live, mature cysticerci were ineffective. However, in the T. taeniaeformis-rodent system, Kwa and Liew (1977) and Ayuya and Williams (1979) used mature metacestodes to obtain ESA and obtained highly significant levels of immunity. There is very little experimental evidence at present in the literature about the protection-inducing antigens found at the different stages of larval development. Thus, whether such antigens in the mature metacestode are identical to those present in oncospheres remains unknown.

Other workers (Table 13) have used the same host species to harbour the parasites used as the source of vaccine as that in which the vaccine was tested, which is the homologous system. The present series of experiments differs from previous reports in that the hosts donating the vaccine material and the immunised hosts were different species (mouse and rat respectively). This was done because relatively more mice than rats could be accommodated in the limited facilities available. This meant that a greater amount of larvae were available and as many larvae as possible were required for *in vitro* studies and vaccine preparation. The available space for rats was mainly utilised for the vaccination studies. The mouse model was not used as an alternative to rats for the vaccination studies because Brandt (1980) had found that the age-limiting factor for challenge infection occurred in the mouse model. Brandt and Sewell (1981a) also observed a strain difference in that rats were much more susceptible to strain M of *T. taeniaeformis* and mice to strain B of that parasite. In his immunisation studies, Brandt (1980) attributed the reduced resistance obtained to the high levels of challenge which he used and/or the heterologous source of the vaccines; suggesting that the mouse strain material may not be able to induce an adequate protection against the rat adapted strain of this parasite used for challenge in the immunised rat. The results of the present studies in which more moderate challenge levels have been used suggests that both factors may have been involved since protection has been obtained but rather less convincingly than in the studies using the homologous system.

Table 13 Comparisons between previous and present studies with regard to the source, production and degrees of protection obtained with ESA derived from T. taeniaeformis

Author (Year)	Source of antigen material	Larval stage and age	Host and route of vaccination	Period (days) and nature of *in vitro* culture	Processing of ESA	Probability of protection
Kwa & Liew (1977)	Rat	Metacestode	Rat subcutaneous with adjuvant	1 serum-free	None	$P < 0.01$
Ayuya & Williams (1979)	Rat	Metacestode > 3 mo old	Rat oral, intra-peritoneal and intra-muscular with adjuvant	1 serum-free	Dialysed and concentrated by vacuum dialysis or Carbowax	$P < 0.01$
Lloyd (1979)	Cat	Oncospheres	Mouse oral and intra-muscular with adjuvant	10 10% normal mouse serum	Centrifuged and concentrated	$P < 0.001$
Rajasekariah *et al.* (1980a)	Cat	Oncospheres	Mouse intraperitoneal and subcutaneous with and without adjuvant	3 serum-free	Sedimentation S/N 3500 *g* S/N 3500 *g* precipitate Not concentrated	$P < 0.001$ $P < 0.01$ $P < 0.001$
Rajasekariah *et al.* (1980b)	Cat Mouse	Oncospheres Metacestode 3 week old 6 mo old	Mouse intra-muscular with adjuvant	3 4-6 serum free 6 serum free	500 *g* S/N 500 *g* S/N 500 *g* S/N 4500 *g* S/N Concentrated by ultrafiltration	$P < 0.001$ $P < 0.001$ $P < 0.001$ NS
Brandt (1980)	Mouse	Metacestode 4-4½ mo old	Rat subcutaneous intraperitoneal	10 1 15 serum-free	1000 *g* S/N Concentrated by lyophilisation	$P < 0.01$ $P < 0.05$ $P < 0.05$
Present study	Mouse	Metacestode 3-10 mo old	Rat intraperitoneal	10-14 serum-free	1000 *g* S/N Concentrated by lyophilisation or dialysis	$P < 0.01$ to $P < 0.05$

The variable degrees of protection obtained with ESA suggests that the concentration of protection-inducing antigens in the used medium varied from batch to batch, despite the attempted use of identical maintenance conditions, one defined medium and the same amount of protein for immunisation. It was not possible to produce and use a single batch of ESA for all the immunisations. Firstly, the protective antigenic capacity of any batch remained unknown until tested *in vivo*. Secondly, the used medium contained so little protein that a very large volume would be required. Thirdly, the stability of ESA was undetermined. In this study, during *in vitro* maintenance conducted to obtain ESA, the gas phase above the liquid medium was air. However Heath (1976) reported that the protective ability of exogeneous antigens was destroyed by exposure to air and this may well have introduced an additional uncontrolled variable. Finally, if an ESA was ever to be applied in field trials using domestic animals, it would be essential to be able to produce repeated batches of protective ESA. Accordingly the effects of the parasite variation shown in Part I could not be avoided. Until optimal *in vitro* conditions are determined and standardized, such inconsistency in immunisation efficiencies using ESA will persist. The accurate measurement of the protection-inducing antigen in the crude ESA would be a definite step forward.

The results with SA also tend to confirm previous observations on artificial immunisation, but in general the protective effects were also less. Kwa and Liew (1977), Ayuya and Williams (1979) and Rajasekariah *et al*. (1980b) immunised rats with saline-soluble extracts of mature metacestodes. The details of the extraction

technique differed mainly with the centrifugal forces used (1500 g, 4500 g and 50,000 g respectively) to obtain supernatants of antigen preparations. All these supernatants stimulated a significant degree of immunity. When sonicated oncospheres were spun at 100,000 g, the capacity of the resulting supernatant to stimulate immunity was considerably reduced but not eliminated (Rajasekariah et al., 1980b). This centrifugal force (100,000 g) was used in this study to obtain SA, and in one attempt where the 100,000 g precipitate was used, nearly absolute protection was attained. This seemed to be in agreement with the findings of Rajasekariah et al. (1980b) where the same precipitate but of oncospheral origin gave almost absolute protection.

An interesting observation was made during an attempt to remove soluble material from the precipitate by repeated suspension with PBS and ultracentrifugation (100,000 g). The protein content in successive supernatants obtained, as determined by a visual assessment using the Bio-Rad protein assay, did not appear to diminish. This is thought to be due to the very high centrifugal forces causing further breaking up of the larval tissues. Thus the repeatedly disrupted tissues continue to release protein into the supernatants. Hence the protective antigenic capacity of SA may partly be attributed to the partial solubilisation effect of ultracentrifugation on the saline-insoluble tissues.

The complex antigenic make-up of both ESA and SA may also have contributed to the relatively lower levels of protective immunity as obtained with these preparations. Since they are crude

preparations, ESA and SA will both contain large amounts of parasite protein consisting of a number of antigens expressing different specificities. When the immune system is stimulated by multi-determinant vaccines as such antigen mixtures, the first antibodies produced will often be directed against the "dominant" antigenic determinant. These antibodies may not be those which can effectively remove or neutralize the parasite. This immunological phenomenon is called "antigenic competition" and is defined as the inhibition of the immune response to one antigen or determinant by the administration of another antigen (Taussig, 1976). This may have occurred in the present study. Wilson (1974) in his contribution to the discussion on the mechanisms of survival of parasites in the immunised host, stated that antigenic competition might follow from the release of a variety of parasite antigens into the circulation, since this might have a suppressive effect on the host's immune response to the protection-inducing antigens.

The absence of adjuvant in these immunisation experiments may have influenced the results obtained. Murray, Robinson, Grierson and Crawford (1979) studied the factors which influence protection which included the use of adjuvant, the quantity of worm protein given, the number of doses employed and the interval between them, and the route of administration of the antigen. Most of the workers mentioned in Table 13 used adjuvant in their immunisation procedures but according to Ayuya and Williams (1979) this does not improve the results in this system.

From the evidence presented here, it is not possible to assess which antigen complex, ESA or SA, is the better product for vaccination. Both antigens seemed to possess similar immunising efficiencies. It is known that these two preparations share many antigenic components (Brandt, 1980). The dye exclusion test and TEM gave evidence of tegument loss from the main larval body. Soluble components released in this way would constitute a somatic component in used medium, hence in the ESA. Also, there are materials meant for excretion and secretion present in the tissues processed to make SA. Nevertheless, the different results obtained in the other studies with ESA and SA (Part I) do suggest that there are real qualitative differences between the preparations.

The insoluble components of the somata are also worth investigating further as there was evidence here of its protective antigenicity.

PART IV

Passive transfer of resistance to *Taenia taeniaeformis* in rats using sera from infected and immunised hosts

CHAPTER 13

INTRODUCTION

Part III established the presence of protection-inducing antigens in both ESA and SA. The aim of Part IV is to determine whether or not these immunisation procedures stimulate the formation of protective humoral factors which can be demonstrated by the passive transfer of resistance using serum from immunised donors to non-immunised recipients.

A further aim was to produce a source of protective antibodies for absorption studies to be described in Part V.

REVIEW OF THE LITERATURE

Passive transfer of resistance using serum from infected hosts

Humoral involvement in cestode immunity has been demonstrated directly by the protective effects of sera from infected hosts following injection into normal recipients. Miller and Gardiner (1932) and Campbell (1938a, b, c) were the first to demonstrate this. They found that serum from rats infected with T. taeniaeformis protected homologous recipients against a challenge infection. Since that time, successful passive transfer with serum from infected donors has been achieved in a variety of taenioid infections (reviewed by Weinman, 1970).

Miller and Gardiner (1934) established that the protective capacity of serum was directly proportional to the degree of infection in donor rats during passive transfer. Campbell (1938a) confirmed this relationship but limited it to 28 days after infection, after which time serum from either light or heavy infections had the same protective capacity. Kwa and Liew (1978) used serum from donor rats harbouring heavy infections of T. taeniaeformis and obtained significant protection in recipients given 1 ml aliquots even with serum dilutions of up to 1:32.

Most of the workers mentioned in this section gave intraperitoneal inoculations of sera to recipient rats and mice except Campbell (1938a, b, c) and Kwa and Liew (1978), who used intravenous injections. Small volumes (0.4 to 3.0 ml) of immune serum or chromatographic fractions of the immune serum were usually used for passive transfer.

To attain complete protection, immune serum was usually transferred on the same day as challenge. Results of experiments on transfer of immune serum at different time intervals (Miller and Gardiner, 1934; Campbell, 1938c; Musoke and Williams, 1975b and Kwa and Liew, 1978) demonstrated that serum transferred up to 5-8 days before and 2-5 days after challenge was still effective in reducing the infection to a level significantly lower than that in control infections.

Isolation of serum immunoglobulins

Several workers have recently confirmed and extended understanding of the mechanism of antibody-mediated protection by employing characterized antibodies in passive transfer.

Leid and Williams (1974) showed that passive transfer of resistance to T. taeniaeformis infection in the rat can be achieved with an immunoglobulin fraction corresponding to the 7Sy2a band in immunoelectrophoresis. This group of immunoglobulins was associated with the first four weeks of infection. Fractionation of sera was by gel-filtration on Sephadex G-200 followed by ion-exchange chromatography on DEAE-cellulose.

Using the same technique of fractionation, Musoke and Williams (1975a) worked on mouse sera collected 28 days after infection and identified 7Sy1 as the immunoglobulin most likely to be responsible for passive transfer of resistance in mice. Musoke and Williams (1975b) also found that the protective capacity of 14 and 21 day serum resided in the 7Sy2a immunoglobulin for the rat system, confirming the findings of Leid and Williams (1974). As

the infection progressed beyond four weeks, the chromatographic fractions found to be effective in passive transfer extended to all those containing 7Sy1 and 7Sy2 immunoglobulins.

The IgM fraction was consistently non-protective in both the mouse and rat models (Leid and Williams, 1974 and Musoke and Williams, 1975a, b). Mitchell, Goding and Rickard (1977) consider that both IgG_1 (non-complement fixing) and IgG_2 (complement fixing) are the subclasses of circulating immunoglobulins mediating resistance in mice and rats. This suggestion is based on their observations that the immunoglobulin involved in decomplemented mice was complement-fixing, and that the protein A-Sepharose column eluate which conferred protection against T. taeniaeformis revealed a mixture of IgG_1 and IgG_2 on analysis.

However, when Musoke and Williams (1976) investigated the antibody-mediated basis of the immune response induced by implanted larvae, fractionating serum obtained from rats implanted with live larvae of T. taeniaeformis for 21 or 28 days, IgG_1 and IgM were both protective. The type of immunoglobulins found to be protective in "implant" serum thus differ markedly from those found to be protective in "infection" serum. This study produced the interesting suggestion that the effective immunogens produced by post-oncospheral developing stages in hepatic infections may differ either qualitatively or quantitatively from those produced by implanted larvae in an abnormal site, in that the parasites in the normal and abnormal sites stimulate antibodies of a different immunoglobulin class.

Passive transfer using serum from immunised hosts

An alternative method of obtaining immune serum is from immunised animals. In the T. taeniaeformis-rodent model this method has not often been used. Serum (1-2 ml) from rats immunised with 180 mg weight of fresh larval material (Campbell, 1938b) or 6 x 1 ml injections of 10% ground larval suspension (Kraut, 1956) was able to protect recipient rats against a challenge infection. The serum was obtained 28 days (Kraut, 1956) and 38 days (Campbell, 1938b) after the first immunising dose. The latter author also immunised rabbits with the same material and was then able to demonstrate that the rabbit serum could confer resistance to rats, thus demonstrating the efficacy of a heterologous sera from immunised animals.

A similar attempt to passively transfer resistance using immunised sera was by Ayuya and Williams (1979). They immunised rats with 1 mg protein of saline-soluble antigen (SSA) or in vitro products (IVP) in adjuvant (Bordetella pertussis) and collected serum 21 days later. Immunisation was performed intraperitoneally for IVP and intraperitoneally or orally for SSA. These sera were not significantly protective against a challenge infection when given to rats although the IVP serum may have conferred slight resistance. However, immune serum taken from rats with an active infection conferred absolute resistance on the recipients. To explain this difference in the protective ability of the two serum types, these authors hypothesised that different protective mechanisms operate in immunity produced by vaccination and by infection.

