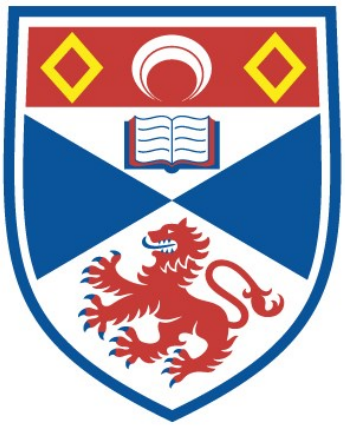
STUDIES ON THE HELMINTH PARASITES OF SOME MARINE BIRDS

Eileen Margaret Shelswell

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1954

STUDIES ON THE HELMINTH PARASITES OF SOME MARINE BIRDS

Investigations into the serological reactions of some digenetic trematodes

A Thesis presented for the degree

of

Doctor of Philosophy

to

The University of St Andrews

by

Eileen Margaret Shelswell

259,622

DECLARATION

I hereby declare that the following Thesis is based on the results of work carried out by me, that the Thesis is my own composition and that it has not been accepted previously for a Higher Degree.

The Research was carried out in the Department of Natural History, in the United College of the University of St Andrews, and in the Serological Museum at Rutgers University, New Brunswick, U.S.A.

CERTIFICATE

I certify that Miss Eileen Margaret Shelswell has spent nine terms at Research work on Studies on the Helminth Parasites of some Marine Birds, that she has fulfilled the conditions of Ordinance No. 16 (St Andrews) and that she is qualified to submit the accompanying Thesis for the degree of Doctor of Philosophy.

University Career and Research Experience.

I entered the University of St Andrews in October 1944 and qualified for the degree of B.Sc. in Botany and Zoology in June 1947. In the following year I read for an Honours Degree in Zoology, but owing to an injury was unable to sit the examinations. In October, 1948, I was appointed Assistant in the Department of Natural History in the United College, and commenced part-time research on the helminth parasites of birds. From September, 1952, I continued this work for one year, at the Serological Museum, Rutgers University, New Brunswick, New Jersey, U.S.A., where I held a Research Fellowship. The results of the research are here presented as a Thesis for the degree of Doctor of Philosophy.

My grateful thanks are due to many people both at the University of St. Andrews and Rutgers University, for guidance and advice during the many phases of this research work. Especially I wish to thank Professor Callan and Mr. Burt for their unfailing interest and assistance throughout the work, and Dr. Boyden and Dr. Stauber, of Rutgers University, for their invaluable help during my visit to the United States of America.

C O N T E N T S.

INTRODUCTION

The original aim of this research was an attempt to correlate the intermediate and adult stages in the life cycle of Cercaria patellae Lebour, 1911, a digenetic trematode. This cercaria is parasitic in the limpet, Patella vulgata Linn., and it was thought that the host of the adult might be some animal feeding on Patella. The more orthodox methods of investigation into the life history of a trematode are not applicable in this particular case, owing to the practical difficulties of keeping sea birds and to the fact that, so far, limpets have not been reared in the laboratory in sufficient numbers or in the stages necessary for experimental purposes. It was decided, therefore, to attempt to solve the problem with the aid of serological methods.

As a guide, an ecological survey of the occurrence of the parasite was undertaken, a survey which confirmed the original theory that the definitive host was a bird. Birds common to the neighbourhood where Cercaria patellae is found

abundantly were examined for trematode parasites. Those found included a fluke which is inadequately described in the literature, and a detailed account of it is given in an appendix to this thesis.

Choice of serological method fell first upon the precipitin reaction, named by Myers (1900) and used extensively by Nuttall (1904) in his investigations into the phylogeny of animals. When foreign proteins (or certain other substances) are injected into the body of an animal, an antiserum is produced which reacts with the solution of the original proteins, forming a precipitate; this can be used as a method for establishing the identity of proteins from different sources. Since all stages in the life cycle presumably contain some protein, or protein grouping, in common, it should be possible to establish the identity of the intermediate and adult forms of any one trematode.

No difficulty was anticipated in applying the precipitin reaction, Wilhelmi (1940), in particular, giving precise directions for a procedure which he claimed would enable the relationships of helminths,

whether adult or larval, to be determined. This technique was followed, the only modification being the substitution of photo-electric measurement of the reaction, which recent workers have found to be more discriminating than the older ring test. A nephelometer, measuring the amount of light scattered by the precipitate at right angles to the incident beam, was built and tested successfully with an anti-human-serum. The antiserum to Cercaria patellae, however, prepared according to the method of Wilhelmi, failed to show any measurable reaction. This method, which uses an antigen from which lipoids have been removed, should give an antiserum which, though highly specific, has a relatively low activity. When the first antisera produced by this method failed to give a positive reaction, attention was concentrated on the production of an antiserum which would give a good homologous test, regardless, in the first instance, of its specificity. A number of different ways of preparing the antigen for injection were therefore tested, in the hope of obtaining a

stronger extract, and injections were given of differing amounts and according to different schedules, as recommended by various workers. All these antisera gave negative results in tests measured by the nephelometer. Finally, anaphylaxis was produced in one of the test rabbits, and this showed, in all probability, that antibodies were being produced. This reaction, however, always difficult to produce in rabbits, cannot readily be used as an exact quantitative method of measurement, even if more suitable animals are used for the tests. It remained to be seen whether some other test might reveal the presence of antibodies circulating in the blood stream of experimental animals.

At this time, an opportunity to visit Rutgers University, New Jersey, U.S.A. arose, and the work was continued there. The logical step seemed to be to explore the serological reactions of trematodes on as wide a basis as possible, using material available locally, with a view to establishing a technique which could be applied to the investigations into the ontogeny of

Cercaria patellae. A number of rabbits and domestic fowl were therefore injected with extracts and suspensions of cercariae from the mud snail, Nassa obsoleta (Say, 1822), and in addition, one rabbit was injected with Cercaria patellae, kindly collected and prepared for me by Mr. Burt in St Andrews. The strengths of the resulting antisera were estimated by the ring test and by some tests on living cercariae (a field which has hitherto been little explored) and more encouraging results were obtained. A few further experiments on anaphylactic shock were also carried out, to investigate the possibilities of using guinea pigs for demonstrating the reaction.

In addition to the above tests, a short investigation was pursued into the production of antibodies during an active infection of a normal or semi-normal host. For this purpose, I used living cercariae of Schistosoma variglandis (Miller and Northup, 1926) for an exporimental self-infection; these schistosome cercariae normally penetrate the skin of gulls.

On my return to St Andrews, only a limited time was available and one of two courses was open: either, to investigate systematically one, or perhaps two, of the many variable factors in the reaction, or alternatively, to attempt to solve the original problem - the life history of Cercaria patellae - with the techniques and material available. The second course was adopted, for it was felt that the experience gained in the attempt would enable an appraisal of the method to be made, and would indicate more clearly the lines along which future work should proceed. Rabbits and domestic fowl were therefore sensitised to Cercaria patellae (and Cercaria B Crewe, 1951, also parasitic in Patella vulgata) by various methods, and, using the ring test technique, some satisfactory reactions were obtained with larval trematodes. Adult flukes proved to be difficult to obtain at this time, but a small supply of one species (an echinostome) and a single specimen of another (a psilostome) were collected. Extracts of these were made and tested against the strongest antiserum to Cercaria patellae, in both cases with negative results. The outcome of these tests was disappointing, but not altogether

unexpected in view of the very small quantity of material available.

PART I

The first part of the thesis comprises morphological descriptions of the cercariae used, and a discussion of certain ecological factors. This is followed by a brief survey of the trematode parasites found in birds, collected at St Andrews mainly between September, 1949, and June, 1952.

Parasites of *Patella vulgata* Linn. and of *Nassa obsoleta* (Say, 1822)

During the course of this work numerous samples of *Patella vulgata* Linn., from the neighbourhood of St Andrews, were examined. As a result of such examinations, the frequent occurrence of *Cercaria patellae* was confirmed, as well as the occurrence of *Cercaria B* Crewe, 1951, a metacercaria (probably that recorded by Crewe, 1951) and a larval cestode, *Cysticercoid D* Crewe, 1951. The latter parasite was found infrequently and never in great numbers. It was noted that the hooks of this cysticercoid agree in number, size and arrangement with those described in *Ophryocotyle proteus* Friis 1869, a parasite of the Herring Gull, the intermediate host of which is hitherto unrecorded. No further detailed examination, however, was made.

It is impossible to recognise an infestation of a limpet with certainty, except by the removal of the shell (Crewe 1951). This is done by cutting round the shell muscle, as close to the shell as possible, with a sharp scalpel. Heavy infestations of rediae of *Cercaria patellae* in the digestive gland

Figure 1

Heavily parasitised limpet, showing white threads in the blood vessels of the mantle.

are visible upon removal, by light scraping, of the pigment over the tunica propria - the membrane covering the visceral mass. Lighter infestations can be distinguished at once after removal of this membranous covering. In the great majority of cases, macroscopic examinations, only, were made of the limpets for the purpose of investigating the occurrence of the parasites. On many occasions Cercaria patellae and Cercaria B were each found accompanied by encysted metacercariae, and in one case a double infestation of Cercaria patellae and Cercaria B was observed in one limpet. Crewe, however, reports only four cases in which Cercaria B was found with metacercariae and none of Cercaria patellae with metacercariae.

Very heavily infested limpets can often be recognised by the appearance of the mantle, before the removal of the shell, (Figure 1). In such cases the mantle is swollen and orange in colour, while small white threads or patches can be seen in the blood vessels. It was found, however, that such appearances do not necessarily indicate infestation only by Cercaria patellae as Crewe states, although Cercaria patellae was responsible for the appearance of infestation in the greater number of limpets

examined; see Table I. In this table, the numbers of limpets infested by Cercaria patellae and Cercaria B include a certain number in which metacercariae were also found. Since such cases have no direct bearing upon the numbers of apparent infestations, the effect being merely additive, they are not listed separately.

Table I.

To show that the appearance of infestation of Patella vulgata is not always caused by Cercaria patellae.

Total No. apparently infested	No. of limpets actually infested.			
	C.patellae	Cercaria B.	Metacercariae only	Uninfest
62	39	13	8	2

Expressing the number of limpets actually infested as percentages of the total apparently infested, the values become:-

63%	21%	13%	3%

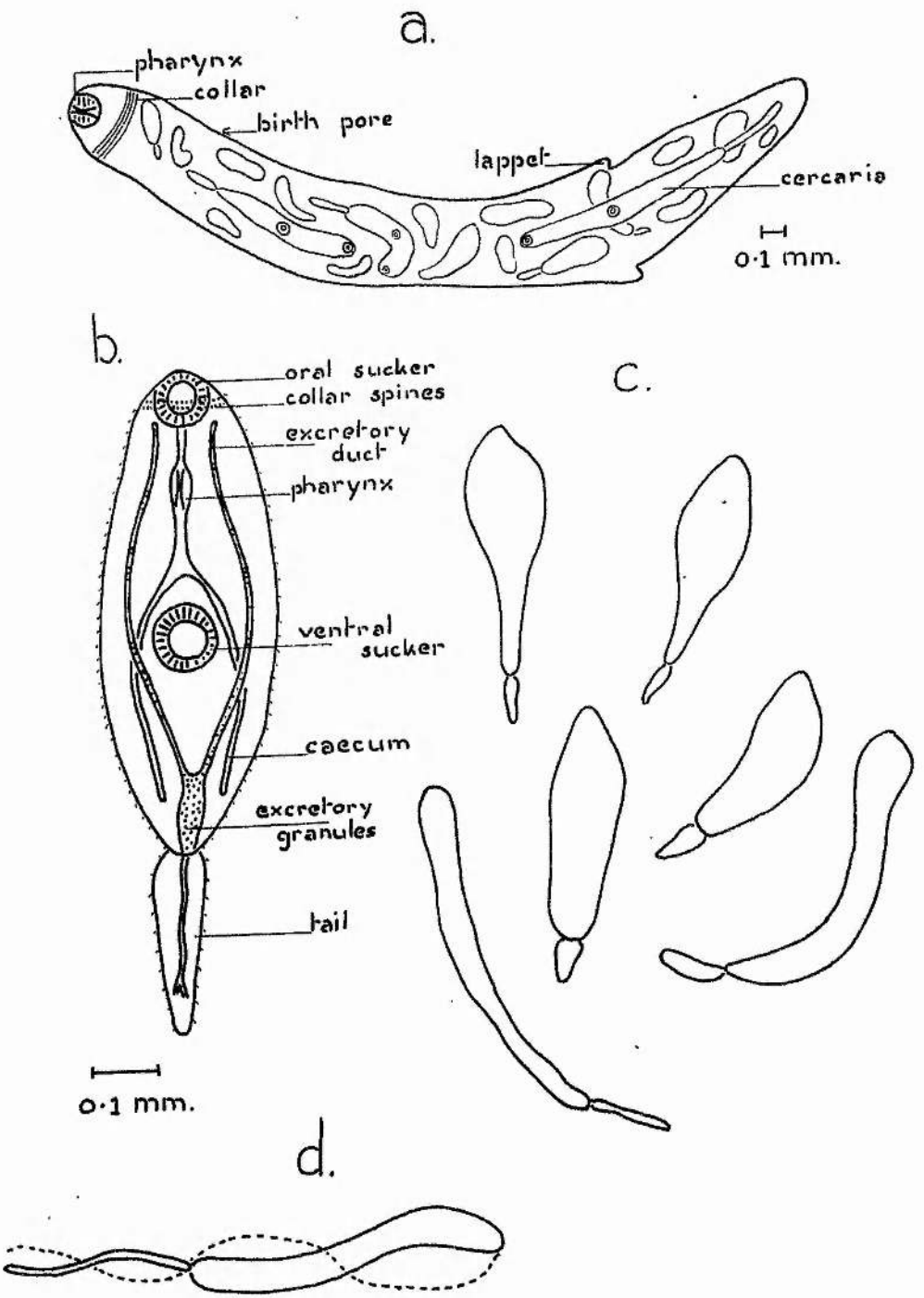
The morphologies of the trematode parasites of Patella vulgata were examined and are described in the following pages.

ECHINOSTOME CERCARIAE.

Cercaria patellae Lebour, 1911. (Figure 2)

The rediae of this cercaria develop in the gonad and digestive gland of Patella vulgata Linn., appearing as distinct white threads in these tissues of the

Figure 2. Cercaria patellae



a). General morphology of a redia.
b). " " " " cercaria.
c). To show the typical variation in form during contractions of the body of a cercaria.
d). To illustrate the eel-like swimming movements of a cercaria.

host. After several generations of rediae, cercariae are produced which emerge by way of the blood vessels in the mantle of the host.

The rediae are large (length 3.45 to 3.9 mm, breadth 0.44 to 0.48 mm), with two well developed lappets situated in the posterior third of the body. A collar is present a short distance from the anterior end, and a birth pore lies posterior to this. At the anterior extremity an oval orifice leads into a well developed pharynx (diameter 0.09 mm); no intestine was observed. A redia may contain developing rediae or cercariae but no individuals were observed to contain both at the same time. When freed from the tissues of the host, they are sluggish and very inactive.

During its fairly short free-swimming existence the cercaria may swim actively with a quick eel-like motion, with body and tail fully extended (Figure 2d), or creep along the substratum by means of its suckers. The body is capable of considerable muscular extension and contraction, and the shape of the body may vary greatly, (Figure 2c).

The form of the cercaria is approximately pear-shaped or oval (length 0.5 to 0.9 mm, breadth 0.13 to 0.25 mm). The tail, also capable of

muscular contortion, is fairly short being about one third of the length of the body. The oral sucker is terminal in position and is slightly smaller than the ventral sucker (diameters 0.066 mm and 0.08 mm respectively); the latter is situated in the middle of the body. The cuticle is spiny with a double crown of very slightly larger spines in the region of the oral sucker. It was not found possible either to count the number of spines in the crown or to measure their size owing to their extreme smallness.

There is a fairly long prepharynx and a small, approximately oval, pharynx. The oesophagus bifurcates a short distance anterior to the ventral sucker and the caeca, narrow simple sacs, extend to the posterior end of the body.

The excretory system includes two longitudinal ducts, extending posteriorly from the region of the oral sucker to the excretory bladder at the posterior extremity of the body. There is also a small duct in the tail leading from the vesicle. Both the ducts and bladder are full of small excretory granules. A few flame cells were seen, but as these did not comprise the total, a plan of arrangement could not be made out.

XIPHIDIOCERCARIAE.

Cercaria armatae Lühe, 1909.