Immunisation of sheep by intramuscular injection of artificially activated embryos of T. hydatigena or T. ovis produced sera which protected recipient sheep against both the homologous and heterologous challenge infection (Blundell, Gemmell and Macnamara, 1968). This confirmed cross-protection between T. hydatigena and T. ovis, and demonstrated that it was at least in part humoral. In a separate study Blundell, Gemmell and Macnamara (1969) reported that this protection and cross-protection were not associated with passively transferable cellular elements. However, Kwa and Liew (1975) transferred peritoneal cells from rats infected with T. taeniaeformis and conferred partial protection to normal recipient rats.

In vivo role of complement

Musoke and Williams (1975b) demonstrated for the first time in vivo that complement played a role in immunity to helminth infection. In their experiment, two groups of rats were challenged with eggs of T. taeniaeformis and inoculated with heat-inactivated 28 day immune (infection) serum. In addition, an anti-complementary factor isolated from cobra venom (CoF) was given to one of the groups, starting 24 hours after challenge infection. Daily circulating complement levels were monitored in this group and were found to be depleted during the five days while CoF was being administered. The five day period was chosen because it was found that the parasite is vulnerable to antibody-mediated attack during this period and also because serum complement levels can only be effectively depleted using CoF for four to five days. At necropsy,

it was found that a significant number of worms had survived in rats given CoF before inactivated immune serum compared to an absolute protection in the group of rats treated only with inactivated immune serum. The results demonstrate that an intact complement system in recipient rats is essential for successful passive transfer of resistance to T. taeniaeformis.

Similar results were obtained by Mitchell et al. (1977) in mice whose complement levels were also depleted by CoF.

CHAPTER 14

MATERIALS AND METHODS

Experimental animals

Donor rats which provided the immune rat sera and recipient rats used for passive transfer experiments were both Sprague-Dawley rats, three to four weeks of age at the start of the experiment. Sheep hyperimmunised to provide the heterologous immune sera were two to five year-old Suffolk and Suffolk crosses.

Preparation of rat sera

Rats were infected as described previously with a total of 800 eggs. It was known (see Part II) that the peak of the antibody response demonstrable by ELISA on serum from laboratory-infected rats occurs approximately 42-49 days post-infection. Therefore, rats were bled 42 days after infection and the resulting serum was designated "immune (infection) rat serum" (IRS).

Uninfected rats of the same age were bled for normal rat serum (NRS).

For preparing immunised rat antisera, 1.5 mg soluble protein of serum-free ESA or SA was injected intraperitoneally into rats. The dose was given in three divided doses at 48 hours interval. Six weeks after the first immunising dose the rats were bled, and the resulting serum was named anti-ESA or anti-SA rat serum respectively.

Preparation of sheep sera

A total of five sheep (A-E) were hyperimmunised to obtain heterologous sera. Two sheep each were used for the production of anti-ESA and anti-SA sheep sera (A and B; C and D respectively). One sheep (E) provided the normal sheep serum.

SA was produced as described in Part II. Before the sheep were hyperimmunised, serum was collected from each of the two sheep (A and B) and inactivated at 56°C for half an hour. These two pools of normal sheep sera were then sterilised by filtration. In each of four petri-dishes, ten five-month old metacestodes were held for 24 hours in 20 ml of defined medium. This medium was then discarded and the larvae transferred to a medium containing 18 ml fresh medium and 2 ml of inactivated sheep serum. Two petri-dishes contained serum from sheep A and the other two contained serum from sheep B. The medium was changed every 48 hours and maintained for 10 days. The maintenance procedure was as described in Part I. All used media containing sera from a single sheep were pooled, filtered through a 0.22 µm pore size membrane filter (Millipore) and concentrated 20 x by dialysis. Five ml each of the two batches of concentrated medium which was estimated to each contain 10 mg soluble protein and 10 mg soluble protein of SA was emulsified in 5 ml Freund's complete adjuvant ("FCA" Difco) by the double-hubbed needle method (Herbert, 1978). The procedure of adding serum to the medium was adopted as it was considered that serum would probably aid the metabolic processes of the larvae.

Sheep were injected intramuscularly with divided doses at four sites on the thigh region of both hindlegs. The two sheep injected with ESA were given ESA from the batch prepared from medium containing their own serum. The control sheep was injected with unused medium emulsified in CFA. Booster injections were given when the primary response in the four immunised sheep had had time to reach a plateau. In this case, the response, as monitored by ELISA reached a plateau after two weeks (Figures 24 and 25) and the booster was given at 63 days. The interval between primary and secondary (booster) injections was intentionally prolonged to minimize the production of antibodies against traces of unwanted material, that is, non-specific antibodies. The booster injection consisted of 10 mg soluble protein of each antigen in sterile saline injected subcutaneously. Six days after the booster injection, the sheep were bled. The immunisation schedule was based on recommendations made by Herbert (1978).

The humoral response of the hyperimmunised sheep was monitored by ELISA and immunodiffusion. In Figures 24 and 25, sheep hyperimmunised with ESA (A and B) produced sera which reacted more strongly to ESA than to SA in ELISA. Sheep hyperimmunised with SA (C and D) however produced sera which reacted to SA in the ELISA much to the same extent as sheep A and B, but reacted weakly with ESA in ELISA, especially sheep D. Overall, sheep given ESA responded better to the hyperimmunisation than sheep given SA.

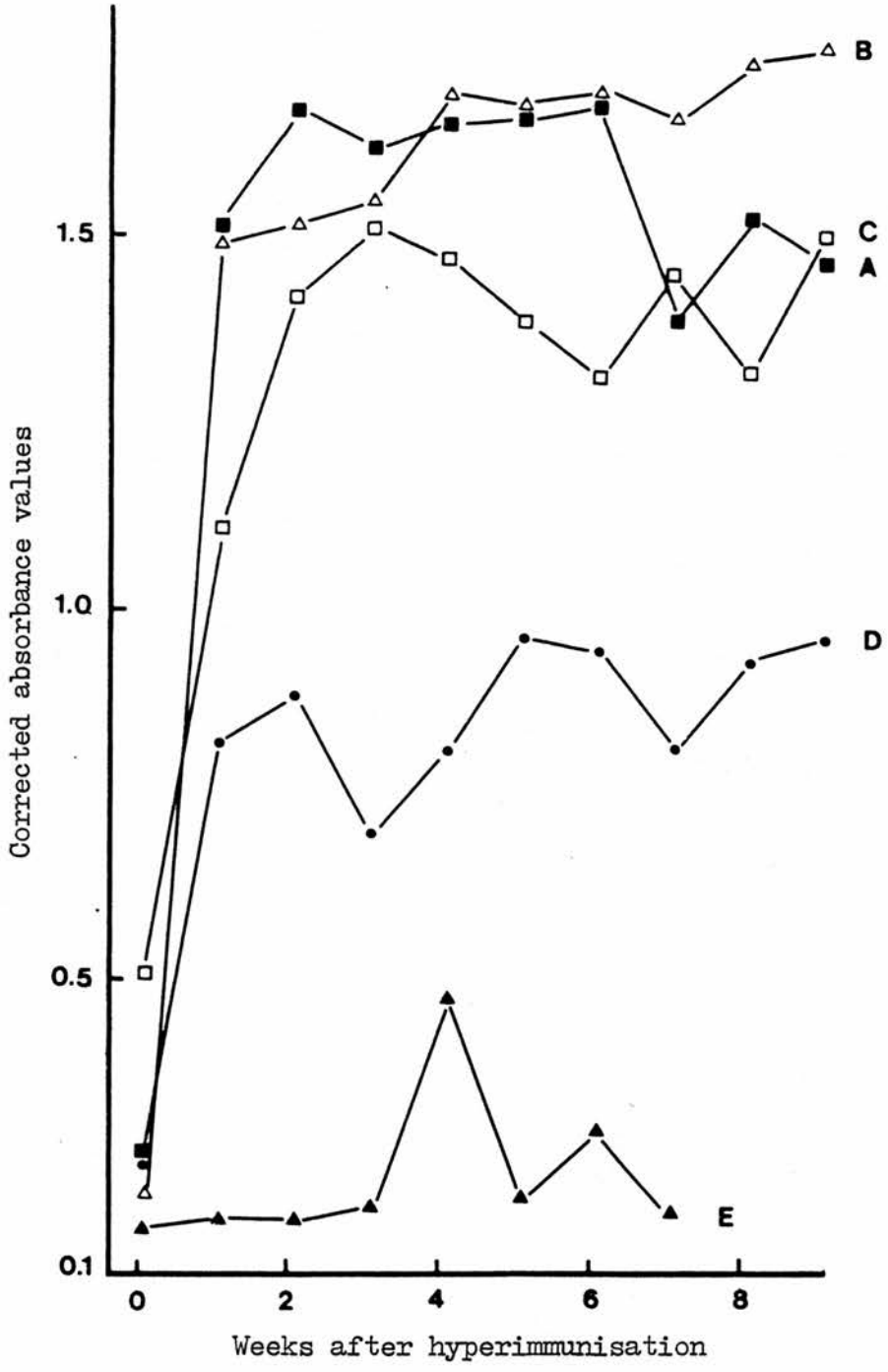

Figure 24 The response of sheep hyperimmunised with ESA (A and B) and SA (C and D) and of a control sheep as detected by ELISA using ESA.
For the corrected absorbance values see Appendix Table 17.

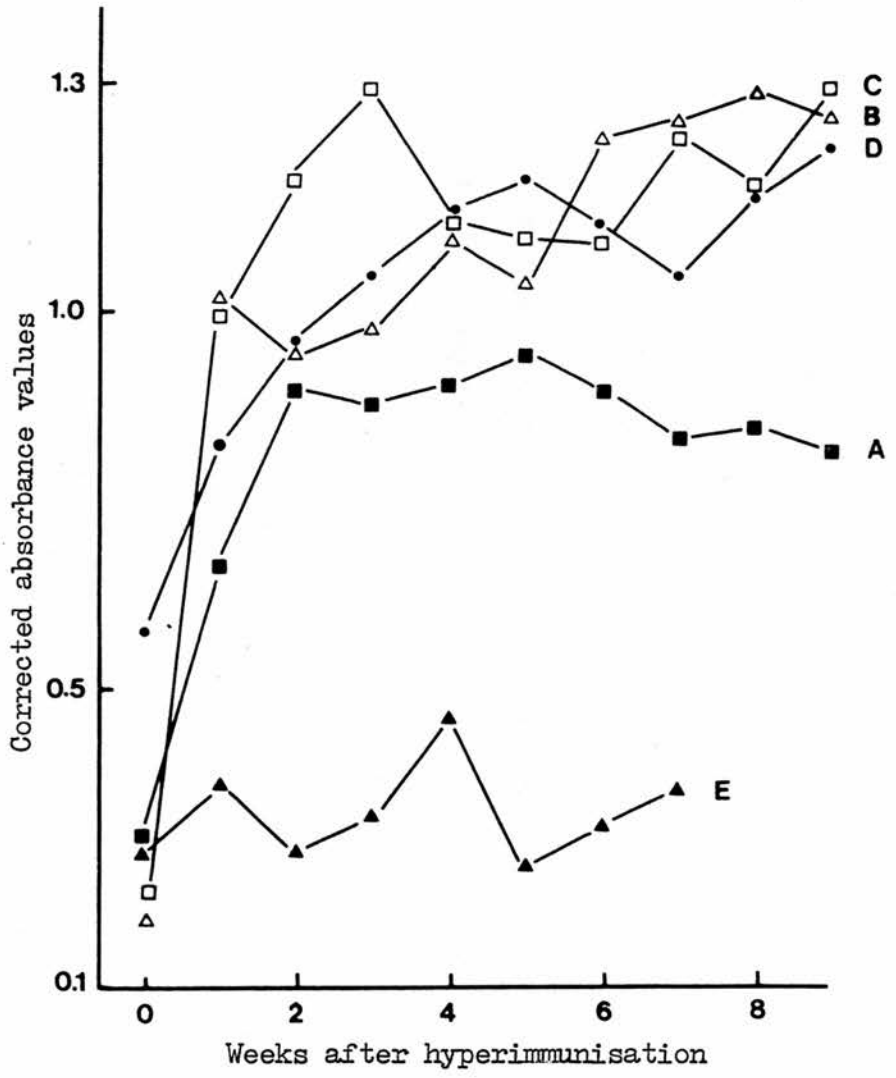

Figure 25 The antibody response of sheep hyperimmunised with ESA (A and B) and SA (C and D) and of a control sheep as detected by ELISA using SA.
For the corrected absorbance values see Appendix Table 18.

Collection and handling of sera

Rats were starved 24 hours prior to bleeding. This step was taken to reduce the lipid content in the serum. Lipids in the serum have been known to interfere with the observation of antigen-antibody reactions *in vitro* and are a source of emboli when injected into animals (Campbell, Garvey, Cremer and Sussdorf, 1970).

Rats were exsanguinated under ether anaesthesia. The skin and muscles of the medial side of the forearm were incised and the arm vein cut with a scalpel. Blood collected in the natural "trough" formed beneath the shoulder and was pipetted into sterile universal bottles. This method of collection was chosen because it was found that more blood was obtained than via heart puncture.

Sheep were bled from the jugular vein via a 1½" 20 G needle into 10 ml vacutainers (Becton, Dickinson & Co.).

The blood from rats and sheep was allowed to clot for 30 minutes at 37°C and then placed at 4°C overnight for clot retraction. Serum was pipetted out into 10 ml tubes and centrifuged at 1500 *g* for 30 minutes at 4°C. The clear serum was pooled, sterilized by passing through a 0.22 µm pore size membrane filter (Millipore), dispensed into 5 ml plastic vials and stored at -20°C until used.

Immunodiffusion

The agar used in the immunodiffusion technique was made up as a 1% solution in barbiturate buffer. Concentrated buffer pH 8.6 was made by dissolving 5.75 *g* diethyl barbituric acid and 3.75 *g* sodium barbitone in approximately 500 ml of hot distilled water,

the solution then being made up to 2 l with distilled water. Five grams of agar (Oxoid No. 2 Ionagar), 0.5 g sodium axide and 50 g sodium chloride was placed in a dry 500 ml bottle, to which was added 500 ml of a mixture comprised of 100 ml of the concentrated buffer and 400 ml distilled water. The bottle was placed in a boiling water bath, in order to dissolve the agar and the molten agar was then dispensed into universal bottles.

Immunodiffusion was performed on microscope slides, using the modified L.K.B. apparatus as described by Sewell (1966). Antigen was placed in the centre well at a concentration of 1mg ml^{-1}. Antibody was placed in the peripheral wells at various dilutions. Incubation of the agar was performed at 20°C for two to four days for the precipitin lines to develop.

The immunodiffusion reactions between sheep sera, which was collected before (week 0) and after (weeks 2, 4, 6 and 8) hyperimmunisation, and the antigens are shown in Table 14. It was observed that sera from sheep A, B and C developed five precipitin lines with both ESA and SA from week 2 onwards. Serum from sheep D which was hyperimmunised with SA did not produce any precipitin line with ESA but its sera samples of weeks 6 and 8 only produced precipitin lines with SA. The control sheep, E, did not produce any precipitin line with either ESA or SA.

Table 14 Immunodiffusion results of sera from sheep hyperimmunised with ESA (A, B), SA (C, D) and from control sheep (E) reacted with ESA and SA.

\+ = positive immunodiffusion reaction
\- = negative immunodiffusion reaction

	Antigen in immunodiffusion: ESA					SA				
Week	0	2	4	6	8	0	2	4	6	8
Sheep A	-	+	+	+	+	-	+	+	+	+
B	-	+	+	+	+	-	+	+	+	+
C	-	+	+	+	+	-	+	+	+	+
D	-	-	-	-	-	-	-	-	+	+
E	-	-	-	-	-	-	-	-	-	-

Fractionation of sera

Simple fractionation of serum for experiments 4 and 5 was achieved by precipitation with saturated ammonium sulphate (SAS). The details regarding the precipitation procedure are given by Hebert, Pelham and Pittman (1973). Various SAS concentrations were employed resulting in 30%, 33%, 35%, 40%, 45% and 50% reaction mixtures of SAS and sera.

Supernatants from the three precipitations and the final precipitate (saline-soluble) were analysed by ELISA for antibody activity, and cellulose acetate electrophoresis was used to analyse the protein composition in the final precipitate.

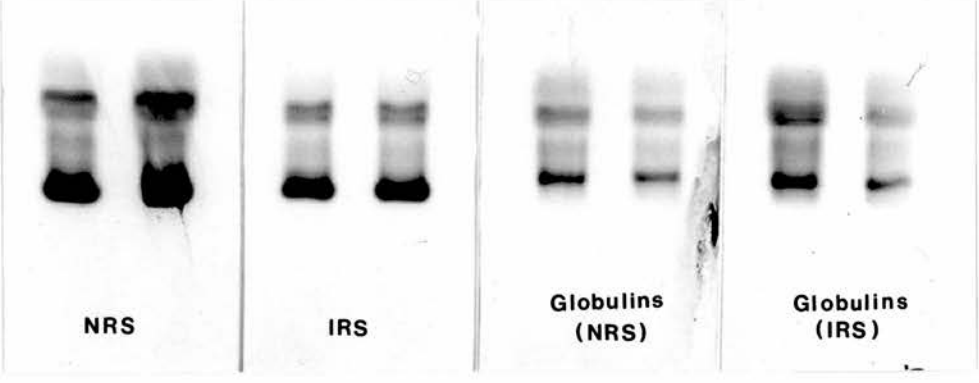

Plate 5 The patterns obtained by the electrophoretic fractionation of rat serum in cellulose acetate strips. Shows albumen depletion in the crude globulin fractions of normal serum (NRS) and immune serum (IRS).

Cellulose acetate electrophoresis

Principle

Electrophoresis is defined as the separation of components in a mixture based on the differing rates of migration of each component in an electrical field. Electrophoresis of serum, a complex mixture of proteins, separates the serum into six fractions visualised after staining with Ponceau-S. These fractions are, in order of decreasing electronegativity; albumin, alpha-1-globulin, alpha-2-globulin, beta-1-globulin, beta-2-globulin and gamma-globulin (Plate 5). Cellulose acetate serves as an inert, porous medium through which the proteins can migrate during electrophoresis and which can be fixed and stained for subsequent analysis.

Method

The fractions precipitated by different percentages of saturated ammonium sulphate were analysed by cellulose acetate electrophoresis for their protein compositions. The equipment, procedures and reagents used were those recommended in the manufacturers operating guide (Application Manual, AM301, Millipore). After processing, the stained protein bands on the cellulose acetate strips were analysed using a Phoroscope Densitometer and the protein concentration of each band calculated.

Analysis of serum fractions

Table 15 shows that the antibody activity, as measured by ELISA, in the final precipitate increased with the percentage of SAS used in the precipitation of immune serum. The antibody activity in the 50% SAS precipitate approximates to that in immune serum.

There was also a loss of antibody activity via the supernatants during the process, greater losses occurring when the percentage of SAS used was low.

Table 15 Detection of antibodies by ELISA using ESA in normal rat serum, immune rat serum, three successive supernatants and the final precipitate after precipitation of immune serum by SAS. Absorbance at 450 nm

Normal rat serum	0.165					
Immune rat serum	1.150					
	Percentage of SAS in SAS-serum mixture					
	30	33	35	40	45	50
Supernatant 1	1.044	1.075	0.935	0.623	0.245	0.186
Supernatant 2	0.461	0.844	0.564	0.382	0.183	0.057
Supernatant 3	0.173	0.545	0.531	0.244	0.109	0.073
Final precipitate	0.057	0.165	0.837	0.875	0.802	0.997

Precipitates obtained by 30%-40% SAS precipitations of immune serum after electrophoresis did not produce visible protein bands on staining. The protein compositions of these precipitates were therefore incalculable. Visible protein bands however appeared for the precipitates produced by the 45%-50% SAS. The percentage composition for each protein band in these precipitates are given in Table 16.

Table 16 Protein composition of precipitates obtained by different percentages of SAS in SAS-serum mixtures and of normal and immune sera. Values expressed in percentage

Percentage of SAS in mixture	Percentage of protein components			
	Albumin	alpha	beta	gamma
45	42	21	9	28
50	44	21		35
Normal rat serum	46	19	35	<1
Immune rat serum	51	26	18	5

The percentage of gamma-globulins seemed to be highest in the fraction of serum isolated by 50% SAS.

Though it is documented that 33% SAS will precipitate fairly pure gamma-globulins from serum (Campbell et al, 1970), the aim here was not so much to purify serum as to obtain the maximum yield of globulins showing antibody activity. Hence the 50% SAS precipitation level was selected to fractionate serum, based on the above ELISA and electrophoresis results.

Administration of serum and challenge infection

Recipient rats were given 1.0 ml or 2.0 ml aliquots of serum, depending on availability. The serum was injected intraperitoneally on the day of oral infection. The number of viable eggs given was 100, viability being based on the percentage of oncospheres which hatched and activated in vitro as described in Part III. The parasites used with rats were of the M strain.

Necropsy and interpretation of results

The rats were killed with ether eight weeks after passive transfer of serum and challenge. The liver was dissected out and the number of cysts containing living larvae were counted.

Either the Students' t-test or a Wilcoxon two-sample test was used for statistical analysis of the number of living larvae, the choice of which being as described in Part III.

CHAPTER 15

EXPERIMENTS

Passive transfer of resistance to T. taeniaeformis in rats by immune homologous sera from infected and immunised donors

Aim

This experiment was intended to confirm the protective activity of immune (infection) rat serum and to investigate that of immunised homologous (rat) antisera. Groups of rats were also vaccinated with ESA or SA to check the immunogenicity of the vaccines used to produce the immunised antisera.

Experiment design

Thirty rats were randomly divided in groups of five. Rats in Groups I, II, and III were given normal rat serum; Groups II and III had previously been vaccinated with 1500 μg soluble protein of ESA and SA respectively 21 days before challenge; Group IV was given anti-ESA rat serum and Group V, anti-SA rat serum; Group VI was given immune (infection) rat serum. Each rat received a 2.0 ml aliquot of the particular serum.

Results

The numbers of living larvae from the livers of rats necropsied eight weeks after passive transfer and challenge are given in Table 17.

The number of larvae per liver was transformed to log $(y + 1)$ where y is the number of living larvae. These transformed data were then analysed by the Students' t-test.

Table 17 The numbers of living larvae in rats passively immunised with 2.0 ml aliquot each of homologous serum from normal, infected or immunised donors, and challenged on the same day with 100 viable eggs

	Group and treatment	Number of larvae per rat
I	Normal rat serum (NRS)	7, 7, 15, 27, 57
II	ESA - NRS	0, 2, 6, 11, 15
III	SA - NRS	0, 1, 10, 14, 18
IV	ESA antisera	0, 0, 1, 5, 12
V	SA antisera	0, 1, 2, 5, 12
VI	Immune (infection) rat serum	0, 0, 0, 0, 0

Rats vaccinated with ESA and SA and given normal rat serum (NRS) on the day of challenge had significantly fewer larvae than the control rats which were given NRS only ($P < 0.05$ and $P < 0.10$ respectively). Rats given the homologous anti-ESA and anti-SA serum were also significantly protected, $P < 0.01$ and $P < 0.05$ respectively, against infection with T. taeniaeformis. Immune rat serum taken from rats with an active infection with T. taeniaeformis conferred absolute resistance on the recipients.

On the basis of these results, NRS was adopted as the negative control and IRS as the positive control in the next study.

Passive transfer of resistance to T. taeniaeformis in rats by immune homologous serum from infected donors and heterologous serum from hyperimmunised donors

Aim

Sheep antisera was studied in this experiment as a possible substitute for rat antisera. The larger volume of serum obtainable from sheep would be an advantage for passive transfer and immunoabsorption work, if sheep antisera were to prove as effective as rat antisera in conferring protection to a challenge infection.

Experiment design

Rats in the negative control group were injected with either normal rat serum (Group I) or normal sheep serum (Group III) and those in the positive control group with immune (infection) rat serum (Group II). Recipient rats in the test groups were injected with the heterologous sheep sera rendered hyperimmune to either ESA (Group IV) or SA (Group V). Each rat received a 2.0 ml aliquot of the particular serum.

Results

The numbers of living larvae in the livers of rats necropsied eight weeks after challenge are given in Table 18.

The larval counts were transformed by log $(y + 1)$ where y is the larval count per liver. On the transformed data, the heterologous (sheep) serum hyperimmune to ESA was found to protect recipient rats against a challenge infection ($P < 0.05$). However, the sheep serum hyperimmune to SA did not confer any protection on the rats. Once again, immune (infection) rat serum gave almost absolute protection.

Table 18 The numbers of living larvae in rats passively immunised with 2.0 ml aliquot each of homologous serum from normal or infected donors, or heterologous serum from normal or hyperimmunised donors, and challenged on the same day with 100 viable eggs

Group and treatment	Number of larvae per rat
I Normal rat serum	5, 6, 8, 16, 39
II Immune (infection) rat serum	0, 0, 0, 0, 1
III Normal sheep serum	2, 4, 5, 11, 28
IV ESA sheep antiserum	0, 1, 3, 6, 7
V SA sheep antiserum	4, 11, 13, 14, 19

Passive transfer of resistance to T. taeniaeformis in rats by globulins isolated from immune homologous serum

Aim

This experiment was performed to investigate the protective ability of globulins isolated from immune (infection) rat serum. This investigation was required prior to absorption by antigens of the protective component in immune globulins.

Experiment design

Rats in the negative control group received 1.0 ml normal rat serum (Group I) and those in the positive control group, 1.0 ml immune (infection) rat serum. Rats in the test groups received globulins isolated from normal rat serum (Group III) or globulins isolated from immune rat serum (Group IV). The globulins were

isolated from rat sera by precipitation with 50% saturated ammonium sulphate, as described earlier. The amount of globulins administered to the recipients was based upon protein content, estimated by the Warburg and Christian method, such that the amount of protein in the globulins was equal to that in 1.0 ml of the neat serum. All sera and its fractions were finally made to the same volume before passive transfer.

Results

The same transformation of data as previously, was employed for Groups I and II in Table 19. The effect of immune rat serum significantly reduced the number of living larvae in the rats ($P < 0.005$) compared to that of normal rat serum. Globulins isolated from immune rat serum clearly conferred absolute protection. These findings confirmed that globulins isolated from immune rat serum managed to retain their protective activity. The results also ensure that globulins isolated from normal rat serum were not protective.

Table 19 The numbers of living larvae in rats passively immunised with 1.0 ml aliquot each of homologous serum from normal or infected donors, or the corresponding globulin fraction and challenged on the same day with 100 viable eggs

Group and treatment	Number of larvae per rat							
I Normal rat serum (NRS)	21,	21,	21,	23,	27,	45,	47,	58
II Immune (infection) rat serum (IRS)	0,	1,	1,	2,	3,	22,	32,	35
III Globulins from NRS	10,	11,	15,	19,	25,	38,	40,	53
IV Globulins from IRS	0,	0,	0,	0,	0,	0,	0,	0

CHAPTER 16

DISCUSSION

It is apparent that passive transfer of immune (infection) rat serum will consistently confer almost absolute protection to recipient challenged rats. This result confirms that of earlier workers who used immune rat serum to passively transfer resistance. Campbell (1938b, c), Kraut (1955), Musoke and Williams (1975b), Heath and Pavloff (1975) and Kwa and Liew (1978) all recorded absolute protection in rats challenged with T. taeniaeformis infection.

A more interesting observation was the passive transfer of resistance using serum from immunised rats, which indicates that the resistance provoked by immunisation has a humoral component. This appears to be the first demonstration of a clear protective capacity for serum produced against metabolic products in a taeniid infection. As already mentioned, Ayuya and Williams (1979) observed a slight, non-significant degree of protection in a similar experiment.

The results obtained using sheep sera are less easily interpreted. The numbers of living larvae harboured by rats given serum from sheep immunised with SA was slightly though not significantly higher than those harboured by control rats given normal sheep serum. This result is similar to that obtained by Ayuya and Williams (1979), who found serum from rats immunised with SSA significantly increased the susceptibility of recipients ($P < 0.05$). These authors suggest that there may be some circulating factors in the

serum which have an enhancing effect on the survival of the parasites, a hypothesis previously put forward by Varela-Diaz, Gemmell and Williams (1972) for T. hydatigena and T. ovis and by Rickard (1974) for T. pisiformis.