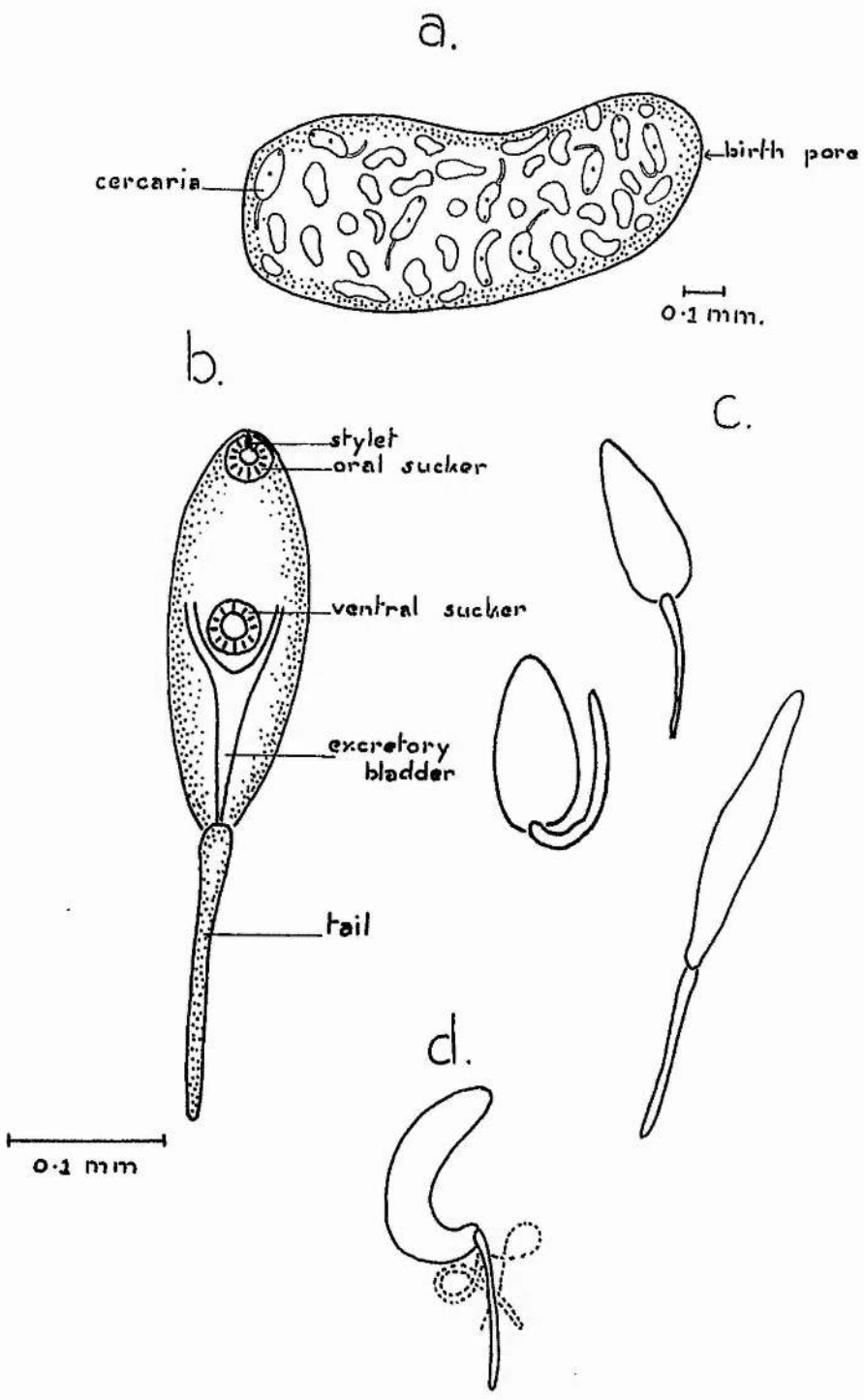
Cercaria B Crewe, 1951 (Figure 3)

Cercaria B inhabits the digestive gland and excretory organ of Patella vulgata Linn. In a limpet harbouring this parasite, the heavily infested region of the digestive gland appears as a bright orange mass of soft, rather loose texture; in lighter infestations the affected area of the digestive gland is cream in colour. The infested masses within the digestive gland consist almost entirely of the sporocysts of Cercaria B which may contain further generations of sporocysts, or cercariae.

The sporocysts are slightly elongate with a terminal birth pore. The length varies from 1.2 mm to 1.5 mm and the breadth from 0.39 mm to 0.44 mm. They are pale orange in colour, this colour being due to pigment cells within the walls of the body. The only movement they exhibit is due to the contained cercariae.

The cercariae emerge during the hours of daylight, by way of the blood vessels of the mantle, and swim very actively. The frequency with which the cercariae emerge was not ascertained as, owing to their small

Figure 3. Cercaria B.



a). General morphology of a sporocyst.
b). " " " " cercaria.
c). To show the typical variations in form during contractions of the body of a cercaria.
d). To illustrate the characteristic swimming movements of a cercaria, effected by a lashing of its tail.

size and the absence of extremely close examination, they were only seen occasionally when they emerged in enormous numbers. This emergence of cercariae was not observed by Crewe. The swimming movement is effected by a ventral curvature of the posterior tip of the body accompanied by a violent lashing of its whip-like tail. (Figure 3d).

The cercaria is small and scarcely visible to the naked eye (length 0.21 to 0.33 mm, breadth 0.045 to 0.16 mm); it is approximately oval in shape and capable of considerable muscular contraction. The tail is long and muscular. The oral sucker is sub-terminal in position and of the same size as the ventral sucker which is situated in the middle of the body (diameter 0.04 mm). The oral sucker bears a small stylet on its anterior border measuring 0.01 mm in length.

The excretory vesicle is Y-shaped, the stem of the 'Y' extending almost to the centre of the body. Further details of structure were obscured by the mass of pale orange pigment cells lying within the walls of the body.

METACERCARIAE

Encysted metacercariae were found throughout the tissues of Patella vulgata, both in limpets otherwise unparasitised as well as in specimens also parasitised by C.patellae or Cercaria B. These metacercariae were examined to ascertain whether they were either C.patellae or Cercaria B or some unknown third species of cercaria.

Observations on the living cysts, under compression from a coverslip, or of sectioned material, revealed little detail of morphological structure. The oral and ventral suckers are prominent, the latter being situated in the middle of the body. The metacercaria lies within a fairly thick-walled cyst (approximately 0.02 mm thick) and the mean internal diameter of the cysts measured 0.133 mm. The mean volume of the cysts, assumed to be spherical, was found to be approximately the same as the mean volume of Cercaria B and about one tenth that of C.patellae. A measurement of the approximate volume of a cercaria was obtained by assuming it to be a prolate spheroid (formed by the rotation of an ellipse about its major axis). The volume of such a figure is given by the formula $\frac{4}{3}\pi ab^2$, where a and b are the major and minor semi-axes respectively.

This formula was applied to the average measurements of Cercaria B and C.patellae and the volumes thus computed, together with those of metacercariae, are given in Table II.

Table II.

The approximate volumes of cercariae and metacercarial cysts.

	Average volume - mm^3
C.patellae	0.0132
Metacercariae	0.00124
Cercaria B	0.00146

The cysts were too small to allow of dissection, but it was found that metacercariae could be liberated by digestion of the cyst wall, if left overnight in a 5% solution of trypsin. The emerged metacercariae had no distinctive characteristics but, apart from the absence of a stylet, they were markedly similar to Cercaria B, both in appearance and measurements.

The conclusion, thus reached, that the metacercariae develop from Cercaria B, was confirmed by evidence obtained from observations on the frequency with which metacercariae were found to be associated with Cercaria B. Examination of various samples of limpets, collected from an area on the East Rocks, St Andrews (see page 22) over a period

of six months, gave the following results:-

Table III.

Association of cercariae and metacercariae

	No. examined	No. with cysts	% infestation by cysts
Total No. of limpets	1,648	149	9.05
No. of limpets with C.patellae	118	13	11.0
No. of limpets with Cercaria B	64	31	48.5

It can be seen from this table that the number of limpets which contain both C.patellae and metacercariae is approximately 11% of all those which are infested with C.patellae. This is not significantly different from the proportion (9%) of the total number of limpets containing metacercariae, and it indicates that the presence of C.patellae has no effect upon the chance infestation by metacercariae, the two forms existing together as a true double infestation. On the other hand, the number of occasions on which metacercariae were found in association with Cercaria B is nearly 50% of all those parasitised by this species of cercaria. This correlation indicates that the metacercariae are those of Cercaria B, having encysted in the limpet in which they have developed.

It should be noted, however, that Cercaria B has been observed to emerge from limpets, and some cercariae, therefore, will encyst in other hosts.

Thus there is both morphological and statistical evidence to support the assumption that the metacercariae found in Patella vulgata are Cercaria B.

In addition to the examination of the parasites of Patella vulgata, trematode parasites of Nassa obsoleta (Say, 1822), the mud snail, from the shores of New Jersey, U.S.A., were collected and examined.[‡] The following cercariae were found with varying frequency:-
Cercariaeum lasium (Leidy, 1891), Cercaria quissetensis (Miller and Northup, 1926), Cercaria setiferoides (Miller and Northup, 1926), Cercaria tenue (Miller and Northup, 1926) and Cercaria variglandis (Miller and Northup, 1926).

It was not possible to distinguish parasitised individuals by external appearances and the presence of an infestation could only be established by observing the emergence of cercariae or by crushing the shell and examining the gonad and digestive gland for rediae or sporocysts. The former method was used

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[‡]My thanks are due to Mr. John McDermott for assistance with the initial identification of these cercariae.

most extensively. Since the cercariae, only, were used for serological studies, detailed examinations were confined to these and reference to earlier stages in the life histories of the cercariae is omitted.

Since all the cercariae examined are adequately described in the literature, comment here is restricted to some observations upon the behaviour of the living cercariae.

CERCARIAE

Cercariaeum lasium (Leidy, 1891) (syn. Cercariaeum lintoni (Miller and Northup, 1926), the cercaria of Zoogonus lasius (Leidy, 1891).

The morphology has been fully described by Miller and Northup (1926).

This cercaria emerges from its host in fairly large numbers during the hours of daylight. When newly emerged, it is active, moving in a leech-like fashion by means of its two suckers. It may also attach itself to the substratum by means of a haptor-like posterior end and raise its body vertically. Since it is tail-less it is incapable of swimming. The cercariae frequently aggregate in large numbers, attaching themselves to others, apparently by either sucker or by the haptor-like posterior end; in this state they tend to adhere

closely to any surface with which they come in contact.

ECHINOSTOME CERCARIAE.

Cercaria quissetensis (Miller and Northup, 1926), the cercaria of Himasthla quissetensis (Miller and Northup, 1926).

This cercaria has been fully described in the original paper and by Stunkard (1938 a). It emerges from the snail during the hours of daylight, sometimes in large numbers, swimming actively. It is negatively phototropic and positively geotropic. When swimming the body is contracted, with the posterior tip and extended tail bent ventrally, while the tail lashes violently from side to side (Figure 4a). It is also capable of creeping along the substratum with a leech-like movement.

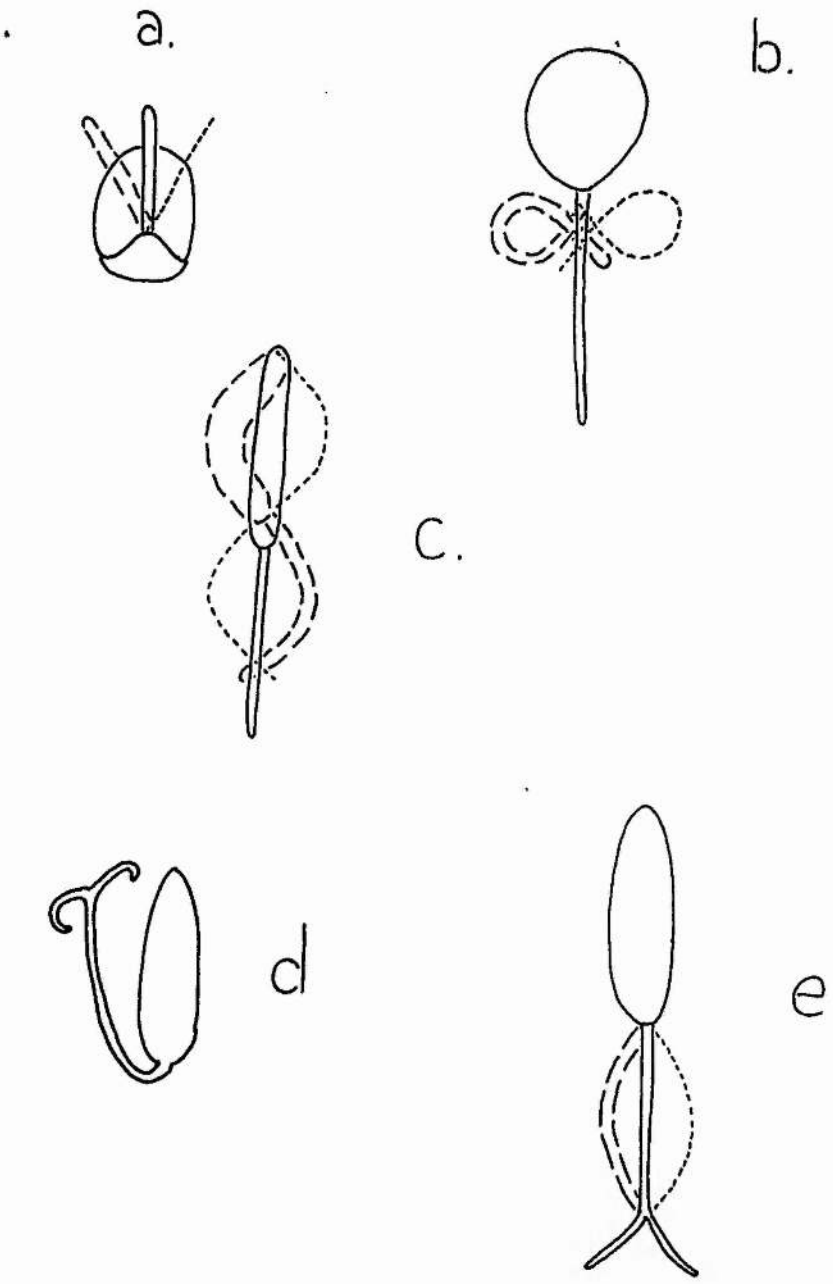
TRICHOCERCOUS CERCARIAE.

Cercaria setiferoides (Miller and Northup, 1926), the cercaria of Lepocreadium setiferoides (Miller and Northup, 1926).

A detailed description of this cercaria has been given by Martin (1938) who investigated its life history. It emerges from its host during the hours of daylight and swims with violent movements. While swimming, the body is contracted and the tail lashes vigorously from side to side forming a figure of

Figure 4.

Diagrams to illustrate the characteristic movements of some cercaria from Nassa obsoleta.



a). Swimming movements of C. quissetensis.
b). " " " C. setiferoides.
c). " " " C. tenue.
d). Characteristic form of C. variglandis attached to surface film.
e). Swimming movements of C. variglandis.

eight (Figure 4b). It exhibits negative phototropism.

GYMNOCEPHALOUS CERCARIAE.

Cercaria tenue (Linton, 1898), the cercaria of Stephanostomum tenue (Linton, 1898).

This cercaria is adequately described by Martin (1939). They emerge in comparatively large numbers during the hours of daylight and swim by a violent lashing of body and tail which, together, appear to form a figure of eight (Figure 4c).

FURCOCERCOUS CERCARIAE.

Cercaria variglandis (Miller and Northup, 1926), the cercaria of Schistosoma variglandis (Miller and Northup, 1926).

An adequate description is given in the original paper by Miller and Northup.

The cercariae emerge from the host somewhat intermittently, usually in large numbers, during the early hours of daylight. They have a characteristic swimming movement moving tail first; the tail itself has the appearance of a plucked wire (Figure 4e). Shortly after emerging, the cercariae attach themselves to the surface film of the water, remaining practically motionless, usually with the

tail bent towards the body (Figure 4d). If disturbed they will adhere closely to any object with which they come in contact.

Ecological and Statistical Survey of the Infestation of *Patella vulgata*

The application of serological methods to the ontogeny of helminth parasites is largely in the experimental stage, and it is necessary, where possible, to obtain confirmatory evidence of any conclusions reached. For this purpose, an ecological and statistical survey of parasitised limpets was undertaken, mainly in the area of St Andrews. Such a survey is unlikely, in itself, to provide conclusive evidence of the relationship of intermediate to adult forms, but it may indicate possible relationships and serve to narrow down the field of research, besides providing confirmation of conclusions reached by other methods. Most of the collections used in this survey were taken from a standard collecting ground on the East rocks at St Andrews, Fife, covering approximately 7,000 square yards, a general view of which is shown in Figure 5. There were available also the results of a series of fifty-one weekly collections, made by Mr. Patrick

Figure 5

General view of the collecting area on the East Rocks, St Andrews.

(see Figures 6 & 7 for closer view of area AB)

B

of the Gatty Marine Laboratory, from similar rocks north of St Andrews pier, about three quarters of a mile north-west of the main collecting area.

Littoral Distribution.

It has been suggested (Nicoll, see Lebour, 1911, and later Crewe, 1951) that the adult form of C.patellae inhabits some sea bird, infestation of the limpet occurring from the faeces of those birds as they feed among the rocks. The simplest test of such a theory is a comparison of the incidence of parasitism between limpets, exposed only at the level of low tide, and those nearer the high tide level and particularly the limpets on any rocks known to be highly frequented by birds.

Three samples of limpets were taken from rocks in the standard collecting ground. The first sample was of five collections, each of 100 limpets, made at various times between November, 1951, and April, 1952, from rocks exposed between half tide and low tide. The second sample was precisely similar to the first, except that the limpets were taken from rocks exposed between half tide and high tide. The third sample, totalling 452 limpets, was of ten separate collections varying from 26 to 55 in each, taken between March and May, 1952, from certain

rocks some distance out but still exposed, as islands, at half tide to high tide. Some of these were cut off from surrounding rocks by channels four to five feet deep even at low tide, and all were know to be much frequented by birds (see Figure 5). The results are shown in Table IV.

Table IV

Percentage infestation of limpets by *C.patellae* Cercaria B and metacercariae collected from different parts of the shore

Rocks Exposed	No. in sample	Percentage infestation		
		C.patellae	Cercaria B	Meta-cercariae
Half to low tide	510	0.82	1.23	3.89
Half to high tide	487	8.82	4.7	10.4
"Island" rocks	452	11.7	6.0	12.8

The variations in the incidence of infestation shown in this table are highly significant, and show that there is a high rate of infestation among limpets in the high tide zone and even higher among limpets on island rocks. Such areas are those in which birds feed as the tide begins to ebb.

Figure 6

Closer view of collecting area AB

(see Figure 5)

These rocks are cut off by an in-coming tide, and on such rocks limpets were found to be heavily parasitised.

Figure 7

Closer view of the rocks in the foreground of Figure 6.

Crewe (1951) mentions that a particularly high percentage infestation is found among limpets in rock pools at Trevone, Cornwall. Most of these limpets (85%), however, were Patella depressa, a species absent from the neighbourhood of St Andrews. A similar survey of P.vulgata in this neighbourhood was not carried out, since the number inhabiting pools was comparatively small, but it was found that limpets from gentle, landward slopes covered with Fucus serratus (see Figures 6 and 7) showed a high rate of infestation. It is reasonable to assume that rock pools and algae have a similar effect upon eggs or miracidia, providing some measure of protection and lengthening the viability of such organisms.

Distribution around the coast.