Serum from sheep immunised with ESA conferred some protection, as the reduction in worm burden was significant compared to control rats given normal sheep serum. These sheep responded well to the hyperimmunisation as manifested by ELISA results and immunodiffusion. The antibody response was against both ESA as well as SA though it appeared to be more sensitive to the former antigen. This observation supports the concept that ESA and SA contain shared components.

No protection was obtained with serum from sheep immunised with SA. It is interesting to note that the weak response of sheep D to hyperimmunisation was detected by both ELISA as well as immunodiffusion. When monitored by ELISA using SA, similar antibody responses were shown by sheep injected with SA and ESA, whereas a higher level might have been expected in the sheep injected with SA. The reasons for this may be related to the complex nature of the SA and the high dosage of antigenic protein material administered. The phenomenon of antigenic competition may have occurred with the immune system of the sheep responding only to the major components in the SA. If these major components are not those associated with the acquisition of protection, this could account for the fact that there was no evidence that anti-SA sheep serum was at all protective.

PART V

Attempts to absorb the protective component from immune serum

CHAPTER 17

INTRODUCTION

The fact that rat serum from an infected animal is highly protective in passive transfer attests that antibodies have a role in acquired immunity to T. taeniaeformis infection. These protective antibodies were induced by the early larval stages, by six weeks after infection. Attempts were made to ascertain whether these protective antibodies can be absorbed by antigens derived from the later larval stages, the mature metacestode. Absorption of a protective antiserum with the appropriate antigen will usually remove the serum's protective capacity. Hence in Part V, it was intended to investigate whether the protective antibodies in immune rat globulins can be absorbed by the protection-inducing antigens in ESA and SA.

For both ESA and SA, three methods of absorption were attempted; the method of Murrell (1971) and two other methods in which the antigens were insolubilised by glutaraldehyde polymerisation or immobilised by attachment to a chromatographic material prior to use for absorbing the globulins. A fourth method was also included whereby globulins were absorbed by direct contact with live larvae. "Absorbed globulins" therefore refers to the globulins after they have been exposed or treated with antigen and "unabsorbed globulins" refers to the globulins that have had no previous contact with antigen in vitro.

After absorption, the globulins were first tested in vitro for antibody activity and then used in vivo in passive protection studies.

REVIEW OF THE LITERATURE

Absorption studies

In attempts to identify effective antigens for serodiagnosis or for inducing protection, workers have absorbed immune serum with various parasite components. The immune sera was usually recovered from rodents infected with T. taeniaeformis and occasionally from animals immunised with products derived from the parasite.

Miller and Gardiner (1932) made an initial attempt to remove the active principle from immune (infection) rat serum by treating it with living worms and powdered worm material. This did not demonstrably affect the protective power of the serum as there was no difference in larval numbers between rats given either absorbed or non-absorbed immune serum and then challenged with eggs of T. taeniaeformis. In a similar attempt, Campbell (1938b) reported success with rat serum taken from an 11-day infection or artificially immunised animals. That is, absorption with ground larval material removed the protective activity of the serum. However, the protective activity in serum from a 28-day infection proved non-absorbable. He hypothesised that the absorbable antibodies in sera from "early" infections or immunised animals are induced by material from disintegrating parasites or parasite tissue. On the other hand, the non-absorbable antibodies in serum from "later" infections are engendered by antigenic materials elaborated by the developing parasite.

In an in vitro study, Murrell (1971) described permeability defects in T. taeniaeformis larvae incubated in immune (infection)

serum. Incubation of larvae (90-120 days old) in immune rat serum resulted in a marked increase in the absorption of radio-labelled compounds including a monosaccharide (glucose), a disaccharide (sucrose) and an amino acid (methionine) plus an enhanced loss of methionine. It seemed that, as a result of exposure to the immune rat serum,the larvae had suffered some loss of selectivity in their permeability to external and internal molecules. This *in vitro* antibody-mediated activity is dependent on complement. This effect of immune rat serum against the larvae was abolished by absorption with a saline extract of the larvae or with their excretory-secretory products. The removal of a single precipitin line demonstrated by immunodiffusion was also observed. This is in contrast to the findings of Campbell (1938b) who had found that antibody of "late" immunity could not be absorbed by worm homogenates. Murrell explained this discrepancy as due to the different methods for preparing the larval extracts.

Hustead and Williams (1977b) later confirmed the work of Murrell, using *T. taeniaeformis* and *T. crassiceps* larvae. They showed that selective control of permeability could be restored by depletion of complement levels *in vitro*. Hence they suggested that the larvae *in vivo* may avoid this adverse effect by liberating anti-complementary factors to deplete complement levels in their vicinity.

Leid and Williams (1974) attempted to absorb the protective activity from immune (infection) rat serum using a variety of techniques. Immune globulins were reacted with either a thick larval suspension or saline somatic extract insolubilized with

glutaraldehyde or polyacrylamide beads. None of these absorption procedures reduced the efficacy of the immune globulin preparation in passive transfer. However, absorption with the thick larval suspension removed all precipitating antibody activity, as shown by double immunodiffusion. To explain the passive transfer results, these authors suggested that the concentration of certain critical antigens in the somatic extracts used for absorption may have been insufficient to effect complete removal of the protective antibodies. They referred to the findings of Rickard and Bell (1971b) who found that the degree of resistance to challenge infection with T. taeniaeformis was dependent upon the duration of implantation of membrane diffusion chambers containing these larvae. Hence Leid and Williams (1974) suggested that if the larvae were maintained in vitro they might release these critical antigens, which would accumulate in the culture fluids, so that these would be effective in absorption.

Rickard and Outteridge (1974) were able to absorb immune (infection) rabbit serum with such culture antigens. The efficacy of the absorption was tested in vitro, the absorbed serum having a reduced lethal effect on activated T. pisiformis oncospheres. However these authors were unable to demonstrate any antibody in the serum of infected rabbits by gel-precipitation or passive hemagglutination with the culture antigen. They therefore expressed doubts regarding any relationship between antibodies measured by serology and protective immunity. This observation tends to support that by Campbell (1938b) who found that precipitins were often not demonstrable in highly protective rat serums and considered that

some protective antibodies were probably induced by antigens which are insoluble in the pH range optimal for in vitro reactions.

To produce species-specific antigens for the immunodiagnosis of larval cestode infections, Craig and Rickard (1981) used affinity chromatography to absorb out cross-reactive antigens. Antigens in parasite extracts which were eluted after binding to the homologous anti-parasite antisera (bovine or ovine) coupled to CNBr-activated Sepharose were then passed sequentially through affinity columns containing heterologous anti-parasite Ig and the "run-through" antigens collected. By these procedures, crude somatic or cyst fluid extracts prepared from T. saginata, T. hydatigena or Echinocossus granulosus were partially purified. The level of cross-reactions to these absorbed antigens in ELISA, using sera from cattle or sheep given heterologous parasite infections, were significantly decreased, compared to the level of cross-reactions which occur when using crude parasite extracts. These authors envisaged that these semi-purified antigens could be useful for immunising mice in the production of hybridoma-derived antibody thereby reducing the chances of producing cell-clones that secrete cross-reactive antibodies.

An interesting finding by Smith, Quinn, Kusel and Girdwood (1981) was that Toxocara canis larvae maintained in vitro at 37°C do not bind antiserum raised against their excretions and secretions as detected by indirect fluorescence. However, under similar conditions but at 2°C, binding was demonstrable by fluorescence on the whole outer surface of the larvae. Later incubation at 37°C

caused a gradual loss of this fluorescence. These authors concluded that the antigens present in ES occur along the whole length of the larval outer surface and turnover at 37°C. It is this antigenic turnover that prevent the attachment of antiserum

CHAPTER 18

MATERIALS AND METHODS

Method of Murrell (1971)

The method of Murrell (1971) for absorbing serum with antigens is briefly as follows; 1 ml concentrated solution of antigen (30-36 mg protein per ml) is added to 10 ml of globulins and incubated in a 37°C water-bath for 30 minutes. After standing at 4°C overnight, the serum is centrifuged at 8000 *g* for 30 minutes at 4°C. The supernatant is then absorbed once more as before. A final centrifugation gives the absorbed serum.

The procedure used differed slightly from this in that the globulin solution was absorbed once only and various combinations of antigens (ESA, SA and insoluble antigen) and antibody dilutions were used.

As mentioned in Part IV, immune rat globulins (IRG) obtained by 50% SAS precipitation of infected rat serum were employed. Normal rat globulins (NRG) served as control. The protein concentration of the globulins and antigens were estimated by the Bio-Rad protein assay.

Absorption of globulins with ESA

ESA was used neat because of its low protein concentration. Unused medium acted as antigen control. IRG was diluted with PBS until it contained the same amount of protein per ml as NRG. Dilutions of IRG and NRG, 1:5, 1:10, 1:15 and 1:20, were then prepared for absorption with an equal volume of antigen. Various combinations of reactants served as controls, that is, NRG and IRG

treated with unused medium or remained untreated. Although the initial dilutions of globulins in the absorption mixture varied, the mixtures were further diluted for the ELISA to give a standard dilution equivalent to a 1:100 dilution of the initial globulin solution.

Absorption of globulins with SA

In this case, the antigen was diluted as it has a high protein concentration. SA was also used neat as well as diluted to 1:5 and 1:15, and treated with globulins in the same manner as ESA. The control reactions were NRG and IRG treated with PBS or remained untreated. The supernatants of the absorption mixtures were diluted to 1:100 of the initial globulin solution and then analysed by the ELISA for detecting antibody activity.

The results of the ELISA on these absorbed globulins and the controls are given in Tables 20 and 21.

Absorption of globulins with an insoluble somatic antigen

As the absorption technique adapted from Murrell's procedure (1971) produced unsatisfactory results, as assessed by the ELISA for detecting antibody, a different antigen preparation was used. The antigens previously used, ESA and SA, were soluble but now an insoluble antigen was employed in the absorption. To prepare this, a homogenised larval suspension was centrifugally washed in PBS at 500 g several times until the supernatant contained little protein. The protein content of the supernatants were monitored by the Bio-Rad protein assay and the antigenic content was detected by

Table 20 ELISA values obtained with globulins after absorption with ESA, using ESA and SA as the detecting antigens. All globulin mixtures made to a final dilution of 1:100 for the assay. Absorbance at 450 nm

Antigen	Globulin	Dilution of globulins	Treatment Unabsorbed (unused medium)	Absorbed (ESA)	Untreated
ESA	NRG	Neat	0.373	0.292	0.220
		1:5	0.236	0.231	
		1:20	0.233	0.228	
		1:50	0.258	0.219	
	IRG	Neat	0.499	0.497	0.432
		1:5	0.338	0.375	
		1:20	0.342	0.378	
		1:50	0.370	0.371	
SA	NRG	Neat	0.384	0.370	0.290
		1:5	0.277	0.223	
		1:20	0.248	0.243	
		1:50	0.308	0.305	
	IRG	Neat	0.634	0.630	0.550
		1:5	0.476	0.432	
		1:20	0.460	0.438	
		1:50	0.550	0.516	

Table 21 ELISA values obtained with globulins after absorption with SA, using ESA and SA as the detecting antigens. All globulin mixtures made to a final dilution of 1:100 for the assay. Absorbance at 450 nm

Antigen	Globulin	Dilution of antigen	Treatment Unabsorbed (PBS)	Treatment Absorbed (SA)	Untreated
ESA	NRG	Neat	0.194	0.211	0.178
		1:5	0.177	0.167	
		1:15	0.181	0.180	
	IRG	Neat	0.277	0.281	0.267
		1:5	0.235	0.236	
		1:15	0.262	0.252	
SA	NRG	Neat	0.251	0.260	0.231
		1:5	0.214	0.191	
		1:15	0.218	0.166	
	IRG	Neat	0.424	0.337	0.446
		1:5	0.373	0.301	
		1:15	0.359	0.315	

the ELISA (Table 22). Repeated washing and centrifuging was used in an attempt to render the precipitate free from soluble larval material.

Table 22 Protein concentration and antigenic load as detected by ELISA in supernatants of 20 centrifugal (500 g) washes from larval precipitate

Number of supernate	Protein content µg/ml	ELISA values for antigens detected by antiserum against ESA	SA
1	194	0.344	0.905
2	154	0.190	0.780
3	150	0.143	0.563
4	140	0.118	0.537
5	150	0.073	0.509
6	140	0.103	0.566
7	128	0.021	0.413
8	114	0.06	0.372
9	85	0.00	0.311
10	75	0.083	0.257
11	52	0.065	0.281
12	57	0.026	0.204
13	112	0.123	0.402
14	85	0.072	0.276
15	57	0.061	0.236
16	48	0.041	0.212
17	40	0.041	0.231
18	59	0.061	0.400
19	64	0.063	0.335
20	42	0.015	0.265

One ml of globulins, NRG and IRG, was reacted with both neat (1 g) and 1:5 (0.2 g + 0.8 ml PBS, $^{w}/v$) larval precipitate. Controls included non-absorbed globulins which underwent the same procedure as absorbed globulins as well as untreated globulins. The mixture of antigen and antibodies was incubated, stored and finally ultracentrifuged. The supernatants were diluted as before until the globulin dilution in the mixture was 1:100, for the antibody detection by ELISA.

Results

The mean ELISA antibody values in globulins (NRG and IRG) after absorption with ESA or SA are given in Tables 20 and 21 respectively. From these Tables, the values of absorbed globulins (reacted with antigen) and unabsorbed globulins (reacted with control solutions) were compared. There appears to be little or no difference in the antibody values between absorbed and unabsorbed globulins at the various dilutions.

In Table 23, where the antibody values of globulins absorbed with a washed larval precipitate are presented, there is a substantial reduction in the antibody values from non-absorbed to absorbed IRG. The reduction was more marked when ESA was used as the detecting antigen in ELISA. In the case of absorbed and non-absorbed NRG, which served as antibody control, there was little difference. Repetition of this experiment produced similar results.

The insoluble larval material used in the second modification of Murrell's method for immunoabsorption was washed as much as it was practical to do so. Table 22 lists the protein concentration

and antigenic load of the supernatants which resulted from the 20 washes. There was the expected reduction in protein load and antigen content. However, the later washes all revealed some protein remaining in the supernatant, mostly SA since there was only a negligible reaction with the anti-ESA serum.