Crewe examined limpets at fourteen places round the coast of Britain and found C.patellae at ten of these and Cercaria B at three. Unfortunately many of his samples are too small for a reported absence of infection to have any significance, particularly in the case of Cercaria B where the proportion of limpets affected was very low. There seems to be no doubt, however, that, in some of these localities, one or both of

these parasites were either absent or present in very small numbers. However, since the location is often not given with any precision and there is no indication of the types of shore from which collections were made, nor of the birds frequenting them, the individual items in Crewe's table are of little importance in drawing ecological conclusions. Crewe's counts, however, showed that out of a total of 3,777 limpets examined, 146, or 4%, were infested with C.patellae (or, considering limpets from infested areas only, 4.8%). This is reasonably close to the figures from St Andrews (5.7% for collections from the standard collecting area, and 5.3% from an area close by - Mr. Patrick's counts) and indicates that, given a suitable littoral environment, C.patellae is widely and fairly evenly distributed round the coasts; this in turn points to the fact that the final host, a bird as indicated in Table VI and following text, is both fairly abundant and widely distributed.

In contrast with the incidence of C.patellae, parasitism by Cercaria B at St Andrews is much more frequent than in any area examined by Crewe (0.3% overall, or .88% from those localities where the parasite was found); the examination of six monthly

collections (1,196 limpets) from the standard collecting ground showed a percentage infestation of 3.1. It would appear that the environment of St Andrews is favourable to Cercaria B, and one possible cause is an unusual abundance of the final host. It must be emphasised, however, that the frequency of occurrence of any parasite is influenced by a number of ecological factors, and a variation in the population cannot be attributed to any particular one unless an extremely close correlation can be demonstrated. Although uniformity of a population probably indicates an approximate constancy in the various ecological factors involved, this is not necessarily the case, since two periodically occurring factors may cancel one another out. Further, if the parasite remains in the mollusc for a period of some years, annual variations will tend to be obscured. The possibility of such variations being obscured completely must surely be slight.

The scarcity of both parasites in certain parts of the coast is shown, not only by Crewe's counts, but by three made in the course of this work, in localities other than St Andrews. Two of these were

at Auchmithie, Angus, collection A being from the vertical surfaces of rocks which offered no resting places for birds, while collection B was from horizontal surfaces nearby, in close proximity to a cliff where hundreds of Herring Gulls were resting. The third collection was made at Baltasound, Unst, in the Shetland Isles. The results of these collections are shown in Table V, where they are compared with figures of percentage infestation at St Andrews.(In the latter, the figure for C.patellae includes a very small number of cases in which metacercariae were also present and that of Cercaria B includes slightly more. The count of metacercariae, however, includes no cases of parasitism by either C.patellae or Cercaria B.)

Table V

Infestation of limpets in certain areas of Scotland and the Shetland Isles.

District	No. of limpets in sample	C.patellae	Cercaria B	Meta-cer-cariae
Auchmithie A	100	0	0	1
Auchmithie B	100	2	0	3
Unst	100	0	0	0
St Andrews mean values	5,124	5.72%	(3.1%)	8.0%

The mean percentage infestation of Cercaria B at

St Andrews (in brackets) is taken from a smaller sample of limpets, a total of 1,196 being examined for this parasite. Although the samples from the different areas were small, there is no doubt that the first and third collections indicate an exceptionally low incidence of infestation by C.patellae. The fact that the Auchmithie B count is low, although very large numbers of Herring Gulls were present, indicates that this bird is probably not the final host of C.patellae. It may be significant that Auchmithie is a small cove in the midst of six miles of cliff, a type of coast also found in Unst. In a comparison with birds found on the East Rocks, St Andrews, those commonly found at Baltasound, Unst, include Herring Gulls, Greater Black-backed Gulls and Oyster Catchers; the presence of Redshanks and Turnstones was not ascertained, but, in general, the shore offers little scope for waders. However, considerably larger samples from more districts would have to be correlated with a rough census of the bird population before evidence of this type could be considered conclusive.

Table VI. The results of examinations of monthly collections over a period of two years.

Year	Month	No. examined	No. infested with rediae	% infestation by rediae	No. infested with cysts	% infestation by cysts
1950	March	210	5	2.4	5	2.4
	April	248	13	5.2	6	2.4
	May	202	16	7.8	6	3.0
	June	229	14	6.1	10	4.4
	July	201	13	6.4	13	6.4
	August	201	6	3.0	22	11.0
	September	-	-	-	-	-
	October	214	23	10.8	10	4.7
	November	196	10	5.1	25	12.8
	December	187	3	1.6	28	15.0
1951	January	198	13	6.6	29	14.6
	February	196	10	5.1	20	10.2
	March	207	6	2.9	35	16.9
	April	198	12	6.1	16	8.1
	May	222	10	4.5	10	4.5
	June	202	10	5.0	11	5.5
	July	203	16	7.8	21	10.3
	August	198	18	9.2	20	10.1
	September	215	16	7.4	18	8.4
	October	201	15	7.5	15	7.5
	November	197	11	5.6	15	7.6
	December	199	15	7.5	17	8.5
1952	January	200	11	5.5	10	5.0
	February	196	12	6.0	20	10.1
	March	197	5	2.6	17	8.6
	April	205	11	5.4	12	5.8
Mean		204.96	11.76	5.72 ± 2.13	16.24	8.14 ± 3.94
Total		5124	294		411	
% from totals				5.72		8.02

Correlation between Occurrence of Infestation and Time.

An investigation was carried out over a period of twenty-six months to find whether the incidence of infestation varied with time. The size of the population of a parasite depends upon a number of factors, but if equilibrium is reached, loss must be balanced by fresh infestations; (loss may occur when an infested host dies or gets rid of its parasites). Equilibrium will not, in fact, be reached unless such factors are reasonably constant. As is seen in Table VI, the population of C.patellae remained almost constant over the whole period and this may well indicate that the source of infestation, the final host - presumably a bird - is present in numbers which remain approximately constant throughout the year. The adult of C.patellae is not likely, therefore, to be Echinostephilla virgula Lebour, 1909, from the Turnstone as suggested by Nicoll (see Lebour, 1911) for the Turnstone is a migrant bird present in St Andrews in large numbers from September to April and completely absent in the late spring and summer. In addition, it was found that E.virgula was only present in Turnstone intermittently. This particular

parasite was frequently present in large numbers in the Turnstones examined between September, 1949, and March, 1951, (five birds out of a total of six examined were infested with E.virgula) but was almost entirely absent from the flocks which wintered in the neighbourhood of St Andrews during the following year and again in 1953; one specimen was found out of twenty birds examined. (No birds were examined during the winter months of 1952).

Records of infestation by sporocysts of Cercaria B were only made during the latter part of this survey and the number of counts is insufficient for statistical purposes. However, complete records of the numbers of limpets infested with metacercariae assumed, now, to be cysts of Cercaria B, were kept throughout the period and these serve to indicate that this infestation, though less constant than that of C.patellae, also showed little regular seasonal variation which could be correlated with bird migration. It may be true, and this seems likely, that the length of time during which metacercariae inhabit a limpet is longer than the time taken for sporocysts and cercariae to mature. Variations in the infestation by metacercariae are, therefore, a less sensitive

indication of any fluctuations in the appearance of the final host.

Method of sampling.

Samples of two hundred limpets were collected at monthly intervals from the standard collecting area and examined macroscopically for parasites. Each collection was intended to be a random sample of the area, individuals being taken from points more or less uniformly scattered. It has already been shown that the incidence of infestation varies over the area and, further, Crewe showed that a heavier infestation exists among large limpets. Care had to be taken, therefore, to make each collection truly representative of the area. The results of this sampling are given in Table VI. In addition, the counts of limpets infested with C.patellae, in samples of 100 individuals collected by Mr. Patrick, were available. These collections extended over one year, but in this case only those limpets infested with rediae of C.patellae were recorded.

The figures thus obtained were analysed to find whether the variations from sample to sample could be accounted as random sampling errors, or whether they showed any systematic trend, in particular, any

seasonal trend. The method adopted was to work out the standard error of percentage infested, in random samples of the size taken, using the formula

$$\text{Standard error of percentage} = \sqrt{\frac{\text{Mean \%} \times (100 - \text{mean \%})}{\text{number in sample}}}$$

Any deviation from the mean percentage infested of more than twice this standard error is significant at the 5% testing level, i.e., it may be expected to occur only once in twenty samples.

With application of this test to the figures of redial infestation in Table VI, the standard error is 1.62, from which it can be seen that all percentages between 2.48 and 8.96 may be accounted as normal sampling errors. The fact that four of the counts - March, October, December, 1950, and August, 1951 - lie outside these limits is probably due to the difficulty of obtaining truly random samples. Application of the same test to Mr. Patrick's counts proved to be equally satisfactory. A more reliable test for regular seasonal variation is to add together all the available counts for each month of the year, thus obtaining larger samples. This is justifiable because the mean percentages for each year's counts - the counts from the first year, the second year and

Mr. Patrick's collections are not significantly different (5.5%, 6.2% and 5.3% respectively; standard error 0.5). These totals are given in the Table VII.

Table VII

The summation of counts over the period of the survey.

Month	No. in sample	Mean % infestation	Month	No. in sample	Mean % infestation
Jan.	800	6.5	July	800	6.75
Feb.	800	6.4	Aug.	800	4.5
March	1,029	3.5	Sept.	715	4.5
April	1,051	6.0	Oct.	815	7.1
May	824	5.3	Nov.	800	5.1
June	931	5.4	Dec.	886	4.6

Mean percentage infestation = 5.5

Limits of random error, P = 0.05
sample of 800 :- 3.9 - 7.1
sample of 1,000 :- 4.1 - 6.9

All counts lie satisfactorily between these limits except that of March which is low, for a reason which is not apparent. There is no evidence of any decline in the infestation during the summer months when winter migrants are absent.

Analysis of the counts of metacercarial cysts may be made only by the first of the two methods described above. The mean values of the two successive years are significantly different

and this invalidates the use of the second method - that of pooling the counts for each month of the period. The limits of random variation, P = 0.05, are 4.2 to 11.8. It will be seen that the first three samples are low. This, however, does not necessarily have any real significance since it is possible that a number of light infestations, especially when associated with heavy redial infestation, were overlooked in the early counts. The group of high counts in the winter of 1950-51 does appear to have a real significance. Such an increase might be due to any of a number of causes, but it cannot be a regular seasonal variation since there is no sign of a similar increase during the following winter. The steady state of the population of metacercariae in the final year of this survey indicates that a state of approximate equilibrium has been reached, with no sign of seasonal variation.

Footnote: My thanks are due to Mr. J. Gray of the Department of Mathematics for reading through this section and checking the statistical conclusion by an independent method.

Trematode parasites from birds.

It has already been shown that the adult form of C.patellae probably inhabits a marine bird, commonly found in the neighbourhood of St Andrews and, more particularly, of the East Rocks and those near the pier. For this reason a morphological survey of the digenetic trematode population of birds common to these areas was undertaken.

The trematodes found were examined, whenever possible, in the living state; otherwise structure and measurements were determined from specimens fixed in Bouin's fluid. During fixation, specimens were either held lightly between two coverslips, or shaken violently before and when adding the fixative to prevent undue contraction of the body.

Whole mounts and serial sections were studied, the former having been stained in either acetic acid alum carmine, Ehrlich's haematoxylin, Gower's aceto-carmine or borax carmine; the latter stain was the least satisfactory. Sections were stained in Heidenhain's or Delafield's haematoxylin and eosin, or Mallory's Triple Stain.

The following table (Table VIII) is a list

Table VIII — Trematode parasites and their hosts.

Turnstone (Arenaria interpres Linn.) - 26 birds examined.

Echinostephilla virgula Lebour, 1909.	(intestine)
Himasthla leptosoma (Creplin, 1829) Dietz, 1909.	(")
Parorchis sp. Nicoll, 1907.	(")

Oyster Catcher (Haematopus ostralegus occidentalis Neumann, 1929)

14 birds examined.

Notocotylus attenuatus (Rudolphi, 1809) Kossack, 1911.	(caecum and rectum)
Psilostomum brevicolle (Creplin, 1829) Braun, 1902.	(intestine)

Herring Gull (Larus cinereus Brisson, 1760; syn. L. argentatus Pontopp.,1763)

7 birds examined.

Cryptocotyle lingua (Creplin, 1825) Fischoeder, 1903.	(intestine)
Diplostomum spathaceum (Rudolphi, 1819) Olsson, 1876.	(")
Gymnophallus deliciosus (Olsson, 1893) Odhner, 1900.	(gall bladder)
Himasthla elongata (Mehlis, 1831) Dietz, 1909.	(intestine)
Spelotrema excellens Nicoll, 1907.	(")

Greater Black-backed Gull (Larus marinus Linn.) - 1 bird examined.

Cryptocotyle lingua (Creplin, 1825) Fischoeder, 1903.	(")
Gymnophallus deliciosus (Olsson, 1893) Odhner, 1900.	(gall bladder)

Black-headed Gull (Larus ridibundus Linn.) - 7 birds examined.

Himasthla leptosoma (Creplin, 1829) Dietz, 1909.	(intestine)

Redshank (Tringa totanus Linn.) - 11 birds examined.

Cyclocoelum mutabile (Zeder, 1800) Stossich, 1902.	(body cavity)
Himasthla leptosoma (Creplin, 1829) Dietz, 1909.	(intestine)

of the birds examined, with the flukes found in each. Adequate morphological descriptions of most of the flukes found are given in the literature (see Dawes, 1946). In the following pages a few relevant ecological facts are given; the order is based upon the key of identification given by Dawes.

Family - DIPLOSTOMATIDAE Poirier, 1886 emend.
Subfamily - Diplostomatinae Monticelli, 1888
Genus - Diplostomum Nordman, 1832

Diplostomum spathaceum (Rudolphi, 1819), Olsson, 1876 emend.

Host - Larus cinereus Brisson.
Intermediate hosts - Limnaea ovata, L.stagnalis and the Rainbow trout.

Six specimens found in the intestine of the Herring Gull.

Family - HETEROPHYIDAE Odhner, 1914
Subfamily - Cryptocotylinae Lühe, 1909
Genus - Cryptocotyle Lühe, 1899

Cryptocotyle lingua (Creplin, 1825) Fischoeder, 1903

Hosts - Larus cinereus Brisson, Larus marinus Linn.
Intermediate hosts - Littorina littorea and a fish.

Present in the intestine of the Herring Gull and the Greater Black-backed Gull; found in moderately large numbers, except in one Herring Gull which harboured thousands.

Family - NOTOCOTYLIDAE Luhe, 1909
Subfamily - Notocotylinae Kossack, 1911
Genus - Notocotylus Diesing, 1839

Notocotylus attenuatus (Rudolphi, 1809) Kossack, 1911

Host - Haematopus ostralegus occidentalis Neumann.

Found infrequently in the caecum and rectum of the Oyster Catcher; present in small numbers.

Family - CYCLOCOELIDAE Kossack, 1911
Genus - Cyclocoelum Brandes, 1892

Cyclocoelum mutabile (Zeder, 1800) Stossich, 1902

Host - Tringa totanus Linn.

Sixteen specimens found on one occasion in the Redshank; present in body cavity, along dorsal walls of thorax.

Family - ECHINOSTOMATIDAE Looss, 1902, emend. Poche, 1926 or Stiles and Hassall, 1926
Subfamily - Himasthlinae Odhner, 1910
Genus - Himasthla Dietz, 1909, emend. Odhner, 1910

Himasthla elongata (Mehlis, 1831) Dietz, 1909

Host - Larus cinereus Brisson

Found in the intestine of the Herring Gull; present in moderate numbers in two of the birds examined.

Himasthla leptosoma (Creplin, 1829) Dietz, 1909

Hosts - Tringa totanus Linn., Arenaria interpres Linn., Larus ridibundus Linn.

Found very frequently in the Redshank and Turnstone, and in one Black-headed Gull; present in intestine and usually in large numbers, 20 to 100 individuals in one host.

Subfamily - unclassified
Genus - Parorchis Nicoll, 1907

Host - Arenaria interpres Linn.

Single specimen found in the intestine of a Turnstone. Position of "collar" spines shown by pits in the cuticle.

The specimen examined agrees closely with the description of P.gedoelsti (Skrjabin, 1924) recorded from the Turnstone, and it is probable that it is a member of this species. Although Skrjabin makes no mention of evidence of 'collar spines' having been present, it is possible that similar destruction of the cuticle had taken place. Prudhoe (private communication) is of the opinion that both the specimen examined here and P.gedoelsti are probably examples of P.acanthus which have lost their crown of spines. Consequently, since it is apparent that further examination of the species of Parorchis found in the Turnstone is necessary, the final identification of the one specimen found during this work has been left uncompleted.

Family - PSILOSTOMIDAE Odhner, 1911, emend.Nicoll,1935
Genus - Psilostomum Looss, 1829

Psilostomum brevicolle (Creplin, 1829) Braun, 1902

Host - Haematopus ostralegus occidentalis Neumann.

Found fairly frequently in the mid-region of the

intestine of the Oyster Catcher; never present in large numbers, usually less than 12 specimens in one bird.

Family - MICROPHALLIDAE Viana, 1924
Subfamily - Gymnophallinae Odhner, 1905
Genus - Gymnophallus Odhner, 1900

Gymnophallus deliciosus (Olsson, 1893) Odhner, 1900

Hosts - Larus cinereus Brisson, Larus marinus Linn.

Found fairly frequently in the gall bladder of the Herring Gull and Greater Black-backed Gull; at most 6 to 8 specimens were found in one bird.

Subfamily - Microphallinae Ward, 1901
Genus - Spelotrema Jägerskiöld, 1901

Spelotrema excellens Nicoll, 1907

Host - Larus cinereus Brisson.

Found in a single Herring Gull; very many in the intestine.

Family in doubt.
Genus - Echinostephilla Lebour, 1909

Echinostephilla virgula Lebour, 1909

Host - Arenaria interpres Linn.

Found frequently in the Turnstones examined between September, 1949 and March, 1951; large numbers (50 to 100) present in the intestine. Only one specimen was found after this period.

A detailed description of the morphology of this fluke is given at the end of this thesis.

In addition to those birds reported examined, one Heron, one Fulmar, one Curlew, two Golden Plovers, two Ringed Plovers and two Purple Sandpipers were also investigated for parasites. Of these, only the Heron contained trematodes but this, as well as the other birds listed here, was an infrequent visitor to the neighbourhood of the rocks, and none of these were considered as possible final hosts.