Table 23 ELISA values obtained with globulins after absorption with an insoluble somatic antigen, using ESA and SA as the detecting antigens. All globulin mixtures made to a final dilution of 1:100. Absorbance at 450 nm.

Antigen	Globulin	Dilution of antigen	Unabsorbed (PBS)	Absorbed (insoluble somatic antigen)	Untreated
ESA	NRG	Neat	0.120	0.107	0.116
		1:5		0.100	
	IRG	Neat	0.347	0.142	0.369
		1:5		0.155	
SA	NRG	Neat	0.197	0.157	0.186
		1:5		0.145	
	IRG	Neat	0.133	0.280	0.366
		1:5		0.274	

Insolubilisation of antigens by glutaraldehyde

It was decided to use insolubilised antigens, ESA and SA, for absorption because the failure of Murrell's method might have been due to the formation of soluble and possibly unstable antigen-antibody complexes (see Discussion).

The method of Avrameas and Ternynck (1969) was followed to insolubilise the antigens. SA was first concentrated by dialysis against polyethylene glycol to give a solution containing 10-40 mg protein per ml in 0.1M phosphate buffer, pH 7.0. One ml of this SA solution was placed in each of five clean glass test tubes and 0.2 ml of various concentrations of glutaraldehyde (25% glutaraldehyde solution, EM scope) in aqueous solution was added dropwise. The test tubes were shaken after each drop so as to disperse the glutaraldehyde thoroughly. A gel formed after all the glutaraldehyde had been added and this was allowed to stand for three hours at room temperature. The different concentrations of glutaraldehyde were used in an attempt to determine the minimum amount which would completely insolubilise SA.

The degree of insolubilisation of the protein was assessed by dispersing the tube's contents in a few ml of water, centrifuging for 15 minutes at 1500 g and then checking the supernatant for protein by the method of Warburg and Christian (1941). If the insolubilisation is complete, no protein should be found in the supernatant.

Three attempts at insolubilising SA by this method were made, the results being shown in Table 24. The first trial produced

inconclusive results, but on this basis it was tentatively inferred that lower concentrations of glutaraldehyde were probably more efficient in insolubilising SA than the higher percentages of glutaraldehyde. Therefore in trial 2, lower concentrations of glutaraldehyde were used and the maximum efficiency of insolubilisation was achieved at 1.5% glutaraldehyde (91.6%). In trial 3, the same percentages were used again but different results were obtained; this time the higher percentages of glutaraldehyde appeared more effective. This discrepancy between trials 2 and 3 was probably due to the different protein concentrations in the SA (24.6 mg ml^{-1} and 42 mg ml^{-1} respectively). However these results failed to indicate an overall optimal concentration for the glutaraldehyde and indicated that complete insolubilisation was not attainable.

Table 24 Protein concentration in supernatants and the percentage of insolubilisation achieved with different concentrations of glutaraldehyde

Number of trial	SA protein content mg ml-1	% glutaraldehyde	Protein in supernatant (mg ml^{-1})	% insolubilisation
1	13.5	2.5	5.68	58
		5.0	8.77	35
		7.5	10.90	19
		10.0	6.40	53
2	24.6	0.5	22.70	7.7
		1.0	8.30	66.2
		1.5	2.05	91.6
		2.5	9.55	61.2
		5.0	16.33	33.6
		7.5	9.68	60.6
		10.0	17.90	27.2
3	42.0	1.5	17.8	57.6
		2.5	14.3	66.0
		5.0	11.6	72.4
		7.5	8.4	80.0
		10.0	10.0	76.2

Affinity chromatography

Principle

In affinity chromatography, sample components are isolated and separated on the basis of their biological specificity, as in antigen-antibody or enzyme-inhibitor systems.

A ligand with specific affinity for the sample components of interest is covalently coupled to a solid matrix. When the sample is applied, only those components with an affinity for the ligand are adsorbed to the gel-matrix, the other components being washed straight through. Elution of the adsorbed components is then obtained by suitably altering the pH and/or salt concentration of the eluent.

Preparation of the immunoadsorbent

The antigens used as the ligand were coupled to a gel-matrix, CNBr-activated Sepharose 4B (Pharmacia), essentially according to the manufacturer's instructions (Affinity Chromatography - principles and methods, 1979).

The antigens, serum-free ESA or SA, were concentrated by dialysis against polyethylene glycol until the protein concentration was 15 mg ml^{-1}. One ml of this solution was made up to 5 ml in coupling buffer, 0.1M $NaHCO_3$ buffer pH 8.3 containing 0.5M NaCl. The antigen solution was coupled to 1 g of gel which had previously been washed and allowed to swell for 15 minutes in 200 ml of 10^{-3}M HCl solution. The ligand-gel mixture was placed in a universal bottle and mixed on a blood cell suspension mixer (Eschmann) for two hours. Unbound material was washed away in the coupling buffer

by filtration. Any remaining active groups were blocked with 1M ethanolamine in coupling buffer, pH 8.0, for two hours. Three washing cycles were used to wash the gel and remove non-covalently bound protein, each cycle consisting of a wash with 0.1M acetate buffer, pH 4.0, containing 0.5M NaCl followed by a wash with 0.1M borate buffer, pH 8.0, also containing 0.5M NaCl. The prepared gel was then ready for use.

Except during the coupling the whole process was performed with the ligand-gel mixture on a hardened filter paper No. 54 (Whatman) on the acrylic plate of a Hartley type 3-piece funnel of 5 cm internal diameter (Whatman).

All the washings from the ligand-gel were concentrated back to the original volume of antigen solution and analysed for protein content by the Bio-Rad protein assay (Table 25). This was to assess the percentage of protein coupled. The percentage of protein that coupled to the gel was 93% and 89% for ESA and SA respectively.

Table 25 Protein content in mg ml^{-1} of antigen solutions for preparation of ligands and of the washes, with estimation of percentage of protein coupled to the gel

	ESA	SA
Original antigen solution	15.00	15.00
Washes - Coupling buffer ($NaHCO_3$)	0.94	1.60
Ethanolamine	0.00	0.05
Acetate buffer	0.00	0.00
Borate buffer	0.00	0.00
Percentage of protein coupled:		
$100 - \left[\frac{\text{Amount of protein lost via washes}}{\text{Amount of protein in antigen solution}} \times 100\%\right]$	93	89

Equipment

Figure 26 outlines the equipment layout for affinity chromatography. Samples and buffers were pumped onto the columns with upward flow using an LKB Varioperpex peristaltic pump 12000. From the column, the effluent flowed through an LKB Uvicord S optical unit 2138 to measure the percentage transmission at 280 nm, which was recorded by an LKB recorder unit 6520 at a chart speed of 60 mm hour^{-1}. Finally the effluent was collected in 5 ml aliquots in 10 ml tubes on an LKB Ultrorac fraction collector 7000.

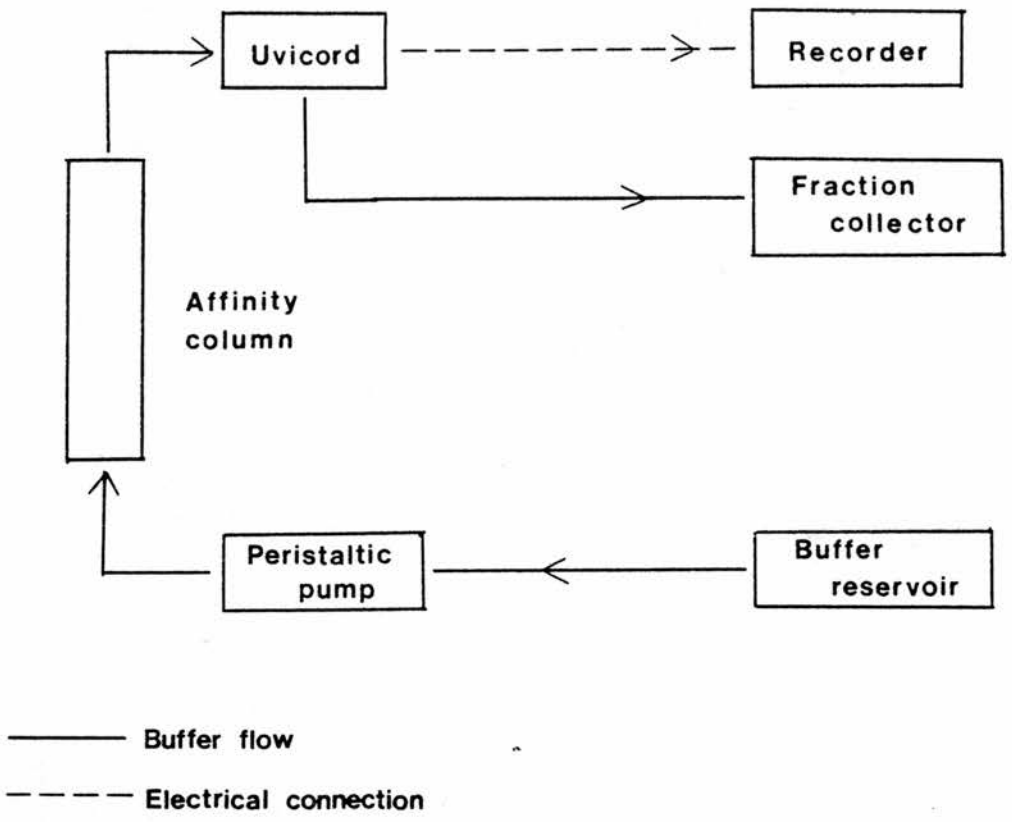

Figure 26 Equipment layout for affinity chromatography.

Procedure

The prepared gel was packed into a Whatman glass column, 0.8 cm internal diameter and 20 cm height. A 3.5 ml gel volume was obtained. After packing the column was linked to the rest of the equipment (Figure 26) and immediately equilibrated with 10 column volumes, 35 ml, of starting buffer, PBS pH 7.6 containing 0.02% sodium azide. The recorder was switched on during the last two volumes of buffer-flow so as to obtain a baseline reading. The flow rate was 2-3 ml cm^{-2} $hour^{-1}$.

The sample, 3-5 ml per application of NRG or IRG, was mixed in 10 ml of the starting buffer and applied to the affinity columns, followed by buffer alone. When the sample was being applied, the flow rate was reduced by half to facilitate binding. The application of globulins produced the first peak. When a steady baseline reading was again achieved, the buffer was changed to 0.05M glycine-HCl solution, pH 2.8. This change of pH and molarity will elute antibodies specifically bound to the ligand, the pH of these fractions being restored by a small amount of solid TRIS (Sigma) placed in the collection tubes. This elution produced a second peak on the recorder chart. The column was again washed with 35 ml of PBS-azide buffer before the next sample application.

When not in use, the affinity columns were stored at $4^{o}C$. Before storage, the columns were thoroughly washed with PBS-azide buffer. The presence of azide in the buffer acts as a bacteriostatic agent.

Handling and analysis of column eluates

The fractions corresponding to the first and second peaks on the recorder chart were pooled separately and concentrated by dialysis against polyethylene glycol to the original sample volume. Samples of the first peak were termed "unbound" globulins and those of the second peak "bound" globulins. These globulins were then analysed for antibody activity by ELISA.

For a trial run, NRG and IRG samples were applied to the affinity columns as a preliminary experiment. The results are shown in Table 26, from which it can be seen that some of the antibody reacting with either ESA or SA was bound to and eluted from the appropriate column. After analysing the results of the trial, samples of IRG intended for use in the passive transfer studies were applied to the two columns on which ESA and SA acted as ligands. The ELISA values for the unbound or "run-through" fractions obtained when samples of this second batch of IRG were applied to the affinity columns are shown in Table 27. These fractions were later used in the passive transfer experiments.

Table 26 ELISA values obtained with unbound and bound fraction of NRG and IRG separated by affinity chromatography, detected by ESA and SA. Absorbance at 450 nm.

Ligand	Fraction	NRG		IRG	
		ESA	SA	ESA	SA
ESA	Unbound	0.066	0.076	0.189	0.206
	Bound	0.060	0.051	0.140	0.156
SA	Unbound	0.046	0.066	0.151	0.134
	Bound	0.069	0.112	0.160	0.196
None	Untreated	0.119	0.130	0.328	0.288

Table 27 ELISA values for antibody in the unbound fractions of IRG after affinity chromatography, in absorbed IRG after larval contact, in incubated IRG, in untreated IRG and in untreated NRG, detected by ESA and SA. Absorbance at 450 nm

Globulins and treatment	ESA	SA
Untreated NRG	0.188	0.162
Untreated IRG	0.786	0.569
Unbound fraction of IRG via ESA column	0.502	0.412
Unbound fraction of IRG via SA column	0.485	0.380
IRG after larval contact	0.665	0.518
Incubated IRG	0.828	0.607

Absorption of globulins by direct contact with live larvae

The recognition that host-protective antigens in oncospheres are probably membrane-associated (Rajasekariah et al., 1980a, b and Rickard and Brumley, 1981) led to the use of a fourth method of absorption. For this, live larvae were used to absorb IRG.

In a sterile manner, 20 larvae from a five month old infection with T. taeniaeformis (B) were collected and washed as for in vitro maintenance (described in Part I). The larvae were then placed in a sterile petri-dish containing 20 ml of IRG which had been sterilised by filtration through a 0.22 μm pore-size membrane filter (Millipore). The petri-dish was sealed, kept at 37°C for six hours and then transferred to 4°C overnight. The next day, the larvae were removed and the serum was refiltered as above and stored at -20°C until use. A control volume of IRG was incubated under

the same conditions but without larvae.

Table 27 shows ELISA values for incubated IRG and IRG after larval contact.