Of the trematodes examined, several may be eliminated from consideration as possible adult forms of C.patellae. Firstly, an echinostome cercaria is indicative of a limited number of families only, these being the Echinostomatidae, the Psilostomatidae and the Philophthalmidae. Secondly, as already indicated statistically, it is improbable that a migrant bird is the final host. Other limiting factors include life histories already known and the rarity of occurrence of any particular fluke. In the present case, however, flukes which might thus be ruled out, are already eliminated by the first two considerations. The morphological factor eliminates all the trematodes examined except Himasthla elongata, H.leptosoma, Psilostomum brevicolle, Parorchis sp. and

Echinostephilla virgula. The last two species are probably eliminated by the fact that the only host in which they were found is a migrant bird. In addition to this, only a single specimen of Parorchis was found during the time of the survey; after March, 1951 one immature specimen of E.virgula was found.

The remaining possibilities are therefore H.elongata from the Herring Gull, H.leptosoma from the Redshank, Turnstone and Black-headed Gull, and P.brevicolle from the Oyster Catcher. As stated previously, the percentage infestation by C.patellae, at Auchmithie, Angus, was found to be remarkably low in the two counts made, in spite of the presence of great numbers of Herring Gulls in the area. There is, therefore, evidence, although not amounting to proof, that the herring gull is not the final host of C.patellae. Although the cercariae of Himasthla are of the same group of cercariae as C.patellae, there are structural differences (particularly in the size of the 'collar spines') which render it improbable that the adult form of C.patellae belongs to this genus. Although no recent work has been published on the life history of H.leptosoma, Lebour (1907) has

described a cercaria, which is probably that of this species, found in the digestive gland of Paludestrina stagnalis. In addition metacercariae of H.leptosoma have been reported from the echinoderm Leptosynapta inhoerens (by Cuénot, 1892) and also in the lamellibranch scrobicularia tenuis (by Villot, 1897); the latter host is considered to be more normal (Dawes, 1946). The cercariae, from Nassa obsoleta, of another species of the same genus, H.quissetensis which is also from the Herring Gull and found in the U.S.A., were examined; there was, however, no opportunity of procuring adults of this species.

Dawes (1946) quotes reports of Psilostomum brevicolle being found in Great Britain only at St Andrews and Lincolnshire. Such facts do not correspond with the widespread occurrence of C.patellae reported by Crewe (1951), but this is not considered to be of great importance since there may be, merely, an absence of published records.

Prior to the statistical analysis, it was thought that the metacercariae found in Patella vulgata were those of C.patellae, and a bird feeding upon limpets was assumed to be the final host. However, since it has been shown that the

metacercariae are probably those of Cercaria B, this assumption is no longer correct. Consequently, as the location of the metacercariae is not known, the frequency of the bird, in the areas known to be highly infested with C.patellae, is the only remaining indication of a possible definitive host.

PART II

The second part includes an account of the serological studies. The methods used are reviewed historically, and ways of measuring the precipitin reaction are investigated. Details are given of the various methods used to prepare antigens and antisera, followed by the results obtained from various tests performed.

Historical introduction to serological methods

When antibody and soluble antigen are mixed together, one of the most striking phenomena is the formation of a precipitate. This "precipitin" reaction was discovered by Kraus in 1897, who observed the formation of a precipitate when adding filtrates of bacteriological cultures to the sera of rabbits previously immunised, by injection, against these cultures.

When the reaction was first used, it was considered to be entirely specific, a precipitate being formed only with the homologous antigen (Kraus, 1897; Tchistovitch, 1899). In 1899, however, Bordet, and a year later, Myers (1900) and Uhlenhuth (1900), found that a precipitate, although slightly decreased in intensity, could also be obtained from closely related antigens (the heterologous reaction). Thus an antiserum produced in a rabbit by the injection of egg white of domestic fowl, was found to react very slightly with the egg white of ducks (Myers, 1900) and pigeon (Bordet, 1899, and Uhlenhuth, 1900), while the antiserum produced by the injection of sheep blood reacted very slightly with that of bullock (Myers, 1900). Nuttall (1904)concluded, from the

results of 16,000 tests with various bloods that precipitation occurred only when the antisera were tested against related forms, and that greater specificity resulted from the use of diluted antisera. The increase in specificity with dilution of antiserum has been confirmed by other workers (Schur, 1904; Wolfe, 1933; Boyden and DeFalco, 1943), Schur going so far as to state that an antiserum may be diluted to the extent where it reacts with the homologous antigen, but shows no reaction with any heterologous antigens. This effect of dilution may explain the apparently discordant results of some of Uhlenhuth's work, for he obtained the heterologous reaction with egg white of birds (1900), but stated later, as a result of work with mammalian sera, that the reaction was specific (1901).

The greater number of serological investigations has been concerned with vertebrate sera, the earlier work being conducted with a view to establishing the specificity of the precipitin test (Myers, 1900; Uhlenhuth, 1900, 1901), and the later, to establishing serological relationships from the point of view of systematic zoology (Nuttall, 1904; Boyden, 1926; Leone, 1948; Eisenbrandt, 1936,1938;etc.).

Much of the serological work with helminth material has been undertaken from a diagnostic standpoint, and it is only comparatively recently that such material has been used for the investigation of natural relationships within a group.

The early investigations were concerned with the investigation of specific antibodies in the sera of parasitised individuals. Thus Isaac and von der Velden (1904) attempted to establish a specific test in cases of human infestations by Bothriocephalus latus, while others (Fleig and Lisbonne, 1907; Weinberg, 1909; Welsh and Chapman, 1908; Bryce, Kellaway, and Williams, 1924) investigated the Hydatid Disease. Sera from patients infested with Echinococcus granulosus were tested for precipitin reactions, against an antigen of the cyst (either the fluid itself or an extract of the membrane) and found to give positive reactions. Using the Bordet-Gengon reaction (a modification of the complement fixation test) those results were confirmed and the work extended to include tests with extracts of Ascaris and Taenia against sera from individuals harbouring these parasites (Guedini, 1907, quoted by Weinberg and Parvu, 1908; the paper is not obtainable in

this country). Similar precipitin tests were carried out on other animals naturally infested with hydatid cysts and also on experimental animals injected with hydatid fluid, but the early results of this work, by Gherardini (1906) and Joest (1906) are quoted by Fleig and Lisbonne (1907) as being negative. (Gheradini's paper is not available in this country, and the reference to Joest's work, by Fleig and Lisbonne, is an inaccurate one as it refers to sheep-pox and not echinococcosis).

By the use of the complement fixation test (Weinberg & Parvu, 1908) and also of the precipitin reaction (flocculation test) (Fleig & Lisbonne, 1907; Hektoen, 1926), it was established that animals injected with extracts of various nematodes and cestodes did produce antibodies; these, however, were found to be not completely specific (Weinberg & Parvu, 1908). It is possible that the discrepancy between the results of the early work by Gheradini and Joest, and that by Weinberg & Parvu, may be accounted for by the fact that the complement fixation test is considered to be more sensitive, though less specific, than the precipitin reaction (Bryce, Kellaway & Williams, 1924).

After an early investigation by Weinberg (1909) into the appearance and demonstration of antibodies in sheep infested with Fasciola hepatica, much of the initial work in the field of serological reactions of trematodes was also conducted for the purpose of establishing diagnostic tests: in particular, for cases of schistosomiasis in which clinical symptoms were absent. Yoshimoto (1910) investigated cases of infestation by S.japonicum, and later sera from patients harbouring Schistosoma haematobium or S.mansoni were used (Fairley, 1919; Cawston, 1921) for tests in the complement fixation reaction against extracts prepared from the digestive glands of infested intermediate hosts (Bulinus contortus and Planorbis boissyi). The test, with antigens from snails infested with schistosome cercariae, was also applied, however, by the latter author, to sheep infested with Fasciola hepatica when it was found that these sera also gave positive reactions. Similar lines of research into schistosomiasis are now being carried on in many places, such as the Wellcome Research Institute where the precipitin reaction (ring test) is used for verification of results (Standen, personal communication).

From the systematic point of view, numerous investigations have been carried out using extracts of various helminth materials in attempts to confirm phylogenetic relationships or to establish new species within the group (Eisenbrandt, 1938; Wilhelmi, 1940; etc.). As pointed out by Boyden (1926), Wilhelmi (1940) and others, a comparison of the serological reactions involved indicates that the precipitin test is the one best suited for phylogenetic determinations of all groups, being a highly sensitive test which can be performed qualitatively or quantitatively. Applying this test, Wilhelmi (1940) and Stumberg (1930) tested the differences of reaction between various ontogenetic stages in the life cycles of helminth parasites, using both definitive and intermediate stages to stimulate antibody production. Both workers found that, although adults produced slightly higher titres in the reaction, the variations were not large enough for the different stages to be considered serologically distinct. (The use of the word 'titre' in serology indicates the end point of the reaction, this being the highest dilution of antigen which will give a visible reaction with an antiserum).

In carrying out any experimental work in the field of serology, careful consideration must be given to three major aspects of the steps involved - the preparation of the antigen, the methods of producing antisera and, lastly, the techniques involved in the measurement of the antigen/antibody reaction. In the first place, the type of material employed in antigen preparations varies considerably. Preparation of antigens from parasitic material involves the use of tissue extracts as opposed to body fluids of vertebrates and some invertebrates. These tissues are evidently more complex in composition than serum, and have not yet been proved to be as reliable in their antigenic properties (Eisenbrandt, 1938). In working with various tissues of Ascaris lumbricoides, Canning (1929) found that each tissue had a distinct specificity of reaction, but most workers have used extracts of whole organisms exclusively. The greatest single factor influencing the specificity of such antigens is the presence of lipoids in the extract. Lipoids, although not antigenic in themselves, serve as haptens, and such haptens may be chemically similar among distantly related species and quite unlike among closely

related organisms. Hence the effect of using extracts from which lipoids have not been extracted, with ether or Bloor's mixture (3 parts ether: 1 part absolute alcohol), is to produce a group reaction, rather than a reaction between species. In addition to the lack of specificity involved in the use of such extracts, the presence of lipoids may cause flocculation of the antigen itself even in a sterile fluid (Eisenbrandt, 1938).

Many workers (Fairley, 1919; Schwartz, 1920; Hektoen, 1926; Campbell, 1936; Wilhelmi, 1940; etc.) have used dried whole organisms, from which saline extracts have been made either with or without lipoidal extraction. Some (Stumberg, 1930, and others) have employed Coca's solution (0.7% sodium chloride, 0.25% sodium bicarbonate, 0.4% phenol) as an extractant with apparently successful results, and normal saline extracts of fresh material have also been used in antigen preparation (Eisenbrandt, 1938). Fairley (1919) and Tanabe (1923), both working with the digestive glands of snails infested with schistosome cercariae, used alcoholic extracts of material, but, as Wilhelmi (1940) points out, such extracts are largely lipoidal in nature and tend to give a non-specific reaction. Besides

the normal use of protein oxtracts for antibody production, Campbell (1936 a and b, 1937) fractionated various components from helminth tissue (of Taenia and Ascaris) and found that polysaccharides stimulated considerable antibody response, and that such antisera were much more specific than those produced in response to extracts of whole worm. Campbell also found that a clear antigen could be obtained by preparing the extract in the usual way - grinding the material in saline and centrifuging the suspension - and then incubating the turbid fluid at a temperature of 45 - 50 C for 45 minutes. This process resulted in the flocculation of the macroscopic particles which settled out, leaving an optically clear solution in which the soluble components had suffered no denaturation.

It is possible, however, to differentiate between antigens prepared from unaltered or 'native' material and those prepared by some distinct process or treatment during which alteration of the material may take place. In contrast to the methods already described, treatment by ultrasonic radiations is included in the latter category. This process has been used fairly extensively in the field of bacteriology (see Kabat and Mayer, 1948; Glick, 1949)

as a means of causing physical disruption of tissue and cells. Relatively little work has been carried out to investigate changes in the serological activity of proteins. Grabar and his co-workers (Grabar and Kaminski, 1950; Grabar, 1951, a and b) have found that egg white is denatured by prolonged ultrasonic treatment. Nevertheless, even after twelve hours radiation, the proteins are still active antigens capable of producing antibodies; the specificity of the antigen, however, is so altered that an antiserum to it is incapable of reacting with the native antigen. Boyden (1953), on the other hand, has found that brief exposure to ultrasonic vibrations (5 to 15 minutes) of a crustacean haemocyanin causes no alteration in the serological reactivity of these proteins.

The technique employed in the production of antisera has varied considerably with different workers. Tchistovitch (1899) found that the rabbit was the most suitable for experimental antibody production, and it has been very generally used, although the domestic fowl has also been used to some extent. The routes of injection of antigen vary with material and workers. Burnet and Fenner

(1949), using bacteriological material, found that, after two series of injections, the strength of the antiserum produced is the same whether intravenous or subcutaneous injections are administered. Wolfe (1935) claims greater specificity obtains from intravenous injections, but it is now generally accepted that there is comparatively little difference between antisera whether produced by subcutaneous, intraperitoneal or intravenous injections. To avoid repeated intravenous injections, Slavin (1950) evolved a method whereby the antigen is absorbed gradually by the rabbit, from a gel of calcium alginate. Sodium alginate and calcium chloride are injected intraperitoneally and the antigen, administered in one or two large intravenous injections, is taken up by the gel formed and absorbed gradually by the rabbit.

Weinberg, (1912) proved that a host is capable of producing antibodies to the products secreted by its parasites as distinct from the substance of the parasite itself, and there is a likelihood that the more active antigenic substances may be present in these secretions. It is also possible that a "normal" host (one in which the parasite is found under natural conditions) may be stimulated to antibody production against metabolic products of a parasite

(active sensitisation) more readily than an "abnormal" host, i.e., experimental animal, is stimulated by the injection of killed organisms, or suspensions and extracts therefrom (passive sensitisation). In recent years many workers (Papirmeister and Bang, 1949; Vogel, 1949; Liu and Bang, 1950; Standen, 1952, and others) have been investigating antisera from individuals actively sensitised to living parasites. In addition, many investigators have been engaged in research into the resistance and hyper-resistance of animals to parasitic infections, and the degrees of immunity established in response to injections of parasitic material into normal hosts (Stumberg, 1930; Campbell, 1936, a; Larsh, 1943, 1951; and others - see Culbertson, 1938). In comparison with these wide-spread fields of research, investigations into antibody-production by the method of passive sensitisation of experimental animals are relatively few, and this may well indicate that 1) the more active antigenic components, of helminth parasites at least, are to be found in some of the secretions from living organisms and, 2) that passive sensitisation of the normal host is more likely to lead to the production of antibodies than will

passive sensitisation of some distantly related animal.

A particular case of active sensitisation of the "normal" host to living parasites, which must be elaborated further, is that known as "swimmer's itch" or "sea bather's eruption" (Cort, 1928; Taylor and Baylis, 1930; Brackett, 1940; Gordon and Griffiths, 1951; and others). The phenomenon is caused by the penetration of the skin by non-human schistosome cercariae, usually those maturing in birds, which do not become established in man but produce an acute dermatitis in the area of penetration. Many such schistosome cercariae, of both fresh water and marine habitats, have been found to cause this phenomenon, and these include Cercaria variglandis (Miller and Northup, 1926) from the mud snail Nassa obsoleta (Say, 1822); (see Stunkard and Hinchliffe, 1952). Olivier (1949) showed that the typical schistosome dermatitis is a sensitisation reaction, an immunity being established in the body of the individual in response to the presence of the cercariae. Once an immunity is established, a further infection causes an immediate inflammatory response at the site of penetration and the cercariae travel no further than the epidermal layers of the skin. It

has been shown recently (Olivier, 1953) that, in previously unsensitised experimental animals, including monkeys, avian schistosome cercariae are able to penetrate the body deeply and migrate to the lungs where they cause small haemorrhages. Thus, it is probable that the initial sensitisation is established during the migrations of the cercariae within the body of their adopted host.

Besides the method of introduction of an antigen, there are other points to be considered when studying the quality of an antiserum produced in response to antigens administered passively. Wolfe (1935), Wolfe and Baier (1938), Wolfe (1939) and Leone (1952) have demonstrated that the greatest specificity is produced by the introduction of a minimal amount of protein in a short injection series, while an increase in the period of time during which the injections are given, as well as an increase in the amount injected, results in an increase in the quantity of precipitate formed with a suitable antigen, but a decrease in the specificity of the antiserum.

The quality of the antiserum naturally depends, also, upon the time at which the experimental animal is bled. Many workers (Wolfe, 1933;

Eisenbrandt, 1938; Wilhelmi, 1940; and others) have bled the animals nine to fourteen days after the last injection, although others (Burnet and Fenner, 1949; Boyden, DeFalco, Toby, personal communications), have found that the maximum antibody production occurs three to seven days after the final injection.

Regarding optimum conditions for the antigen/antibody reaction, the effect of temperature, hydrogen ion concentration, the salinity of the fluid and the length of the incubation period of the reactants are all points to be considered, and Wolfe (1933), Baier (1933) and others have reported fully on the effects of these conditions. In general, a temperature of 37°C for twenty to sixty minutes and a pH of approximately 7 are the most suitable conditions, while, when dealing with rabbit antisera, a low salinity (a 0.9% solution of sodium chloride is commonly used) gives optimal aggregation of antigen and antibodies. The effect of saline upon fowl antibodies is the reverse, an increase in salt concentration causing an increase in the reaction (Goodman and Wolfe, 1952).

The first semi-quantitative test used to record the results of the precipitin reaction was the ring

test, devised by Ascoli (1902), in which antiserum is layered below antigen in a small test tube, the formation of a turbid ring at the interface being indicative of a positive reaction. Such a test may be either qualitative or quantitative, measurement being achieved by estimating the end point or titre of the reaction - i.e. the greatest dilution of antigen capable of producing the ring at the interface of the two fluids. Such a method, having the advantages of measuring amounts of precipitate almost imperceptible to the naked eye and of using very small quantities of fluid, seems to be the most widely employed at the present time, (Eisenbrandt, 1938; Wilhelmi, 1940; Standen, personal communication; and others). Two other quantitative methods of measuring the reaction have been used. In one of these, a volumetric method, originated by Nuttall in 1904 and elaborated by Boyden and Baier (1929), the precipitate is allowed to flocculate and settle out. As used by Boyden and Baier, the fluid is drawn into a thrombocytocrit, and the volume of the precipitate noted as it settles out. The other method, which was first used by Libby in 1938, employs a modification of the nephelometer invented by Richards (1894, 1904) and used by him in atomic weight determinations. The principle of

such a method is that when a beam of light is passed through a turbid solution, some of the light is scattered, and this scattered light may be compared with a standard (Richards) or measured directly with photo-electric cells (Libby). This method was, perhaps, foreshadowed by Uhlenhuth (1901) who noted that the precipitate was especially distinct when viewed across transmitted sunlight. The sensitivity of the photo-electric cell, the use of readings taken over the whole range of the reaction and its measurement in situ, renders Libby's method more accurate as compared with the ring test or the volumetric method. It has been emphasised by many workers that to make the ring test fully quantitative, the strength of the antigen must be known, the crucial factor being the minimum concentration of antigenic substances which will produce a positive reaction. For practical purposes, the measurement of protein or nitrogen concentration is generally considered to be sufficient, estimation of the latter being made by Kjeldahl's method (Franceschelli, 1909). Measurement of antigenic concentration is less important with the use of a photo-electric instrument, since this allows an accurate measurement over the

full range of the reaction and, provided the readings do cover the entire range, reliable comparisons can be made regardless of the concentration of antigen (Boyden and DeFalco, 1943).

Dean and Webb's method (1926) is one of measuring the rate of precipitation, in which the amount of antiserum is held constant and the amount of antigen varied. These workers found in several tests, each with an antiserum of different strength, that the ratio (dilution of antigen):(dilution of antiserum) was approximately constant for the point at which the most rapid precipitation occurred; this is known as the zone of optimal proportions. The photo-electric method of Boyden and his school measures the amount of precipitate formed when a constant amount of antiserum is added to successively decreasing amounts of antigen. The maximum amount of precipitate, which is a measure of the quantity of antibody present, is formed near the point of optimal proportions; on either side of this, the reaction gradually decreases to zero, formation of a precipitate being inhibited by excess antigen.

The single end-point reading of a ring test, which measures the sensitivity of antibodies in the reaction, is taken in the zone of antibody excess, whereas the readings taken by Libby's photo-electric instrument cover the entire range of the reaction, from the zone of antigen excess to the zone of antiserum excess. These readings fall on a curve, obtained by plotting the intensity of scattered light against dilutions of antigen, which increases, from zero in the region of excess antigen, or pro-zone, to a maximum near the point of "optimal proportions" or "neutralisation", to zero again in the region of excess antibody, or post-zone. Provided that the reaction is complete, i.e., the curve extends to zero on either side of the maximum, accurate collations can be made between different antigens, by comparing the total area of each curve.

In the ideal case of measuring reactions between single antibodies and single antigens, the maximum amount of precipitate formed would give an adequate measure of each reaction. Since, however, the measurement invariably comprises a composite reaction of numerous systems (Cohn and Pappenheimer, 1949; Wetter et al., 1952), the optimum

concentration producing the maximum amount of precipitate in each individual reaction may well be different. In these cases the maximum in a graphic curve of the whole reaction may be considerably flattened and, in extreme cases, more than one maximum may be obtained (Boyden and DeFalco, 1943). As a result, measurement of the entire reaction, rather than a single point on it, is essential in obtaining an accurate estimation of the precipitin reaction. If a single pure antigen can produce numerous antibodies (see Boyd, 1947), it follows that the use as antigens of complete organisms such as trematodes, must give rise to a vast complexity of antibodies. The antigenic composition of helminths is virtually unknown (Eisenbrandt, 1938) and, in addition, the problem is further complicated by the fact that there is an enormous variation among experimental animals in their capacity to produce antibodies.

Other methods for the evaluation of antisera besides the use of the precipitin reaction include agglutination tests and the effect of immune sera upon living organisms. The first

of these tests is used extensively in many fields of serology, but its application depends largely upon the size of organism or particle involved in the reaction. Liu and Bang (1950), during investigations directed towards developing a test for infestation by Schistosoma mansoni, found that agglutination of cercariae occurred in immune sera of monkeys, but that the agglutinins present reacted only upon living cercariae.

Before the effects of immune sera upon living organisms can be discussed, mention must be made of the effect of normal sera upon cercariae. The 'cercaricidal' action of normal sera was first described by Culbertson (1936) who tested the effects of a variety of vertebrate sera upon several different cercariae. Of all the sera tested, only that of cat lacked a lethal effect upon the organisms. One amphistome cercaria (Diplodiscus) was found to encyst in contact with normal serum, but generally the cyst walls of the metacercariae disintegrated. The same year, Tabangui and Masilungen (1936) investigated this toxic action of various vertebrate sera upon the cercariae of Schistosoma japonicum and a distomid cercaria. These workers confirmed Culbertson's findings that cat serum lacked the lethal effect

and reported that this was also absent in rabbit serum.

The earliest reports of the effects of immune sera upon parasites were concerned with the formation of a precipitate in and around various species of nematode eggs, larvae and adults. (Culbertson, 1938; Otto, 1939; Taliaferro, 1940; Sarles, 1930; Olivér-González, 1940; and others). The first reports of a similar effect upon trematodes was by Papirmeister and Bang (1948) who described the formation of a more or less transparent sheath round the cercariae of Schistosoma mansoni one to three hours after being in contact with sera from infected monkeys or humans. The sheath appears first round the tail, or at the junction of body and tail, and then spreads anteriorly until the whole body of the cercaria is surrounded. This formation is ascribed to a precipitin reaction. A year later Vogel and Minning (1949 a) and b)) described the same "Hullenbildung" effect with the three species causing schistosomiasis in man. Standen (1952) found that besides this sheath formation in immune sera, some normal sera of humans, cats, dogs, horses and all cattle may cause the appearance of

a similar sheath of precipitate round the cercariae. However, this reaction is inhibited completely after inactivation of normal sera (heating to 56°C for 30 minutes to destroy complement), while the true precipitin reaction is only retarded by such treatment of immune sera. The formation of this precipitin sheath appears to protect the cercariae from any cercaricidal action of the serum itself.

The application of the various methods reviewed here is described in the following pages.

Materials used

All the cercariae described previously were used both for antibody production and for the preparation of test antigens. Of the adult flukes found, only Himasthla leptosoma and Psilostomum brevicolle were available when the final tests were carried out; extracts of these were made for use as heterologous test antigens.

The parasites were extracted from their hosts in the following ways. Cercariae and rediae of C.patellae, and cercariae and sporocysts of Cercaria B were obtained from Patella vulgata by removing the shell and teasing out the parasitised areas of the limpet in sea water. The rediae of C.patellae appear as conspicuous

white threads throughout the digestive gland and gonad, development of the latter frequently being almost entirely suppressed by the infestation. The distribution of Cercaria B, however, is more localised, discrete masses appearing among the digestive gland of the limpet. These are pale yellow to bright orange in colour, depending upon the extent of the infestation, and consist almost entirely of sporocysts. Rediae of C.patellae and sporocysts of Cercaria B were obtained in fairly large numbers from heavily parasitised limpets. They were obtained in a reasonably uncontaminated state by repeated washings in sea water followed by removal of the remaining extraneous matter with a pair of forceps or pipette. Active cercariae of C.patellae were obtained occasionally as they emerged into the surrounding sea water in the dish containing the limpet. It was found that limpets could be kept alive in the laboratory for two or three days only, and during these days cercariae were emitted infrequently, although, at such times, they appeared in considerable numbers. The cercariae were removed, by means of a pipette, to a test-tube and washed thoroughly by repeated

changes of sea water. If necessary - i.e. if the cercariae were swimming actively - the tube was chilled, but not frozen, in the refrigerator, a process which caused the cercariae to fall to the bottom, allowing removal of most of the sea water. The activity of the cercariae was restored, on being returned to room temperature, even when they had been kept two or three days at low temperatures.

Collection of material from Nassa obsoleta was confined, in the main, to the collection of the cercariae as they emerged from living snails. Large collections of Nassa were kept alive for periods of up to five weeks in seven gallon glass jars containing some sea water, at a temperature of 2° - 4° C. When required for experimental use, fifty or a hundred individuals were isolated in small jars, each jar containing 2-4cc of sea water, and kept at room temperature. Usually, after twelve to twenty-four hours, infested snails were identifiable by the cercariae discharged; (no cercariae were discharged if the temperature was below 12°C). Thereafter, only the infested snails were kept alive for as long as cercariae were emitted. With frequent changes of water and an occasional meal of small pieces of molluscs,

some snails continued to emit cercariae, intermittently, for four to five weeks.

The cercariae, usually discharged in large numbers, were collected daily and washed in sea water as described above. If not used immediately, they were placed in tubes in a small quantity of sea water and kept in a frozen condition until required.

The adult trematodes were extracted from their hosts as soon as possible after the latter had been shot. The flukes were washed thoroughly in 0.9% saline and once rapidly in distilled water before being frozen and stored at a low temperature.

Before details of the various preparations of antigens and antisera used in this work can be given, the techniques of measuring a precipitin reaction are first described.

Photo-electric measurement

a) Development of a suitable instrument.

It has just been shown (p. 61) that the measurement of the total amount of precipitate formed in the antibody/antigen reaction provides

a much higher degree of accuracy in comparison between homologous and heterologous tests than does the ring test. The simplest and most sensitive method of obtaining such an estimation is the photo-electric measurement of turbidity. At an early stage in the work, some experiments were carried out with a view to finding the most suitable photo-electric method and apparatus for measuring the amounts of precipitates.

The general principle underlying such optical methods is that when a beam of light is passed through a turbid solution, some of the light is scattered. It is possible either to measure the consequent reduction in intensity of the original beam (turbidimetry) or to measure directly the intensity of the scattered light - the Tyndall light of the colloid chemist (nephelometry).

In the earliest instruments used for such measurement of precipitates, reliance had to be placed upon visual comparison. Thus, in the nephelometer employed by Richards (1894), a tube illuminated from the side was viewed axially, and the resulting luminosity compared with a standard by varying the length of the tube illuminated. The development of photo-electric

instruments has since rendered such methods obsolete, and it is now possible to obtain a measure of any precipitate from a reading on a galvanometer.

Such instruments have, on occasion, been used in serological work. Thus Lanni (1946) used a commercial colorimeter as a "turbidimeter" and Baier (1947) a specially constructed instrument, the "microdensitometer", in the same manner; Libby (1938) designed a "photronreflectometer" - now termed the "photroner" - to measure the amount of light scattered by serological precipitates. A number of models of this instrument are now in regular use.

The most suitable method for measuring the type of precipitates likely to be encountered in this work had to be found. In investigations of this nature, it is essential to have a readily reproducible turbidity for the purpose of testing various instruments. Numerous tests, made on a variety of substances, showed that a constant turbidity was difficult to maintain when using inorganic precipitates, owing to changes in the size of the individual particles. Colloidal suspensions of sulphur, and of lead carbonate

stabilised in a 1% solution of gelatine, were used with some degree of success, but finally kaolin was found to provide a reliable and reproducible turbidity if prepared by a standardised method. A quantity of kaolin was shaken with water and allowed to stand in a tall vessel for twelve hours, when the still cloudy supernatant suspension was poured off from the larger particles which had settled out. The suspension was diluted in known proportions and each solution thus obtained showed a turbidity which remained reasonably constant for half an hour; each dilution could be regenerated at any time by shaking.

Instruments measuring transmitted light.

In the ideal case, the light (I_t) transmitted by a turbid solution is given by the formula $I_t = I_o^{-\alpha c d}$ or $\alpha c d = \log \frac{I_o}{I_t}$, where I_o = the intensity of the incident light, α = constant depending upon the scattering power of individual particles, c = the concentration of the turbid material and d = the thickness of the layer (i.e., cell). With other factors held constant, c is proportional to $\log \frac{I_o}{I_t}$, which is the optical

density of the liquid. This is similar to Beer's Law for coloured solutions in which the optical density is proportional to the amount of absorbing material in the solution.

The instrument used here for the experimental measurement of turbidities by the reduction of transmitted light was a Unicam colorimeter. The principle of such an instrument is that light passing through an optical glass cell, or cuvette, containing a clear, colourless solution falls on a photo-electric cell and generates a current in a galvanometer circuit. A second cuvette of identical dimensions, containing the coloured or turbid solution is then substituted for the control, and the diminished deflection read on the galvanometer scale, calibrated in units of optical density.

The effective range of this instrument was found to be between optical densities of 0.05 and 1.0: kaolin suspensions of 0.04 to 1.8 g/litre give these values. Readings could be made beyond this range but were then liable to more than 10% error. The lower limit of the instrument is set by the instability of the readings at this end of the galvanometer scale. The upper limit, set by

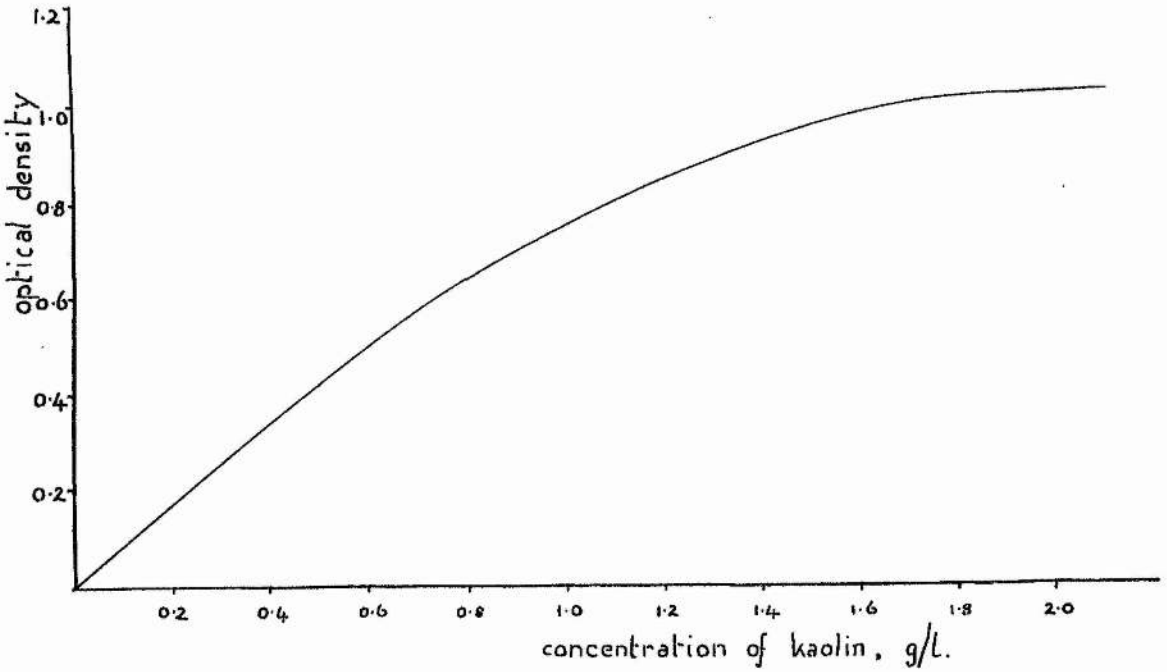


Figure 8.

Representing the turbidity of suspensions of kaolin, as measured in a 'Unicam' colorimeter (turbidimeter) in units of optical density.

the small amount of light transmitted at high concentrations, might be extended by the use of thinner cuvettes.

For the purpose of measuring the area of a curve representing the entire range of the precipitin reaction, it is important that the deflections of the galvanometer should be directly proportional to the concentration of the suspension. It is seen from Figure 8 that the graph, representing measurements of turbidity of a suspension of kaolin, departs from a straight line at concentrations greater than 0.7 g/litre.

In using an instrument such as a colorimeter, a reduction of intensity of the incident beam is caused either by the absorption of light by a coloured solution, or by loss of light due to scattering in a turbid solution. It is important, however, when measuring one effect to ensure that there is no interference from the other. Thus any colour, such as that of slightly haemolysed serum, in turbid solutions would interfere seriously with measurement of a precipitate, by increasing spuriously the optical density. This source of error can be obviated to a great extent by the use of a control of saline

and serum, and also by the use of an appropriate (red) filter as the following figures show:-

Optical density of a clear, but haemolysed serum measured against water - "blank".

No filter	Blue filter	Green filter	Red filter
.34	.73	.77	.07

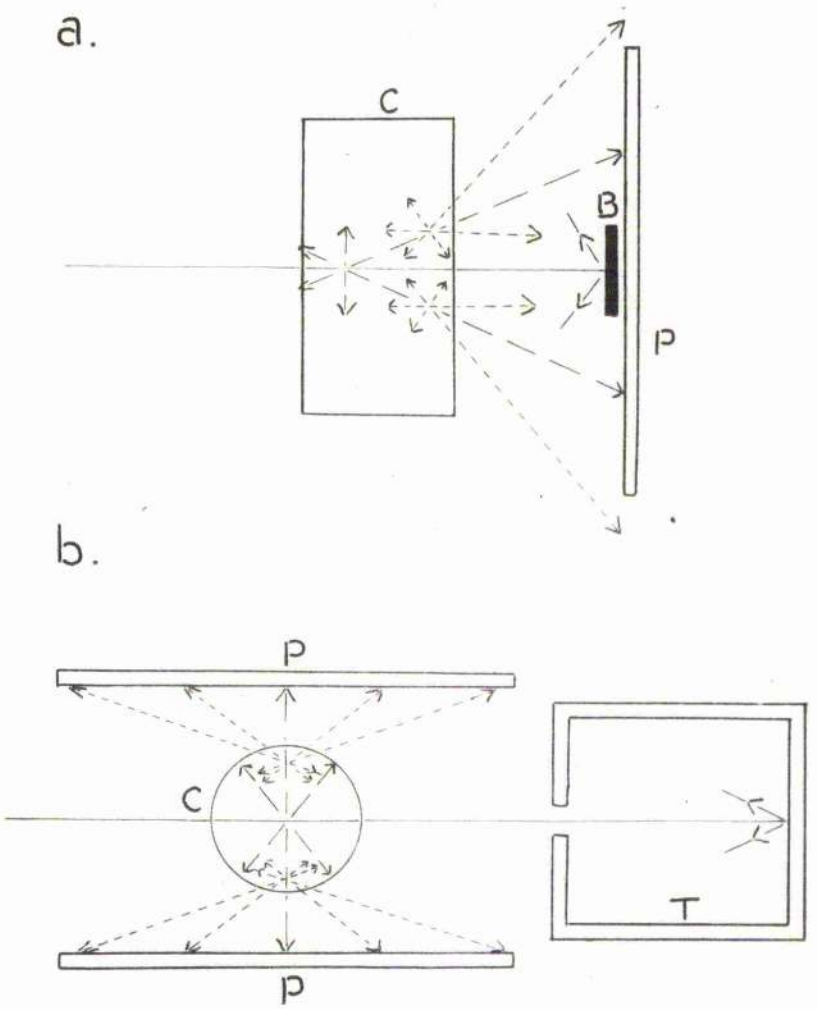
White, blue and green light are all absorbed by the red coloured serum and high density readings are obtained. It is clear, therefore, that unless suitable precautions are taken, the error from this factor can be very considerable.