Estimation of the efficacy of absorption

From the data given in Figures 14 and 15 for the standard antigen concentration of 10 μg soluble protein per ml, in Part II, can be derived the data in Figure 27, by subtracting the results for negative serum from that for the positive serum. Figure 28 shows the same data (as for Figure 27) after transformation - there is an approximate straight line relationship between √absorbance and $\log_{10}$ (dilution). Figure 28 also shows fitted regression lines, which can be used to re-express the apparent antibody contents of the absorbed globulins in Table 27 in terms of dilution rates instead of absorbance values. The calculations are set out in Table 28. From Table 28 it appeared that the unbound fraction of IRG after being through ESA or SA columns have about 19% and 17% antibody content respectively relative to the unabsorbed/ untreated IRG, detected by ESA in ELISA. The antibody content for IRG after larval contact was only about 53% relative to untreated IRG. Overall, the proportion of the initial antibody content remaining in the absorbed IRG was more when SA was used as the antigen in ELISA.

This use of the regressions implies that the lines may be extrapolated beyond the range of the data in Figure 27. This is not strictly justifiable but it almost certainly provides a better estimate of the antibody content than a simple direct comparison

between the results expressed as absorbance values. Hence this procedure gives a better indication of the efficacy of the absorption methods used.

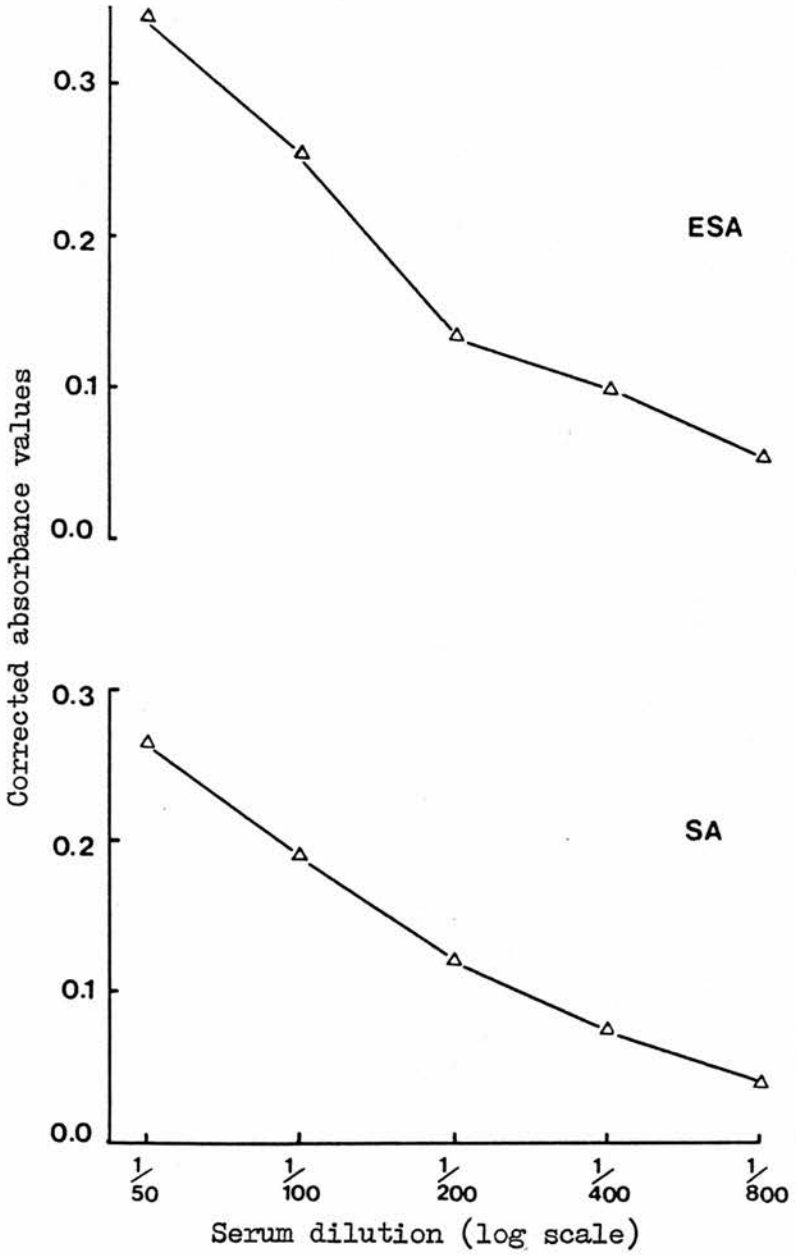

Figure 27 Titration of specific absorbance by a pooled serum from infected rats against a soluble protein concentration of 10 μg ml^{-1} for ESA and SA. In each case the result given by the same dilution of a pool of normal rat serum has been deducted from the raw value obtained with the infected rat serum.

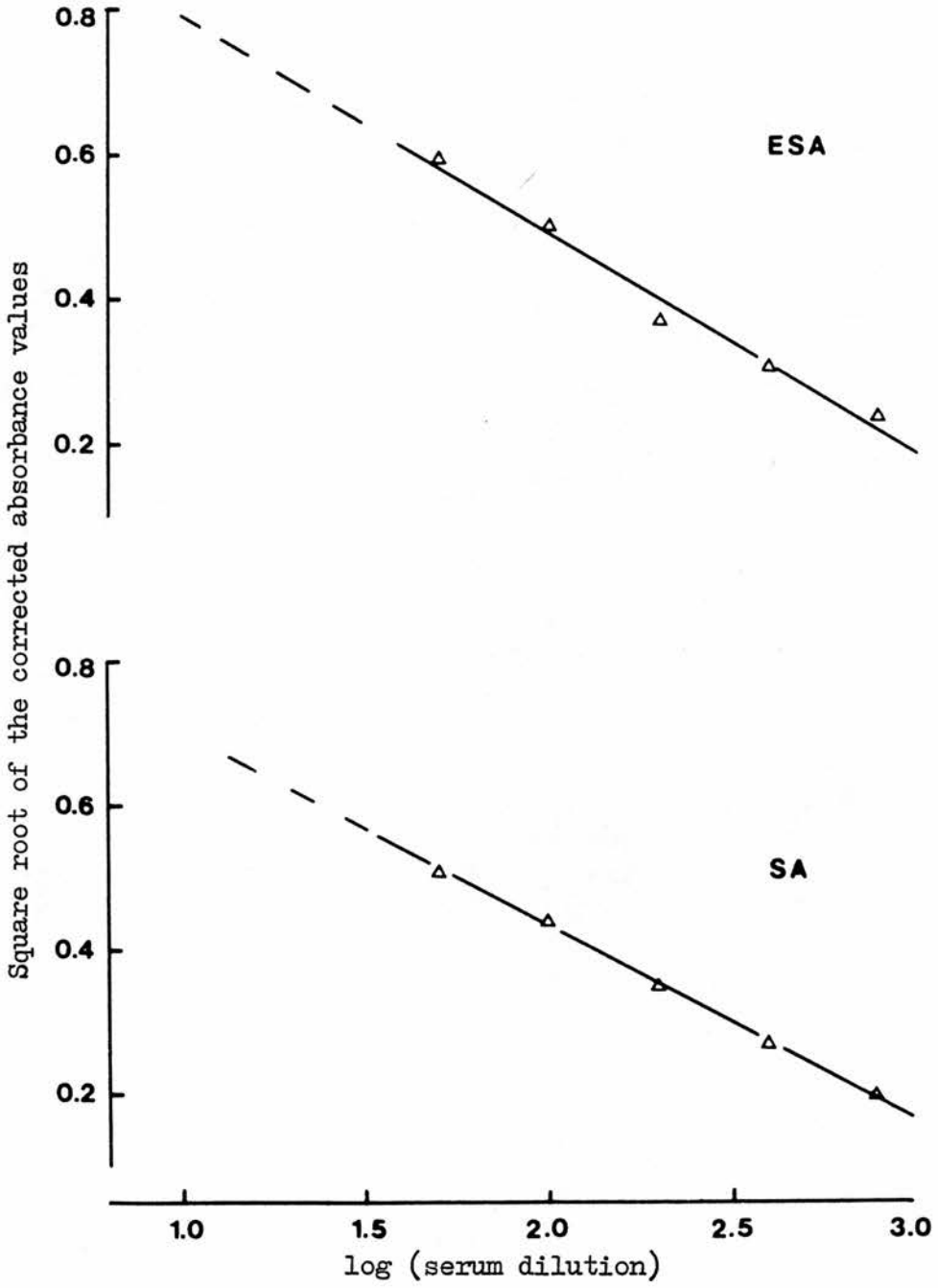

Figure 28 Results following square root transformation of the corrected absorbance values shown in Figure 27. The lines show the best fit regressions and the broken lines represent the extrapolations of these lines.

Table 28 ELISA absorbance values for absorbed IRG less those for NRG and the relative estimated antibody contents calculated by extrapolation from the linear regression shown in Figure 28, obtained from the data in Figures 14 and 15 in Part II

ESA

	Absorbance value	√Absorbance = y	$\log_{10}$ dilution $= \frac{y - a}{b}$	dilution	* %
Untreated IRG	0.598	0.773	1.048	11.2	100
IRG - ESA	0.314	0.560	1.766	58.4	19
IRG - SA	0.297	0.545	1.818	65.8	17
IRG - larvae	0.477	0.691	1.327	21.2	53

SA

	Absorbance value	√Absorbance = y	$\log_{10}$ dilution $= \frac{y - a}{b}$	dilution	* %
Untreated IRG	0.407	0.638	1.227	16.8	100
IRG - ESA	0.250	0.500	1.749	56.1	30
IRG - SA	0.218	0.467	1.874	74.9	22
IRG - larvae	0.356	0.597	1.383	24.2	69

*Apparent antibody content as percentage of that in IRG, a intercept of the regression b regression coefficient

CHAPTER 19

EXPERIMENT

Passive transfer of resistance using absorbed immune globulins

Introduction

An experiment was undertaken to determine whether the immunoabsorption methods employed were removing a sufficient proportion of the protective components from the immune globulins to abolish or reduce the protective effect. If immunoabsorption methods are so effective, a passive transfer test should show resistance in rats given unabsorbed immune globulins (as demonstrated in Part IV) and nil or reduced resistance in rats given absorbed immune globulins.

It was appreciated that to carry out such a study using the relatively poorly absorbed sera obtained in the preliminary work was somewhat premature, but since lack of time prevented further *in vitro* studies, it was decided to assess the efficacy of the techniques used so far in an *in vivo* study, even though the absorption was far from complete.

The immunoabsorption methods chosen for the *in vivo* test were affinity chromatography and direct contact with live larvae.

Experiment design

Forty-five male Sprague-Dawley rats were randomly divided into four groups (I-IV) of ten and one group (V) of five. Rats in the various groups were given 1 ml of the following material.

Group I : unbound globulins from affinity chromatography with an ESA ligand;

Group II : unbound globulins from affinity chromatography with an SA ligand;

Group III : globulins after direct contact with live larvae;

Group IV : globulins treated in the same manner as for those in Group III but without larval contact;

Group V : saline

Rats in Group V served as a control for the challenge infection, that is, this group provided a check on the viability of the eggs used.

Results

Results in Table 29 show clearly that, except perhaps for one rat in Group III, IRG protected the rats against the challenge infection, despite any of the three absorption procedures. The results in Group V show that the challenge was infective.

Table 29 The numbers of living larvae in rats passively immunised with 1 ml of immune globulins (IRG) which have been treated with ESA, SA or live larvae, or with incubated IRG or saline, and challenged on the same day with 100 viable eggs

Group and treatment	Number of living larvae
I IRG–ESA	1, 0, 0, 0, 0, 0, 0, 0, 0, 0
II IRG–SA	0, 0, 0, 0, 0, 0, 0, 0, 0, 0
III IRG–live larvae	89, 2, 0, 0, 0, 0, 0, 0, 0, 0
IV IRG	0, 0, 0, 0, 0, 0, 0, 0, 0, 0
V Saline	193, 60, 152, 202, 103

CHAPTER 20

DISCUSSION

One problem in assessing immunoabsorption or immunoadsorption methods is not knowing the degree to which antibody activity in serum should be reduced after absorption. Immunodiffusion, as used by Murrell (1971) might have been a better serological test since the removal of one or more precipitin lines from the reactions with absorbed globulins compared to unabsorbed globulins would reveal that absorption had occurred. Immunodiffusion was considered as an assessing parameter but as even serum from infected animals did not produce precipitin lines with either ESA or SA, it could not be so employed. This finding is similar to those by Campbell (1938b) and Rickard and Outteridge (1974), who were unable to demonstrate antibody by precipitation, using serum from infected animals. Hence the assessment of the efficacy of absorption had to be based on the values indicated by ELISA.

Absorption Method 1 (Murrell's) was not employed since it did not reliably reduce the level of antibodies detected <u>in vitro</u>. Method 2, using glutaraldehyde to insolubilise the antigens in SA was eventually abandoned. This was mainly because the technique requires a large amount of protein, some of which is probably wasted because it is inside the aggregate and so not exposed to the antibodies. This was a particular problem with ESA, as the neat solution is very low in protein content. The technique is more suitable for working with serum proteins.

By Murrell's method, there was little difference in the ELISA values between unabsorbed and absorbed IRG when ESA and SA was used in the absorption. There are at least two possible explanations for this. Firstly, there might have been far too little antigen present to absorb all the corresponding antibodies. However the observations made in Part II which indicated that the antigen concentration in ESA are high relative to the antibody concentration in serum also suggest that this is unlikely. Secondly, the antigen-antibody reaction may have produced soluble immune complexes. Indeed the failure to demonstrate immune precipitation suggests that this is so. If the antibody is of low avidity, these complexes may dissociate relatively readily, or some of the antibody in the complexes may be free to react with the antigen on the ELISA plate. This would cause a high reading, masking the occurrence of absorption. The 8000 g centrifugal force used was perhaps insufficient to precipitate such immune complexes. If this latter explanation is true, a higher centrifugal force might help in precipitating the immune complexes.

However, when Murrell's method was employed using an insoluble somatic antigen (a washed larval homogenate), the activity of the globulins reacting with ESA in ELISA was reduced substantially compared to that in the unabsorbed sample. It seemed that the antibodies which had absorbed to the larval extract were removed from the IRG sample along with the insoluble antigen. This may suggest that the method is unsuitable if soluble antigens are used with ELISA as the test for assessment. Therefore it was decided to insolubilise these soluble antigens so that antibodies which

react with them would be immobilised to the static antigen. This should eliminate the presence of immune complexes in absorbed IRG samples.