Instruments measuring light scattered.

Rayleigh's formula (1871) for the intensity of light scattered (I_s) in a direction making an angle β with the incident ray, is $I_s = I_o \frac{f c v}{\lambda} (1 + \cos^2\beta$ where I_o = the intensity of the incident beam, f = constant, c = the concentration of the scattering material, v = the volume of each particle and λ = the wave length of light. This equation shows that if the other quantities are kept constant, I_s is proportional to c. The final term, $(1 + \cos^2\beta)$, varies from 2 when $\beta = 0^\circ$ or 180° to 1 when $\beta = 90^\circ$, so that the intensity of light scattered in line of the incident beam is twice as great as that scattered at right angles to it.

The measurement of scattered light is known as 'nephelometry'. The terminology, however, applied to instruments measuring this light, varies; although the name 'nephelometer' is frequently used, alternatives to this are common. In Libby's 'photroner', measurement is made of the light scattered in a forward direction (where ρ, the angle between the incident beam and the path of the scattered light, is small). In this instrument, the incident beam of light normally - i.e., if the cuvette contains a clear, colourless solution - falls upon a black (null) spot in the centre of the photo-electric cell; with a turbid solution, however, some of the light is scattered on to the sensitive surface and, up to certain concentrations, the readings obtained on the galvanometer are directly proportional to the turbidity of the solution. It was noted by Baier (1947), in the course of experimental comparisons between his own microdensitometer and the photroner, that a definite saturation point existed - i.e., a point at which the readings on the galvanometer no longer increased with increased turbidity. In the upper ranges of the latter

Figure 9.



Key

—— = incident beam of light

– – – = primary scattering ..... = secondary scattering

C = cuvette P = photo-electric cell

T = light trap B = black (null) spot

Diagrams to illustrate the principles underlying the design of two instruments measuring the intensity of scattered light, and to emphasise the advantage of the use of photo-electric cells of large area.

a). The Photroner. **b). The Nephelometer.**

instrument it was found that the greater the turbidity, the smaller the deflection of the galvanometer; this effect is due to secondary scattering. In an extreme case, a suspension so turbid as to be opaque will give a reading of zero. This saturation point is well known to users of the photroner who employ thin cuvettes (internal width approximately 0.5cm) in order to keep within the effective working range of the instrument. The saturation effect is also reduced if the sensitive area of the photo-electric cell is large; in this way much of the light which is secondarily scattered will eventually reach a photo-sensitive surface, (Figure 9a).

An alternative method for the measurement of turbidity (a 'nephelometer') consists in placing photo-electric cells in line with the incident beam. By using two such cells connected in parallel, one on each side of the cuvette, the disadvantage of the reduced intensity of the scattered light is offset by the larger area of photo-sensitive surface (Figure 9b); the saturation effect is probably reduced and the instrument is simpler, mechanically, to construct.

Trial models of each type, one on the principle of the photroner - a "black spot" instrument - and one on the principle of measuring light scattered at right angles to the incident beam - the nephelometer - were constructed and tested with suspensions of kaolin. Both instruments showed the saturation effect, but it was less marked in the nephelometer. In both instruments the lower limit of the readings on suspensions of kaolin, which could be made with a 10% accuracy, was 0.02 g/litre, this limit being set by the amount of stray reflected light. In a second model of a nephelometer, in which a "light trap" was constructed (the incident beam passed through a hole in the back of the apparatus to another blackened box where it was completely absorbed), the lower limit was increased to give readings on a kaolin suspension of 0.002 g/litre. Readings made on a photroner, with the same kaolin suspensions, showed that the lower limit of this instrument was the same - 0.002 g/litre.

A comparison of the instruments tested.

Table IX shows a comparison of the limits of several photo-electric instruments. Between these limits, readings which are proportional to

the concentration can be made to within an accuracy of 10%. Each instrument can, in fact, be used beyond such limits without much loss of accuracy, since a slight error in a single reading (at either end of the scale) makes little difference to the total area enclosed by the curve representing the precipitin reaction. In each case the upper limit could be extended (but not beyond the saturation point) by the use of a calibration curve.

Table IX

Effective limits of various photo-electric instruments.

(The figures are concentrations of kaolin in g/litre)

Instrument	Lower	Upper	Saturation point
Unicam colorimeter	0.04	0.7	infinity
"Black spot"	0.02	0.2	0.8
Photroner	0.002	0.125	0.5
1st nephelometer	0.02	0.6	3.2
2nd nephelometer	0.002	0.6	3.2

The performances of four of the instruments over a wide range of kaolin suspensions, are shown in Figures 10 and 11. Measurements of an actual precipitin reaction (human serum x anti-human serum) ranged from values equivalent to 0.005 to 0.175 g/litre of kaolin suspension. Of the instruments tested, only the second nephelometer and the photroner are able to include a range such as this

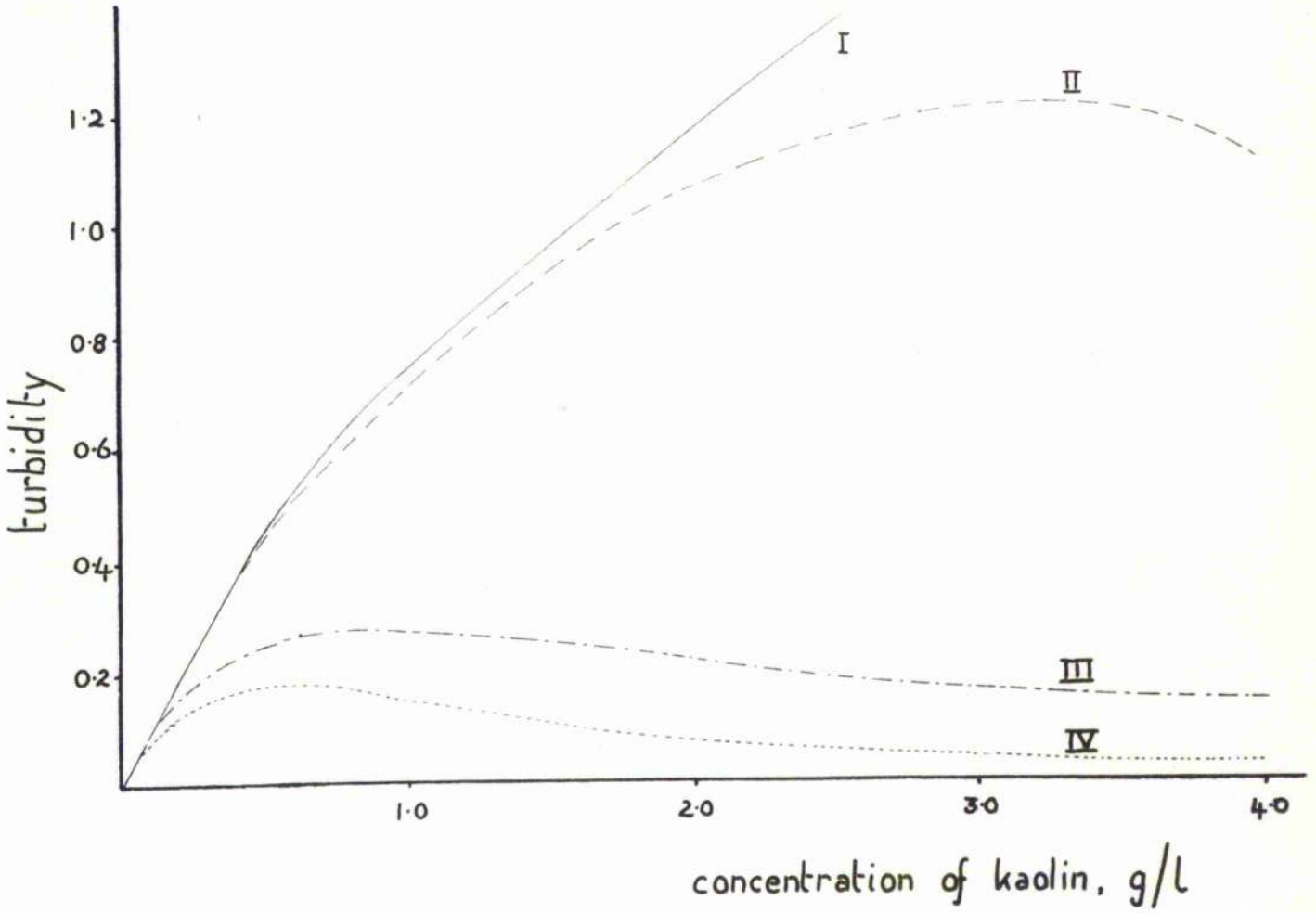


Figure 10.

To illustrate the performance of four photo-electric instruments measuring turbidity of high concentrations of kaolin.

I. Colorimeter.
II. Nephelometer.
III. 'Black spot' instrument.
IV. Photroner.

within the working limits of their scales.

Owing to the liability of photo-electric cells to fatigue and also to variations in light source, some standard is required for each instrument. In the case of the colorimeter (turbidimeter) measuring transmitted light, this is attained by adjustment to give a full scale deflection with a control (water or serum and saline, etc.). In the photroner, a beam of light falls on the photo-sensitive surface when the cuvette containing the test solution is not in place. By means of this beam, adjustment, similar to that in the colorimeter, can be made to give a full scale, or constant, deflection of the galvanometer. When the specially constructed cuvette is in place, this control beam is cut out. For the nephelometer, a standard solution giving a constant deflection was employed. A solution of kaolin might have been used, but an alcoholic solution of eosin (0.100 g /litre) proved to be more stable and was readily reproducible. Since, in the first two instruments, standardisation is achieved by adjustment of the incident beam, the readings obtained are direct - i.e., already standardised. In the third instrument, however, it is necessary to relate

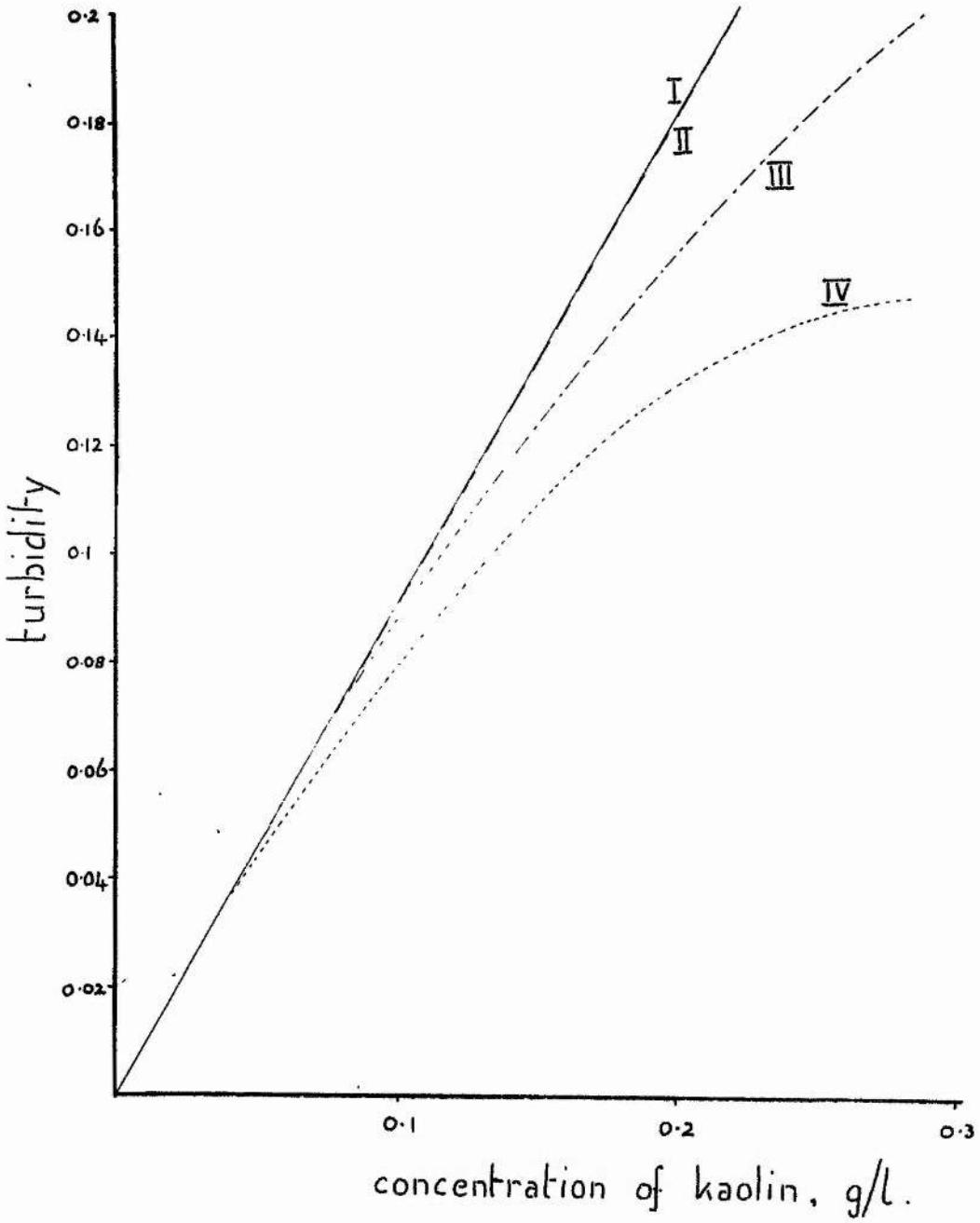


Figure 11.

To illustrate the performance of four photo-electric instruments measuring turbidity of medium concentrations of kaolin.

(see Figure 10.)

each test reading to that of the standard solution. In the system adopted, a turbidity giving a deflection equal to that of the standard had a value of 1. The standardised readings were calculated by dividing the reading of the test solution by that of the standard.

The nephelometer finally adopted is illustrated in Figures 12 and 13. The light source was a 12 volt 36 watt lamp run from a step-down transformer, the voltage being kept constant by a stabilising transformer unit. This light was focussed by a lens (diameter 3.75 cm), associated with an iris diaphragm, and passed through a cuvette into a light trap. The cuvette was flanked on either side by photo-electric cells ("Eel", 3 cm. in diameter), situated exactly parallel to the beam and equidistant from it. In some of the preliminary experiments, square-sectioned cuvettes were used, but in the final instrument carefully matched standard buffer test tubes (13.5 cm x 1.4 cm) were substituted with only a slight loss of sensitivity at the lower end of the scale. By using these tubes it was possible, without great expense, to test a number of solutions, comprising a series of antigen dilutions, in the containers in which they were being incubated.

Figure 12

The nephelometer.