Affinity chromatography appeared to result in improved absorption with soluble antigens, as assessed by in vitro evaluation (Table 28). The assessment of the efficacy of absorption, based on antibody values was interpreted in the light of the titration results shown in Figures 14 and 15 in Part II. These values indicated that the absorption of IRG with ESA and SA had probably reduced the antibody content to 19% and 17% respectively of the initial antibody content in unabsorbed IRG, as detected with ESA in ELISA, and to about 30% and 22% respectively as detected with SA (Table 28). The antibody contents in globulins after direct contact with larvae were 53% and 69% as detected by ESA and SA respectively. The overall results seemed to suggest that antibodies reacting to ESA were more efficiently removed than those reacting to SA by both absorption methods used (Methods 3 and 4).

The presence of antibodies detected by both ESA and SA in the bound globulins which were eluted (Table 26) provides conclusive evidence that some antibodies had bound to the ligands. It was hoped that the unbound fraction would confer less protection against the challenge infection, because the "protective" component had been partially or completely removed. However the results of the passive transfer test (Table 29) show this was not so as, although from Table 28 it is seen that there was a reduction in antibody activity of IRG after affinity chromatography with ESA or SA as

ligand, the unbound IRG fraction still conferred almost absolute resistance. This finding is similar to that of Leid and Williams (1974), who were unable to absorb out the protective antibodies from immune (infection) rat serum with metacestode-derived T. taeniaeformis antigen, despite removal of all precipitating antibody activity as monitored by immunodiffusion.

Kwa and Liew (1978) showed that serum from rats infected with T. taeniaeformis, diluted up to 1:32 gave significant protection ($P < 0.01$) in passive transfer at 1 ml aliquot per rat. At dilutions of 1:64 and greater, there was no longer significant protection. Applied to this study, such titrations of the unbound fractions used in passive transfer studies might have shown some difference in protection levels compared to similar titrated doses of whole IRG. In the light of this it is not surprising that the absorbed fractions which appear to have a reduced antibody content were still fully protective. Clearly the degrees of absorption achieved in this study were far from complete and it might have been better to have used diluted immune globulins in the passive transfer test. Again, however shortage of time has prevented this further investigation. Alternatively recycling the globulins through the same or a different immunoabsorbent might have removed all the protective effect especially if more than one antigen is involved in protecting rats against T. taeniaeformis infection.

IRG which had direct contact with live larvae had the highest residual antibody activity of all IRG treated samples. However, in passive transfer this was the only absorbed globulin fraction

which may have possessed any protective capacity - and this only on the basis of the results in one or perhaps two rats. This lends a little support to the general finding that there is a poor correlation between antibody detected at serology and protective power of a serum. The findings of Smith *et al.* (1981) seems pertinent to this method of absorption. These authors showed that antiserum against ES attached to the surface of *Toxocara canis* larvae *in vitro* only at 2°C. At 37°C, this attachment did not occur due to the release or turnover of ES at this temperature. This suggests that it might have been better to have carried out the entire absorption process of Method 4 at a low temperature or to have used killed larvae.

The failure of affinity chromatography to remove the protective components from IRG, as manifested by results of the passive transfer experiment, probably had several causes. Firstly, the amount of protection-inducing antigen acting as ligand may have been insufficient to remove all the activity. The ELISA results using the same antigen as in the ligand, with the unbound fraction suggests that this is so. Recycling the material through the column several times should solve this problem. Secondly, protective components specific for components in the alternate antigen would not be removed. To do this would necessitate further application onto the alternate ligand. Thirdly, protective antibodies may be present which are not absorbed by either ESA or SA. Perhaps further absorption with insoluble somatic antigen or intact metacestodes might remove any residual protective activity. The various treatment procedures suggested to completely remove the protective

activity of the globulins are based on the assumption that immune rat globulin includes both protective and non-protective antibodies reacting against ESA, SA and probably against other antigens.

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A P P E N D I X

Appendix Table 1

Part I - First Experiment

Corrected mean absorbance values at 450 nm for ESA and SA by ELISA for various dilutions of serum-free used medium.

	Dilutions of used medium					Unused medium
	Neat	1:5	1:25	1:125	1:625	Neat
ESA	0.547	0.515	0.436	0.371	0.265	0.045
SA	0.502	0.461	0.236	0.083	0.050	0.060

Appendix Table 2

Part I - Second Experiment

Mean absorbance values at 450 nm for ESA and SA by ELISA for various dilutions of serum-free used medium, with and without the presence of serum i.e. A_2 in B_2 and A_1 in B_1 respectively.

		Dilution					
		1:1	1:2	1:6	1:16	1:40	1:100
ESA	A_1 in B_1	0.727	0.626	0.544	0.485	0.466	0.434
	A_2 in B_2	0.714	0.665	0.575	0.523	0.453	0.402
SA	A_1 in B_1	0.735	0.627	0.572	0.464	0.323	0.304
	A_2 in B_2	0.827	0.709	0.526	0.446	0.353	0.324

Appendix Table 3

Part I - Third Experiment

Corrected mean absorbance values at 450 nm for ESA and SA by ELISA for used medium in which 3 month and 10 month old metacestodes were maintained

Day of maintenance	ESA		SA	
	3	10	3	10
1	0.916	0.889	0.346	0.355
2	0.860	0.937	0.369	0.363
3	0.819	0.602	0.393	0.289
4	0.843	0.773	0.471	0.345
5	0.668	0.670	0.435	0.224
6	0.601	0.649	0.248	0.235
7	0.628	0.406	0.321	0.297
8	0.390	0.601	0.184	0.340
9	0.436	0.399	0.196	0.159
10	0.557	-	0.247	-
11	0.596	-	0.307	-

Appendix Table 4

Part I - Fifth Experiment

Corrected mean absorbance values at 450 nm for ESA and SA by ELISA for the following used media; Group I (sealed vessels, serum-free), Group II (unsealed vessels, serum-free), Group III (sealed vessels containing serum) and Group IV (unsealed vessels containing serum)

	Group	Day of maintenance								
		3	5	7	9	11	13	15	17	19
ESA	I	0.175	0.230	0.162	0.163	0.170	0.179	0.101	0.142	0.206
	II	0.163	0.141	0.150	0.177	0.253	0.239	0.178	0.133	0.217
	III	0.197	0.228	0.272	0.143	0.120	0.188	0.151	0.186	0.244
	IV	0.281	0.276	0.221	0.191	0.177	0.184	0.141	0.227	0.203
SA	I	0.144	0.156	0.123	0.129	0.161	0.106	0.033	0.067	0.229
	II	0.063	0.057	0.033	0.0	0.0	0.048	0.088	0.064	0.140
	III	0.134	0.182	0.169	0.080	0.129	0.096	0.213	0.318	0.376
	IV	0.181	0.143	0.189	0.151	0.177	0.223	0.251	0.184	0.326

Appendix Table 5

Part I - Sixth Experiment

Corrected mean absorbance values at 450 nm for ESA and SA by ELISA for 3 groups of used media (DT_1, DT_2 and DT_3) in which larvae having damaged teguments were maintained

	Group	Day of maintenance						
		3	5	7	9	11	13	15
ESA	DT_1	0.151	0.140	0.126	0.113	0.138	0.090	0.231
	DT_2	0.305	0.249	0.255	0.142	0.257	0.240	0.271
	DT_3	0.265	0.234	0.206	0.325	0.276	0.147	0.250
SA	DT_1	0.076	0.018	0.051	0.016	0.046	0.021	0.119
	DT_2	0.073	0.084	0.089	0.065	0.094	0.060	0.238
	DT_3	0.136	0.206	0.110	0.137	0.121	0.120	0.147

Appendix Table 6

Part I - Seventh Experiment

Corrected absorbance values at 450 nm for ESA by ELISA for three types of used media, namely: Group I (serum-free), Group II (containing SBCS) and Group III (containing FCS).

Group	Number of replicates	Day of maintenance										
		3	5	7	9	11	13	15	17	19	21	23
I	4	1.292	0.953	0.822	0.794	0.604	0.932	0.718	0.627	0.579	0.578	0.597
		1.134	0.751	0.851	0.761	0.652	0.640	0.605	0.562	0.609	0.607	0.539
		1.868	0.914	0.662	0.682	0.633	0.559	0.626	0.515	0.532	0.650	0.500
		1.639	0.785	0.733	-	-	0.497	0.579	0.602	0.452	0.565	0.710
	Mean	1.483	0.851	0.767	0.745	0.630	0.657	0.632	0.576	0.543	0.600	0.586
II	5	0.678	0.740	0.479	0.759	0.791	0.740	0.604	0.574	0.650	0.602	0.550
		0.936	0.675	0.510	0.876	0.760	0.672	0.570	0.528	0.620	0.581	0.513
		0.848	0.627	0.725	0.945	0.684	0.657	0.500	0.543	0.580	0.603	0.599
		0.652	0.672	0.718	0.806	0.718	1.070	0.672	0.637	0.582	0.672	0.597
		0.831	0.790	0.758	0.884	-	0.608	0.559	0.578	0.633	0.595	-
	Mean	0.789	0.700	0.638	0.854	0.738	0.750	0.581	0.572	0.613	0.610	0.565
III	5	0.810	0.836	0.633	0.521	0.679	0.410	0.408	0.420	0.359	0.439	
		0.939	0.810	0.630	0.649	0.724	0.378	0.415	0.452	0.424	0.495	
		0.753	0.716	0.599	0.578	0.417	0.385	0.434	0.448	0.390	0.446	
		0.945	0.706	0.646	0.723	0.357	0.452	0.479	0.438	0.401	0.608	
		1.069	0.657	0.557	0.592	0.415	0.378	0.421	0.441	0.485	0.503	
	Mean	0.903	0.745	0.613	0.612	0.520	0.400	0.431	0.440	0.412	0.500	

Appendix Table 7

Part I - Seventh Experiment

Corrected absorbance values at 450 nm for SA by ELISA for three types of used media, namely: Group I (serum-free), Group II (containing SBCS) and Group III (containing FCS).

Group	Number of replicates	Day of maintenance										
		3	5	7	9	11	13	15	17	19	21	23
I	4	0.498	0.291	0.925	0.890	0.427	0.442	0.706	0.519	0.480	0.609	0.596
		0.288	0.158	0.275	0.838	0.792	0.721	0.455	0.524	0.553	0.555	0.512
		0.633	1.030	0.341	0.415	0.366	0.284	0.412	0.555	0.508	0.680	0.496
		0.970	0.722	0.868	0.787	-	0.374	0.587	0.474	0.468	0.556	0.660
	Mean	0.597	0.550	0.602	0.732	0.530	0.455	0.540	0.518	0.502	0.600	0.566
II	5	0.567	0.762	0.353	0.252	0.684	0.853	0.413	0.400	0.609	0.592	0.684
		0.817	0.781	0.102	0.635	0.792	0.245	0.501	0.306	0.447	0.495	0.588
		0.401	0.289	0.491	0.881	0.361	0.713	0.431	0.362	0.543	0.424	0.662
		0.330	0.755	0.799	0.730	0.676	0.978	0.490	0.503	0.404	0.640	0.596
		0.683	0.880	0.690	0.759	-	0.586	0.364	0.507	0.465	0.506	-
	Mean	0.560	0.700	0.487	0.651	0.628	0.675	0.440	0.415	0.493	0.531	0.632
III	5	1.036	1.058	0.787	0.840	0.893	0.635	0.393	0.405	0.334	0.427	
		0.771	0.805	1.001	0.724	0.789	0.646	0.600	0.591	0.614	0.673	
		0.475	0.560	0.513	0.664	0.641	0.457	0.645	0.403	0.557	0.634	
		1.004	0.623	1.072	0.978	0.207	0.656	0.439	0.626	0.578	0.725	
		0.609	1.108	0.706	0.423	0.365	0.255	0.295	0.468	0.577	0.607	
	Mean	0.708	0.830	0.816	0.726	0.580	0.530	0.474	0.500	0.532	0.613	

Appendix Table 8

Part I - Seventh Experiment

Values of pH for used media without serum (Group I) and with serum i.e. FCS (Group II) and SBCS (Group III).

Day of maintenance	I	II	III
0 (unused medium)	7.70	7.85	7.85
3	7.40	6.90	7.15
5	7.25	6.95	7.20
7	7.15	6.95	7.10
9	7.35	6.95	7.20
11	7.40	7.10	7.35
13	7.45	7.00	7.10
15	7.45	6.90	7.05
17	7.45	6.95	7.10
19	7.45	6.90	7.00
21	7.30	6.80	7.10
23	7.50	7.10	-

Appendix Table 9

Part II - First Experiment

Chequer-board titrations of sera from uninfected mice and from mice infected with T. taeniaeformis (B) against ESA and SA in ELISA. Twofold dilutions of pooled sera against the antigens at five soluble protein concentrations. Relative absorbance at 450 nm.

Antigen concentration (μg ml^{-1})		Serum dilution 1/50	1/100	1/200	1/400	1/800
				Negative serum		
ESA	1	0.100	0.084	0.075	0.060	0.050
	5	0.150	0.139	0.125	0.107	0.100
	10	0.140	0.134	0.115	0.105	0.085
	15	0.160	0.145	0.125	0.112	0.100
	20		0.140	0.110	0.103	0.090
SA	1	0.120	0.089	0.065	0.049	0.040
	5	0.110	0.096	0.075	0.056	0.050
	10	0.120	0.108	0.090	0.077	0.065
	15	0.125	0.113	0.090	0.073	0.060
	20	0.135	0.122	0.100	0.085	0.070
				Positive serum		
ESA	1	0.474	0.400	0.262	0.214	0.158
	5	0.575	0.513	0 365	0.304	0.232
	10	0.606	0.529	0.364	0.299	0.231
	15	0.601	0.520	0.358	0.301	0.222
	20	0.628	0.543	0.380	0.298	0.228
SA	1	0.448	0.372	0.266	0.203	0.169
	5	0.522	0.459	0.289	0.275	0.247
	10	0.528	0.475	0.272	0.292	0.244
	15	0.556	0.481	0.273	0.312	0.206
	20	0.579	0.508	0.289	0.321	0.228

Appendix Table 10

Part II - First Experiment

Chequer-board titrations of sera from uninfected rats and from rats infected with T. taeniaeformis (M) against ESA and SA in ELISA. Twofold dilutions of pooled sera against the antigens at five soluble protein concentrations. Relative absorbance at 450 nm.