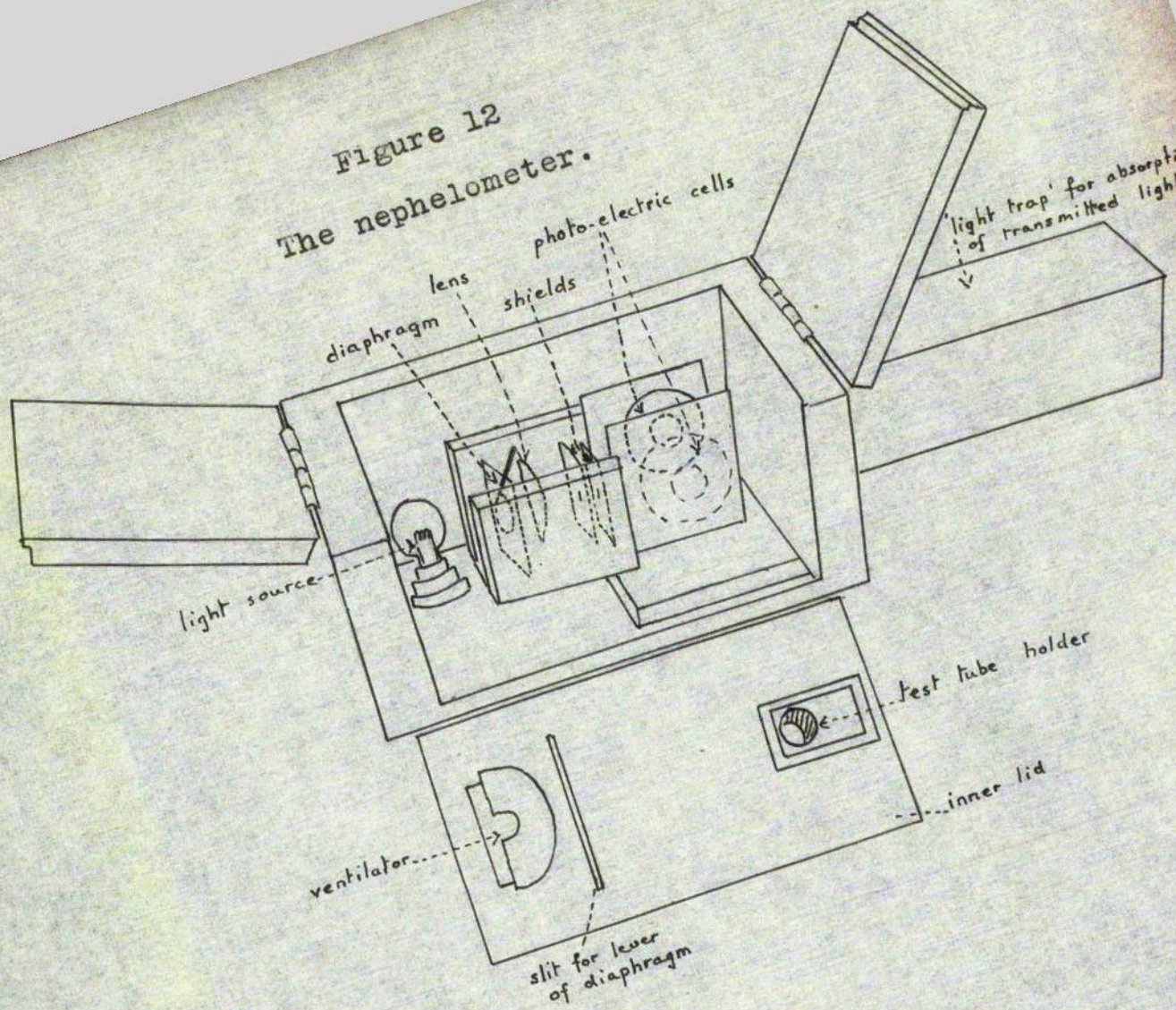
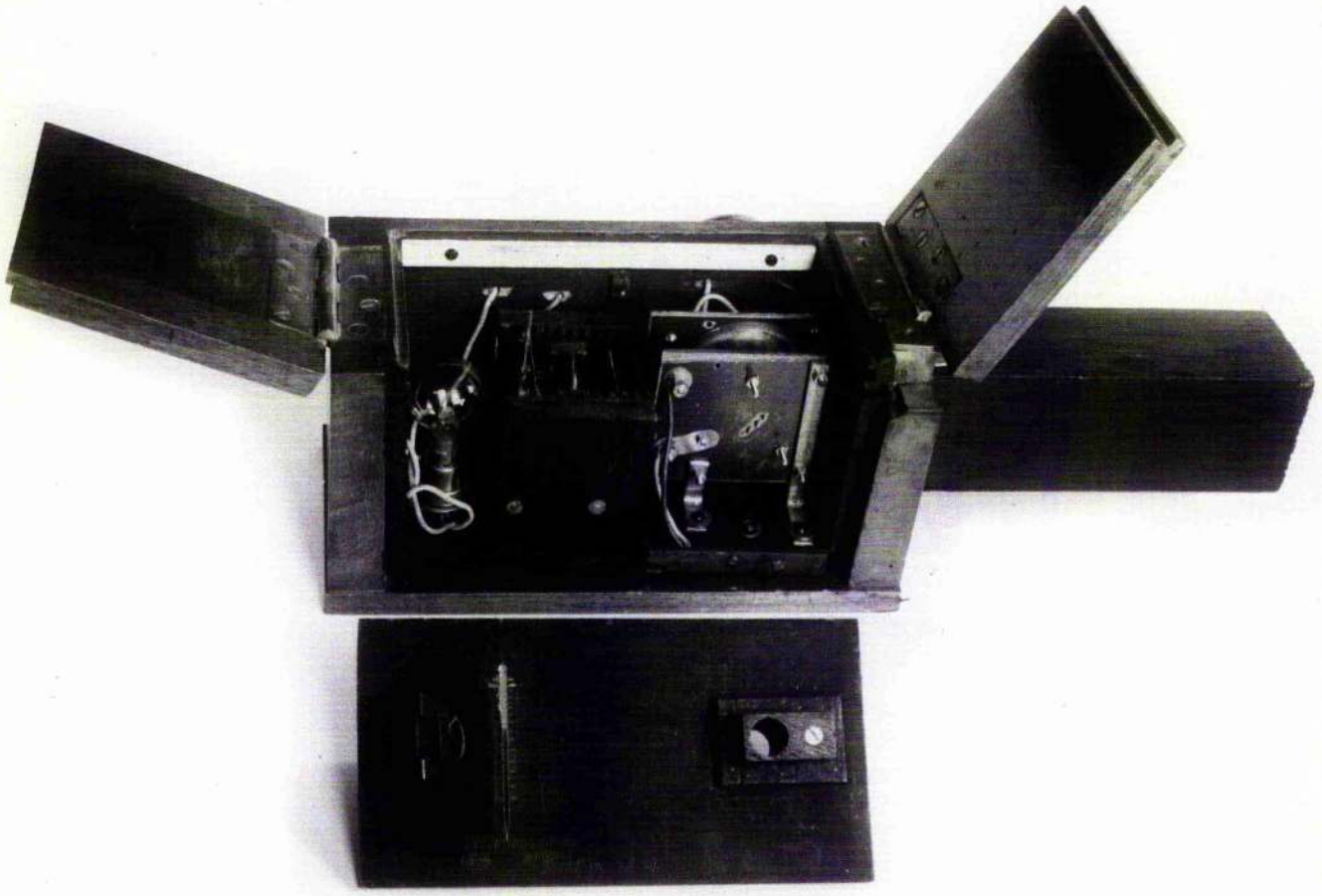
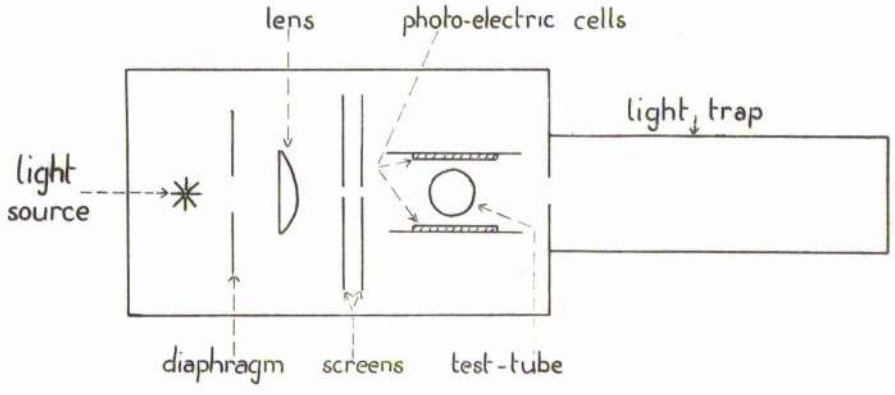


Figure 13

Diagrammatic representation of the nephelometer.




lens
photo-electric cells
light trap
light source
diaphragm
screens
test-tube

b) Measurement of a precipitin reaction.

To carry out a test measuring the reaction between antibody and antigen, 0.3cc antiserum were added to 2cc antigen of the required dilutions in each of eleven tubes and to a control of saline, each tube being mixed thoroughly by shaking. All dilutions of antigen, throughout this work were prepared by placing two volumes of antigen, of suitable concentration, in the first tube and one volume of saline in each of the others. Half the fluid in the first tube was then transferred to the second, mixed thoroughly with the saline in that tube, half of this volume being transferred to the third tube, and so on. This gave a geometric progression of "doubling dilutions". These fluids were measured in the nephelometer immediately after mixing and again after a suitable period of incubation at 37°C; this varied from 20 minutes to several hours. The difference between the two sets of readings, each reading having been reduced to the standard scale, is the "net standard turbidity"; this could be plotted against the tube number of the antigen

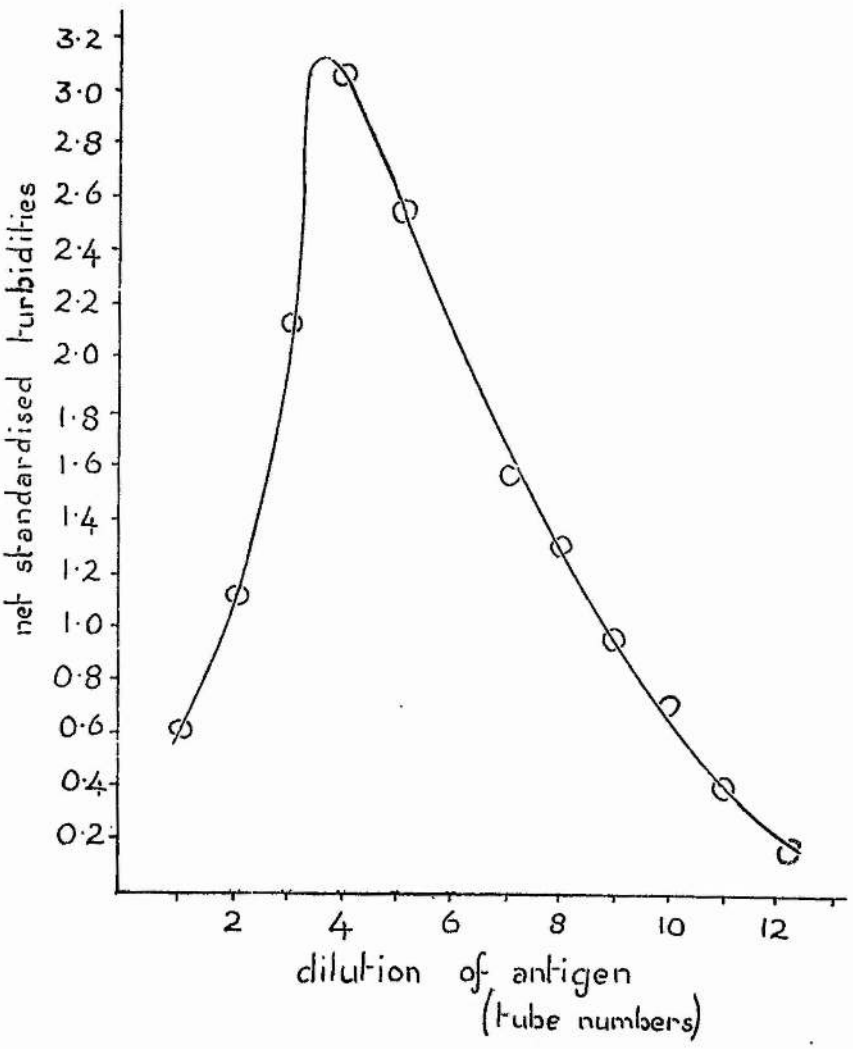


Figure 14.

Human serum x anti-human serum.

Turbidities of antigen - antiserum plotted against doubling dilutions of antigen.

dilutions, which is proportional to the logarithm of the dilutions.

As a test of this method of measurement of the precipitin reaction, an anti-human serum was produced in a rabbit by intravenous injections over a period of fourteen days. 9.0cc of human serum was given in six equal doses, a test bleeding carried out ten days after the final injection and the animal bled completely two days later. (A rabbit which had received 3.5cc human serum over a period of ten days provided only a very weak antiserum). Several homologous tests were carried out using full strength antiserum with a series of dilutions of antigen in 0.9% saline. The results of one of these tests are given in Figure 14, in which antigen dilution is plotted against the net standardised turbidities. It is seen that the reaction is almost complete (i.e., the readings decrease approximately to zero) in the region of antibody excess, but that it is incomplete in the region of antigen excess. To obtain an entire curve, the test would have to be carried out using a more dilute antiserum, but it was apparent from this trial reaction that, with an adequate antiserum, no particular

difficulty would be encountered in measuring the reaction by this method.

c) Application of the method to antisera to C.patellae.

The technique having proved satisfactory, attempts were made to apply it to antisera induced in rabbits by the injection of C.patellae. In the first experiments, lipoid-free extracts were employed, as previously recommended by Wilhelmi (1940); these, however, contained rather less than the optimum concentration of antigen and when no reaction was observed, attention was focussed on increasing the strength of the antisera, by preparing more effective antigens and injecting them in larger quantities. These antigens included living and freshly killed rediae, extracts made in a variety of ways - by grinding, by grinding and centrifuging and by grinding, centrifuging and filtering through paper and bacteriological filters. (See page 102). The test antigens were also increased in concentration up to the limit where their turbidity interfered with measurement. In no case was a definite reaction detected. In two cases, measurable turbidity developed after three to five hours' incubation, but in each case bacterial action in the tubes was found to have

masked any serological reaction which might have been present.

Negative results were also obtained with two other helminths, Cercaria B and Fasciola hepatica, when testing sera from animals previously injected with these. A summary of the various trials is included in Table XIII (page106), and they are not further elaborated here.

At a later stage in the work, antisera produced in ways similar to those described above, were shown, by other tests, to contain active antibodies. It was therefore concluded that nepholometric measurement, excellent though it is for antisera to vertebrate and some invertebrate body fluids, was unsuitable, in its present form, for work with antisera to helminths produced by these methods. To confirm this, final tests of the method were made with two antisera (R 28 and a pooled fowl serum F 3 - F 7, see Table XIII) which gave a good reaction as measured by the ring test, (titres 1:56,000 and 1:5,120,000 respectively). The results of these experiments were almost identical, and one (R 28) is reported in Table X; for comparison, the results of the test of anti-human serum x human serum are also given. (0.3cc anti-serum was added to each of ten dilutions of antigen

and to a saline control making a total of 2cc of fluid in each tube. The tubes were incubated at 37° C and readings taken at twenty minute intervals.)

Table X

Measurement by nephelometer of two precipitin reactions

I. Anti-Cpatellae - serum (R28) x homologous lipoid-free extract; full strength antigen = 0.001 g nitrogen/cc

II. Anti-human - serum (R2) x human serum; full strength antigen = 0.005 g N/cc approx.

Tube dilution No.	Net standardised turbidities			
	I			II
	20 mins.	40 mins.	60 mins.	20 mins.
1 (full strength)	0.050	0.082	0.092	0.62
2	0.044	0.067	0.074	1.12
3	0.036	0.046	0.065	2.13
4	0.035	0.040	0.048	3.60
5	0.023	0.031	0.040	2.55
6	0.006	0.003	0.002	2.11
7	0	0	0.002	1.58
8	0	0	0	1.32
9	0	0	0	1.06
10	0	0	0	0.71
11 (saline)	0	0	0	0

It will be seen that, with the anti-C.patellae serum a detectable increase in turbidity occurred in the first five tubes (the standard deviation of the instrument being about 0.005) but that the highest reading is only about 3% of that obtained in the test of anti-human-serum x human serum. It will also be seen that the whole of the reaction with C.patellae lies in the zone of excess antibody, and

it is therefore impossible to assess the amount of reaction by computing the area of a curve plotted from the readings.

Measurement by ring test

Measurement of the precipitin reaction by ring test requires much simpler apparatus than does the photo-electric method, but probably demands a more skilled technique in setting up a test and reading the result. It is the more widely used of the two methods and proved to be more sensitive in this particular field of research.

In carrying out one of these tests, twelve small test tubes (2.5 x 0.5 cm) were placed in a rack and 0.1cc of doubling dilutions of antigen was introduced into each of the first eleven; the twelfth contained a similar quantity of salt solution as a control. About the same volume of antiserum of constant strength was layered, slowly, below each dilution of antigen and also below the salt solution, by means of a capillary pipette. The tubes were incubated at a temperature of 37°C and readings then taken at intervals of twenty mimutes. The appearance of a slight but definite precipitate at the

interface indicated a positive reaction. Sometimes faint fugitive precipitates appeared early but later disappeared; only those remaining could be regarded as true rings. The precipitate increased in amount and appeared in successively higher dilutions during the period of incubation. The end point, or titre, of the reaction is the greatest dilution capable of forming a visible precipitate at the interface. The clarity of the "ring" of precipitate depends largely upon the skill with which the test has been set up - i.e., upon the degree of admixture between the two layers. An experienced worker can continue to take readings over a period of two, or even three hours, if the tubes are kept at a uniform temperature.

When the test is conducted in the above manner, only a very small quantity of antigen is required - 0.2cc suffices for a complete test. Nevertheless, if only a minute quantity of material is available (e.g., a single fluke) it may not be possible to prepare even this volume of extract at a suitably high concentration. An attempt was therefore made to develop a micro-technique in which only 0.02cc of antigen would be required. Lengths were cut

from narrow glass tubing (0.25cm internal diameter), one end of each being drawn out to a tip about 0.15 cm internal diameter. Volumes of 0.01cc of the doubling dilutions of antigen were placed in separate drops on one watch glass and similar volumes of antiserum on another. By bringing the tip of the tube into contact first with the antigen and then with the antiserum, these were drawn up, successively, by capillary attraction, and if this was done carefully, a sharp interface was maintained between the two layers. By using the antiserum in this manner, there was no possibility of contaminating the main stock. It was found, however, that the very faint precipitin rings encountered in this work were extremely difficult to see in these tubes and, as time did not permit of further trials with clearer glass and special illumination, the method was not pursued further.

Some workers have assessed the results by measuring the thickness of the rings; this was found to be impracticable, however, since there was too much variation with the degree of admixture of the layers. The commonly adopted method, and that used here, is to assign a number

of '+' signs to the results according to the intensity of the precipitate - a purely subjective method. In all the tests carried out, the intensity of the rings never increased to more than a reaction, although I have observed considerably stronger rings (designated ++++) obtained in ring test measurement of vertebrate sera.

The results of a typical ring test are given in Table XI in which the increase in titre over a period of one hour is shown. The tube numbers 1 - 11 refer to the increasing dilutions of antigen; tube no. 12 is the saline control.

Table XI

Results of a typical ring test, showing the increase in titre with time.

Tube numbers of antigen dilutions.	1	2	3	4	5	6	7	8	9	10	11	12
Anti-C.lasium-serum												
20 minutes	+	+	+	+	+	+	-	-	-	-	-	-
40 "	++	++	++	+	+	+	±	-	-	-	-	-
60 "	++	++	++	++	+	+	+	±	-	-	-	-

The homologous test antigen was prepared by centrifuging a suspension of previously frozen cercariae.

In the incubation method adopted, the two layers tended to merge after one hour, and

accurate reading of the results was often no longer possible. The great majority of titres given in this report are those read at twenty minutes, there seldom being any great difference between these readings and later ones.

The rate at which the rings develop is dependent upon the temperature, which should be standardised to within a few degrees. Tests carried out at room temperature take longer to come to completion, although, as the tubes are not subjected to changes of temperature, convection currents are avoided and the interface remains distinct for a longer period.