Antigen concentration ($\mu g\ ml^{-1}$)		Serum dilution				
		1/50	1/100	1/200	1/400	1/800
				Negative serum		
ESA	1	0.060	0.048	0.040	0.037	0.030
	5	0.090	0.080	0.070	0.065	0.060
	10	0.095	0.083	0.080	0.077	0.070
	15	0.090	0.086	0.080	0.071	0.070
	20	0.090	0.086	0.080	0.075	0.070
SA	1	0.095	0.090	0.085	0.085	0.085
	5	0.115	0.108	0.105	0.103	0.100
	10	0.115	0.113	0.115	0.119	0.110
	15	0.160	0.154	0.130	0.129	0.120
	20	0.220	0.179	0.150	0.117	0.090
				Positive serum		
ESA	1	0.307	0.214	0.162	0.107	0.075
	5	0.423	0.332	0.231	0.175	0.120
	10	0.429	0.337	0.217	0.176	0.133
	15	0.409	0.305	0.224	0.176	0.123
	20	0.430	0.319	0.230	0.175	0.131
SA	1	0.308	0.239	0.182	0.148	0.119
	5	0.353	0.271	0.220	0.175	0.143
	10	0.377	0.306	0.233	0.193	0.159
	15	0.382	0.320	0.256	0.212	0.176
	20	0.392	0.327	0.259	0.217	0.183

Appendix Table 11

Part II - Second Experiment

Serum glutamate dehydrogenase assays of two groups of mice infected with T. taeniaeformis (B). The enzyme values are expressed in U l^{-1}.

Days after infection	Mice	
	BKW	CF_1
0	2.85	2.38
1	2.86	2.78
3	2.38	2.38
5	2.38	12.70
7	24.34	10.88
14	9.52	8.00
21	5.24	5.44
28	4.76	2.86

Appendix Table 12

Part II - Second Experiment

Circulating antigen in pooled sera samples from mice infected with T. taeniaeformis (B) detected by ESA and SA antisera in ELISA. The values are corrected units of absorbance at 450 nm.

Days after infection	ESA	SA
0	0.125	0.000
1	0.259	0.000
3	0.299	0.000
5	0.403	0.372
7	0.140	0.025

Appendix Table 13

Part II - Second Experiment

Corrected values of absorbance at 450 nm obtained in ELISA for detecting antibodies using ESA and SA, with pooled serum samples from two groups of mice infected with T. taeniaeformis (B).

Days after infection	CF_1		BKW	
	anti-ESA	anti-SA	anti-ESA	anti-SA
14	0.159	0.000	0.080	0.255
21	0.444	0.359	0.252	0.484
28	0.419	0.576	0.165	0.314
35	0.893	0.711	0.370	1.015
42	0.995	0.617	-	-
49	0.981	0.892	0.501	0.562

Appendix Table 14

Part II - Third Experiment

Circulating antigen in the plasma of rats infected with T. taeniaeformis (M) detected by ESA and SA antisera in ELISA. The values are corrected units of absorbance at 450 nm.

Days after infection	ESA		SA	
	Mean	Range	Mean	Range
0	0.025	0-0.070	0.015	0-0.080
1	0.016	0-0.045	0.015	0-0.104
3	0.066	0.042-0.100	0.000	0
5	0.065	0-0.136	0.0054	0-0.043
7	0.022	0-0.084	0.006	0-0.04
14	0.020	0.015-0.026	0.000	0
21	0.033	0-0.074	0.042	0-0.141
28	0.000	0	0.057	0.017-0.085
35	0.020	0-0.086	0.013	0-0.048
42	0.054	0-0.237	0.007	0-0.040

Appendix Table 15

Part II - Third Experiment

Corrected values of absorbance at 450 nm obtained with plasma samples from rats infected with T. taeniaeformis (M), in ELISA for detecting antibodies using ESA. The number of living larvae in the liver of each rat is also included.

Rat No.	Number of larvae in liver	Weeks after infection										
		0	2	3	4	5	6	7	8	9	11	12
1	3	-	-	-	-	-	0.365	0.640	0.483	0.401	0.387	0.361
2	4	-	0.102	0.140	0.440	0.448	0.552	0.631	0.616	0.736	0.610	0.009
3	7	0.047	0.011	0.088	0.267	0.367	0.409	0.447	0.442	0.467	0.505	0.451
4	10	-	-	0	-	0.167	0.280	0.260	0.230	0.300	0.250	0.241
5	12	0.012	0	-	-	0.431	0.450	0.602	0.547	0.600	0.567	0.600
6	13	-	0	0.125	0.153	0.422	0.424	0.458	0.458	0.666	0.464	0.521
7	14	-	-	-	0.248	-	0.592	0.590	0.589	0.592	0.617	0.603
8	22	-	-	-	-	0.345	0.590	0.651	0.630	0.580	0.549	0.541
9	25	-	0.016	0.116	0.190	0.383	0.610	0.523	0.483	0.522	0.500	0.489
10	28	0.014	-	-	-	0.323	0.447	0.417	0.422	0.420	0.439	0.420
11	28	-	-	-	0.085	0.212	0.379	0.387	0.308	0.226	0.245	0.166
12	50	-	0.036	0.185	0.392	0.305	0.616	0.636	0.545	0.514	0.472	0.582
13	51	-	0	-	0.115	0.235	0.489	0.443	0.377	0.361	0.318	0.335
14	54	-	0	-	0.105	0.228	0.288	0.304	0.250	0.223	0.186	-
15	54	-	-	-	-	-	0.679	0.675	0.569	0.481	0.423	0.405
16	57	-	0	0.068	-	-	0.459	0.546	0.301	0.473	0.473	0.462
17	67	0	0	0.242	0.406	0.434	0.590	0.569	0.595	0.504	0.554	0.511
18	124	-	-	-	-	-	0.862	0.842	0.721	0.728	0.763	0.695
19	135	-	-	-	0.590	-	1.018	0.795	0.675	0.639	0.606	0.505
20	179	-	-	-	0.432	0.653	-	0.542	0.377	0.348	0.348	0.516
21	225	0.010	0	0.281	0.564	0.990	1.023	0.702	0.952	0.558	0.532	0.536
22	279	-	-	0.017	0.245	0.800	0.843	0.605	0.478	0.377	0.365	0.365
23	326	-	-	-	0.243	0.343	0.826	0.692	0.611	0.608	0.587	0.620
24	600	-	-	-	0.554	0.737	0.962	0.826	0.678	0.649	-	0.623
Mean absorbance value (< 100 larvae)		0.018	0.016	0.120	0.240	0.330	0.483	0.516	0.461	0.474	0.444	0.418
Mean absorbance value (> 100 larvae)		0.010	0	0.149	0.438	0.704	0.922	0.715	0.642	0.558	0.533	0.551
Total mean absorbance value		0.016	0.015	0.126	0.314	0.435	0.598	0.574	0.514	0.500	0.468	0.459

Appendix Table 16

Part II - Third Experiment

Corrected values of absorbance at 450 nm obtained with plasma samples from rats infected with T. taeniaeformis (M), in ELISA for detecting antibodies using SA. The number of living larvae in the liver of each rat is also included.

Rat No.	Number of larvae in liver	Weeks after infection										
		0	2	3	4	5	6	7	8	9	11	12
1	3	-	-	-	-	-	0.340	0.158	0.122	0.147	0.175	0.182
2	4	-	0.075	0.674	0.152	0.217	0.254	0.437	0.390	0.361	0.349	0.078
3	7	0	0	0	0.022	0.054	0.050	-	0.129	0.145	0.222	0.245
4	10	-	-	0	-	0	0.212	0.265	0.254	0.233	0.279	0.254
5	12	0	0	-	-	0.134	0.114	0.293	0.251	0.313	0.344	0.346
6	13	-	0.071	0.055	0.055	0.113	0.210	0.252	0.253	0.298	0.320	0.359
7	14	-	-	-	0.117	-	0.380	0.427	0.422	0.422	0.523	0.508
8	22	-	-	-	-	0.038	0.143	0.144	0.166	0.163	0.224	0.266
9	25	-	0	0.018	0.038	0.100	0.235	0.201	0.082	0.207	0.144	0.487
10	28	0.022	-	-	-	0.095	0.062	0.268	0.404	0.488	0.609	0.634
11	28	-	-	-	0	0.012	0.194	0.234	0.235	0.169	0.298	0.333
12	50	-	0.117	0.162	0.243	0.151	0.533	0.566	0.499	0.532	0.559	0.572
13	51	-	0.060	-	0	0.057	0.447	0.302	0.255	0.300	0.283	0.324
14	54	-	0	-	0.017	0.160	0.314	0.313	0.285	0.273	0.230	0.353
15	54	-	-	-	-	-	0.395	0.534	0.475	0.501	0.496	0.486
16	57	-	0	0	-	-	0.184	0.230	0.070	0.261	0.311	0.275
17	67	0	0.0153	0.085	0.112	0.198	0.530	0.535	0.598	0.590	0.659	0.776
18	124	-	-	-	-	-	0.673	0.699	0.670	0.663	0.644	0.689
19	135	-	-	-	0.261	-	0.596	0.605	0.537	0.541	0.559	0.647
20	179	-	-	-	0.205	0.377	-	0.517	0.512	0.431	0.562	0.686
21	225	0	0.0075	0.229	0.216	0.422	0.586	0.476	0.602	0.436	0.411	0.548
22	279	-	-	0.046	0.076	0.372	0.536	0.493	0.357	0.251	0.340	0.333
23	326	-	-	-	0.231	0.347	0.764	0.821	0.716	0.650	0.704	0.771
24	600	-	-	-	0.287	0.583	0.789	0.651	0.591	0.453	-	0.567
Mean absorbance value (< 100 larvae)		0.005	0.034	0.124	0.076	0.102	0.270	0.322	0.288	0.318	0.354	0.381
Mean absorbance value (> 100 larvae)		0	0.0075	0.138	0.213	0.420	0.657	0.609	0.569	0.489	0.538	0.606
Total mean absorbance value		0.004	0.0314	0.127	0.127	0.191	0.371	0.410	0.370	0.368	0.402	0.447

Appendix Table 17

Part IV

Corrected mean values of absorbance at 450 nm obtained in ELISA for detecting antibodies using ESA, with sera from sheep hyperimmunised with the larval antigens and from control sheep.

Weeks after hyperimmunisation	Hyperimmunised with:	Sheep A ESA	B ESA	C SA	D SA	E Sham
0		0.255	0.209	0.515	0.245	0.160
1		1.501	1.482	1.095	0.809	0.170
2		1.665	1.506	1.411	0.870	0.172
3		1.608	1.540	1.500	0.693	0.191
4		1.635	1.678	1.465	0.795	0.471
5		1.652	1.647	1.379	0.956	0.196
6		1.675	1.678	1.302	0.930	0.288
7		1.378	1.665	1.441	0.805	0.180
8		1.515	1.728	1.312	0.919	
9		1.465	1.748	1.492	0.947	

Appendix Table 18

Part IV

Corrected mean values of absorbance at 450 nm obtained in ELISA for detecting antibodies using SA, with sera from sheep hyperimmunised with the larval antigens and from control sheep

Weeks after hyperimmunisation	Sheep				
	A	B	C	D	E
Hyperimmunised with:	ESA	ESA	SA	SA	Sham
0	0.303	0.192	0.244	0.567	0.275
1	0.662	1.015	0.988	0.823	0.367
2	0.893	0.935	1.168	0.959	0.283
3	0.867	0.967	1.287	1.037	0.325
4	0.896	1.096	1.098	1.128	0.457
5	0.944	1.026	1.091	1.167	0.259
6	0.887	1.220	1.077	1.097	0.313
7	0.825	1.244	1.217	1.041	0.361
8	0.842	1.284	1.164	1.151	
9	0.806	1.247	1.287	1.210	

Names and Addresses of Commercial Companies

Beckman-RIIC Ltd., High Wycombe, U.K.
Becton-Dickinson (U.K.) Ltd., Wembley, U.K.
Bio Rad Laboratories Ltd., Watford, Hertfordshire, U.K.
BDH British Drug House Chemicals Ltd., Poole, Dorset, U.K.
Cambridge Medical Company, Cambridge, U.K.
Dow Corning, Midland, Michigan, U.S.A.
EMscope Laboratories Ltd., Ashford, Kent, U.K.
Endecotts Ltd., London, U.K.
Eschmann Bros. & Walsh Ltd., Shoreham-by-Sea, Sussex, U.K.
Fisons Scientific Apparatus, Loughborough, U.K.
Gibco Europe Ltd., Paisley, Scotland, U.K.
Gilson, Villiers-Le-Bel, France
Glaxo Laboratories Ltd., Greenfield, U.K.
Greiner Labor Technik (Dynatech Laboratories Ltd.), Billinghurst, U.K.
Hellma (England) Ltd., Westcliff-on-Sea, U.K.
Hughes & Hughes Ltd., Romford, Essex, U.K.
ICI Imperial Chemical Industries Ltd., Macclesfield, U.K.
LKB Instruments Ltd., South Croydon, U.K.
Microflow Ltd., Fleet, U.K.
Miles Laboratories Ltd., Slough, U.K.
Millipore Ltd., London, U.K.
MSE Measuring Scientific Equipment Ltd., Crawley, U.K.
Nordic Immunological Laboratories, Tilburg, The Netherlands
Oxoid Ltd., Basingstoke, U.K.
Pharmacia Fine Chemicals AB, Uppsala, Sweden
Philips Analytical Dept., Pye Unicam Ltd., Cambridge, U.K.
Pye-Unicam Ltd., Cambridge, U.K.
Sarstedt W. (U.K.) Ltd., Leicester, U.K.
Scientific Suppliers Co. Ltd., London, U.K.
Sigma Chemical Co. Ltd., Poole, Dorset, U.K.
Sterilin Ltd., Teddington, U.K.
Vickers Instruments Ltd., York, U.K.
Virtis - The Virtis Co. Inc., New York, U.S.A.
Whatman Ltd., Maidstone, U.K.