The formation of rings is influenced not only by the time and temperature of incubation, but also by the concentration of salt and the pH value of the aqueous fluid in which the antigen is diluted. It has been shown by several workers that, with rabbit antisera, a decrease in salt concentration favours ring formation but that if the concentration is too low, unspecific rings are formed in all tubes including the control. Boyden has found that the best concentration is 0.2%; in the tests performed here, however, physiological saline gave adequate results, with less risk of the appearance of

unspecific rings which occasionally formed with a 0.2% salt solution. The use of sea water (in which the cercariae were stored) as a diluent for the antigens was found to be disadvantageous, since the high concentrations of sodium chloride (2.5% to 3%) inhibited the reaction almost completely. In the tests with fowl antisera, however, the effect of salt upon the reaction is the reverse (Goodman and Wolfe, 1952). An increase in the concentration of the salt causes a slight increase in the reaction until a limit (approximately 10% salt) is reached beyond which the formation of rings is unspecific. It was found that the optimum concentration for tests with fowl sera was 2.25% and this was used in all such tests.

The effect of altering the pH of the reaction was investigated in several tests, using a variety of antisera and their homologous antigens. The range of values, over which the tests were carried out, was small since the effect of variation over wider ranges has been fully reported by other workers. It was found that the titre of the reaction was not altered to any great extent by a change in the pH value from 6.8 to 7.3, except in one or two tests with antisera from fowl in which

a slightly higher titre was obtained with the lower value.

As a result of these observations, final tests with antisera from domestic fowl were carried out with 2.25% solutions of sodium chloride, buffered to a pH of 6.8; with rabbit sera 0.9% sodium chloride was used, at a pH of 7.3.

As a qualitative method, the ring test was found to be extremely sensitive and good results were obtained with it in reactions where the nephelometer gave little or no response. One reason may be that, as the two layers diffuse slowly into one another, all possible proportions of antigen to antiserum will be encountered in one tube and the reaction will be apparent where these proportions are an optimum. Further, as the antigen and antibodies are precipitated out, more material will diffuse in to replace them in the reaction zone and the concentration of precipitate will gradually be built up. Conditions for obtaining a ring are therefore less critical than those for obtaining a good precipitate in "bulk mixing". The method of ring test is therefore invaluable for preliminary work in this or related fields.

If the test is to be quantitative, conditions must be very carefully standardised. In addition to the factors mentioned earlier, changes in the size of tube or in the type of illumination may cause a variation in the titre. Such factors are likely to be constant in any one series of measurements, but the results of two different series may not be strictly comparable. Even when all conditions have been carefully controlled, there still remains a considerable subjective element in the determinations of the end point. Rings in the higher dilutions were often faint and ill-defined and the end point far from clear. (In this respect, the tests with helminths compared unfavourably with similar tests involving vertebrate sera). The ring test also suffers from a certain apparent lack of precision. In a series of doubling dilutions, a difference of one tube in reading the end point will result in a difference of 100% in the titre of the reaction. This is less serious than it seems at first sight, since the range of dilutions normally covered is enormous, titres varying between 1:5 and 1:2,000,000 being found in the literature. The difficulty would be overcome if results were quoted on a logarithmic scale, similar to the pH scale used for hydrogen ion concentration (pH = - log

hydrogen ion concentration). The values quoted above would then become 0.7 and 6.3 (= - log 1/5 and - log 1/2,000,000), since "p A/G" = log antigen dilution; doubling dilutions would result in a constant difference of 0.3 units (log 2 = .3), a figure which represents the precision of the test, as carried out in this work. Since dilutions are almost invariably prepared as a geometrical progression, the method appears to be all the more logical. However, the more usual method of quoting "dilutions" is now so firmly established that it will be adopted here, although it is worthwhile to remember that large differences in titres are inherent in the test, and do not necessarily reflect correspondingly large differences in the relationships of the materials used. In some cases, values on the logarithmic scale (p A/G) are quoted in addition, and direct comparison may be made between the two expressions of results.

The quantity determined in the ring test is, in the first instance, based upon the dilution of the test antigen, taking the concentration of the first tube as unity. (This method has been used in some of the qualitative tests performed in this work.) For comparative measurements, however, it

is essential to refer the dilutions to a more definite standard. In using body fluids of closely related animals, the standard may be taken as the undiluted serum etc., but in dealing with aqueous (in this case, saline) extractions of solid material, the concentrations of various extracts may differ very appreciably, and a more definite standard is required.

The most commonly adopted practice, and one which is frequently used by authors, including those working with sera of fairly constant composition, is to quote the concentrations of antigen in terms of the nitrogen content of the extracts. Wilhelmi (1940), however, pointed out that not all nitrogenous substances are antigenic, while polysaccharides and other carbohydrates, which contain no nitrogen, are able to stimulate antibody production. He suggested that a better measure of the effective concentration was the total dry-weight of material in solution. In fact, neither method can give an exact measure of the antigenic quality of the extract and, as the ratio of nitrogen to dry-weight probably varies but slightly among the different materials used, it makes little difference which standard

is employed . Determinations by both methods were made in this work; on the basis of analyses of C.patellae and Cercaria B, which indicated that each gram of dried material contained approximately 0.1 g nitrogen, it was simple to convert from one scale to the other with a sufficient degree of accuracy.

Wilhelmi calculated the concentration of substances in solution from the differences in the dry-weight of the solid material before and after extraction with saline. It was found, in practice however, at least with the small amounts of material used in this work, that the method was liable to give very erroneous results. The residue cannot be separated from the filter pad which must therefore be weighed with it, and the thick Seitz filter pads, being slightly hygroscopic, are very difficult to weigh with precision. Further, the pad becomes impregnated with salt and it is very difficult to wash this out completely or to allow for it. Finally, any loss of material during manipulation completely vitiates the result.

The more direct procedure was therefore adopted of drying (*in vacuo* or at 110°C) and weighing a small sample of the final extract and subtracting from this the weight of the salt, which was determined by titration with silver nitrate (and corrected, in the case of buffered saline, for the phosphate content). Since a Stanton aperiodic balance, with a sensitivity of 0.01 mg per scale division was available (the property of the Department of Chemistry, St Andrews), measurements with quantities of solution as small as 0.01cc, and containing about 0.01 mg nitrogen, could be made. The method was quick, certain, and reasonably accurate, provided the weight of salts did not exceed the weight of organic matter. It broke down on two occasions, once when the quantity of organic material was exceedingly minute (an attempt was being made to make an extract from a single fluke), and once when cercariae had been suspended in sea water of uncertain composition. On these occasions a very rough assessment of the concentration was made by estimating, as accurately as possible, the volume of the fluke and of the mass of cercariae, and assuming that they contained approximately 1% nitrogen.

When nitrogen estimations were made, they were carried out by Koch and McMeekin's (1924) modification of Kjeldahl's method, which was found to be simple and reliable. 'In this method, the sample of material is treated with hydrogen peroxide after digestion with sulphuric acid, and a solution is obtained which is sufficiently clear to be evaluated with Nessler's reagent without the necessity for distillation.

The ring test was used successfully in both qualitative and quantitative tests upon antisera produced; the former tests indicate the presence of antibodies in a serum, and the latter measure the sensitivity of the antisera. Before the application of the test is discussed further, however, details are given regarding the methods of preparation of antigens and antisera.

Preparation of antigens

Antigens are used for two purposes - for sensitising the experimental animals and for testing the antisera produced. Antigens for both purposes are generally identical except in the matter of concentration, though this need not necessarily be so. Very little work has been done

on the biochemical composition of trematodes, and the antigenicity of the various fractions (i.e., the ability of these fractions to stimulate antibody-production) is unknown.

During the course of this work, a number of estimations were made by the use of the modified micro-Kjeldahl technique, of the whole material of C.patellae and of various extracts therefrom. It was found that in approximately 1 cc of fresh material (average dry weight 130 mg) the total nitrogen content was 12.5 mg. When the same quantity of rediae was extracted with an equal amount of saline, the centrifuged extract contained 7 mg nitrogen (mean of fifteen determinations on extractions made in various ways). Another, but more dilute extract was then taken, filtered through successively finer filters and analysed at each stage; these results are given in Table XII and Figure 15. The turbidities, in units equivalent to g.kaolin/cc, measured at the same time, were determined on the Unicam colorimeter (optical density being converted into turbidity by using the curve in Figure 8 as a calibration curve).

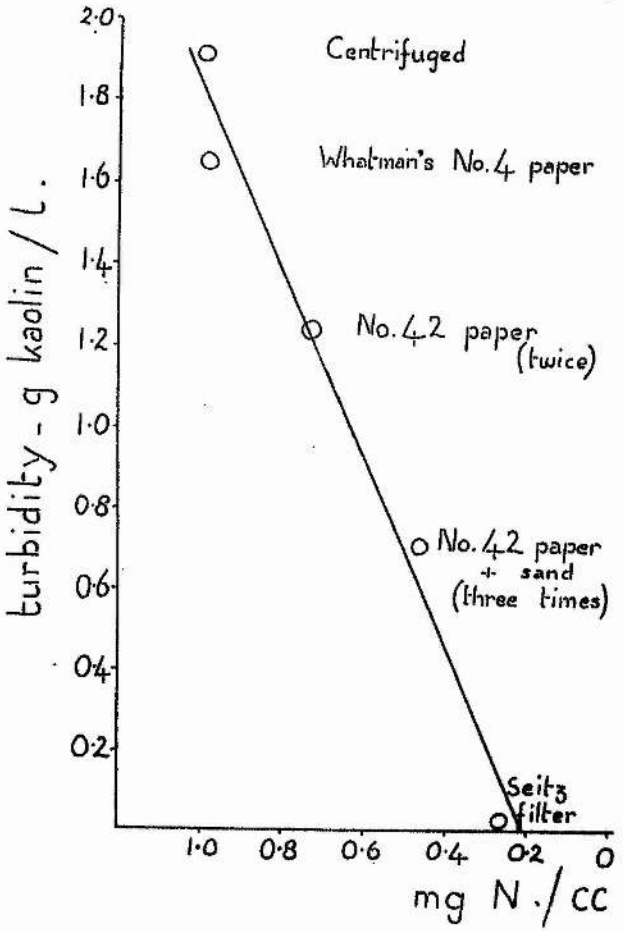


Figure 15.

To illustrate the effect of different filters on the turbidity and nitrogen content of an extract of Cercaria patellae.

Table XII

The effect of filtration upon an extract of C.patellae

Methods, used accumulatively	mg N/cc	Optical Density	Turbidity
Centrifuged	.99	1.14	1.92
Filtered thro' No.4 paper	.98	1.03	1.65
" twice " "42 "	.73	.94	1.24
" 3 times" "42 " and a layer of sand	.46	.56	.71
Seitz filter	.26	.025	.03

The turbidity of the extracts is seen to be due to colloidal material which comprises 75% of the nitrogen content of the centrifuged extract, only 25% of which, that passing through the Seitz filter, is in true solution. The proportion of this nitrogen which is combined in protein was not ascertained. Eisenbrandt (1936), however, found that in nematodes, approximately 50% of the total nitrogen is non-proteinous, as compared with a negligible amount in mammalian sera. If Eisenbrandt's finding is applicable to trematodes, 12.5% of the total nitrogen in the centrifuged extracts can be assigned to protein in true solution, or a total of 1 mg nitrogen per cc of whole rediae. Thus a very rough apportionment of the distribution of the nitrogen content of trematodes may be obtained. The proportion of protein-

nitrogen among colloidal suspensions and insoluble material is not necessarily the same as that in solution, and it is not possible by this method to distinguish between proteinous and non-proteinous nitrogen in these portions of a suspension.

Protein nitrogen in solution	7%	Filtered extract	Centrifuged extract	Whole organisms
Non-protein nitrogen in solution	7%			
Total nitrogen in colloidal suspension	42%			
Total nitrogen in insoluble material	44%			

Simple tests upon the total fat content were also made during the preparation of lipoid-free extracts and it was found that this constituted, very approximately, 20% of the whole material. That these lipoids are partly in the form of an emulsion of globules in the centrifuged colloidal solutions, was shown by the great reduction in turbidity which followed extraction of the lipoids with ether.

One may assume that the proteins and perhaps some carbohydrates present will stimulate antibody-production if injected into experimental animals, antibodies which are likely to be highly specific

in their action. Lipoids, although not antigenic in themselves, may, in combination with proteins or even carbohydrates, also cause antibodies to be produced. These, however, tend to be somewhat unspecific owing to the fact that biochemical correspondence among fatty substances covers a much wider field in the animal kingdom than do proteins and carbohydrates. As a result, a test involving the reaction of lipoids, in addition to the other fractions of an extract, may not be of the specificity required to differentiate between closely related genera and species. To produce an antiserum of extreme specificity, all antigenic fractions likely to lead to a group reaction should be eliminated, but in an unexplored field, removal of lipoidal material should be sufficient in the first instance. There appear to be two methods of eliminating lipoids from the reaction. The first is to inject an extract from which the lipoids have already been removed; the resulting antisera could then be tested against a similarly prepared extract, or less successfully perhaps, against the whole extract of the organism. An alternative method is to inject the whole extract, regardless of a possible lack of

specificity of the antiserum produced, and to test this against a test-antigen from which the non-specific lipoids have been removed. (An additional method, but one not used here, involves the adsorption of the non-specific antiserum with an extract of lipoids; this leaves only the specific antibodies to react in the test).

There are two main advantages of the second method. Firstly, stimulation of the experimental animal to a variety of antigens, regardless of their specificity, may cause an increase in the production of antibodies to the particular antigens to be tested. Secondly, the preparation of a lipoid-free antigen for the purpose of a series of injections requires a quantity of material which is large compared with that which suffices for a test. In dealing with helminths, this is an important practical consideration since large quantities of material are seldom available.

For the above reasons most of the injections were made with whole extracts, either filtered or centrifuged, or with suspensions of whole or ground-up organisms, prepared from living, preserved or dried material; lipoid-free extracts

Explanations to Table XIII

Rabbits 1 - 26 received injections in several series.

" 26 - 30 and R 543 - 545 received injections in two series.

Fowls 1 - 2 received injections in two series.

" 3 - 7 " " " one "

Rabbits 1 - 21 were bled 10 - 14 days after final injections.

" 22 - 30 and fowls 1 - 7 bled 4 - 7 days after final injections.

Anaphylaxis - antigenic extracts administered intravenously to produce shock at varying intervals after sensitising doses.

The nitrogen determinations in R 4, R 5, R 6, R 7 and R 30 were calculations from the dry weights.

In R 9 and R 10 the micro-Kjeldahl method was used.

In all other cases the nitrogen content has been estimated roughly from the approximate volume of whole material used and the proportion of nitrogen known to pass through various filters.

Abbreviations

I.V. intravenous

Subcut. subcutaneous

I.P. intraperitoneal

TABLE XIII Preparation of Antisera

Anti-serum No.	Material	Method of Preparation of antigen	Total mg. nitrogen injected approx.	Route of injection	No. of injections	Duration of experiment (days)	Method of test	Result
R.1.	Human serum	Fresh	(3.5 cc)	I.V	5	20	Photo-electric	✓
R.2	Human serum	Fresh	(9 cc)	I.V.	6	24	Photo-electric	✓
R.4	Cercaria patellae	Wilhelmi's method	1·1	I.V.	3	15	Photo-electric	0
R.5 R.6 R.7	C.patellae	Wilhelmi's method	2·2	I.V.	6	28	Photo-electric	0
R.8	C.patellae	Suspension of living rediae Suspension of freshly ground rediae	25	subcut.	7	40	Photo-electric	0
R.9.	C.patellae	Freeze-thawed extract, Berkefeld filtered	2	I.V.	5	20	Photo-electric	0
R.10	C.patellae	Freeze-thawed extract, Berkefeld filtered	4	I.V.	17	105	Photo-electric	0
R.11	C.patellae	Freeze-thawed extract, paper filtered	38	I.V.	12	54	Photo-electric	0
R.12	C.patellae	Living rediae + metabolic products (whole digestive gland and gonad of infested limpet)	-	I.V.	12	52	Photo-electric	0
R.14	Fasciola hepatica	Freeze-thawed extract of flukes, Berkefeld filtered	6	I.V.	6	22	Photo-electric	0
R.15	F.hepatica	Freeze-thawed extract of flukes, paper filtered	24	I.V.	12	50	Photo-electric	0

Table XIII (continued)

Anti-serum No.	Material	Method of Preparation of antigen	Total mg. nitrogen injected approx.	Route of injection	No. of injections	Duration of experiment (days)	Method of test	Result
R.17	Cercaria B	Freeze-thawed extract of sporocysts, paper filtered	20	I.V.	11	52	Photo-electric	0
R.19	C.patellae	Suspension of freshly ground rediae	10	I.V.	7	42	Photo-electric	?
R.21	C.patellae	Paper-filtered extract of rediae Suspension of freshly ground rediae	20	I.V. subcut.	9	40 40 + 28	Photo-electric Anaphylaxis (1 cc extract)	0 ++++
R.22	C.patellae	Suspension of formalin-preserved rediae	50	I.V. + subcut.	19	70 70 + 30	Ring test Anaphylaxis (1·5 cc extract)	0 ±
R.23	Cercariaeum lasium	Suspension freeze-thawed cercariae Suspension cercariae incubated in rabbit serum Suspension of living rediae	3·6 0·2 3·3	I.V. + subcut I.V.	12 1 10	180	Living cercariae Ring test	? ✓
R.24	Cercaria tenue	Suspension freeze-thawed cercariae Suspension of living cercariae	2·3 5·4	I.V. + subcut	6 9	155	Ring test Living cercariae	? ?
R.25	Cercaria setiferoides	Suspension freeze-thawed cercariae Suspension of living cercariae	2·4 2·0	I.V. I.V. + subcut	3 3	90	Ring test Living cercariae	✓ ?
R.26	Cercaria variglandis	Suspension Freeze-thawed cercariae Suspension living cercariae	·7 ·9	I.V.,I.P.+ subcut. subcut.	9 5	78	No material available for test	
R.27	C.patellae	Suspension of living cercariae	1·1	I.V.	5	26	No live material available for test	
R.28	C.patellae	Homogenised suspension of rediae	65	I.V. + subcut.	7	55	Ring test Photo-electric	✓ ✓
R.29	Cercaria B	Homogenised suspension of sporocysts	65	I.V. + subcut.	9	55	Ring test	?
R.30	C.patellae	Wilhelmi's method	40	I.V.	4	12	Ring test	0

Table XIII (continued)

Anti-serum No.	Material	Method of Preparation of antigen	Total mg. nitrogen injected approx.	Route of injection	No. of injections	Duration of experiment (days)	Method of test	Result
G.3	C.patellae	Suspension of formalin-preserved rediae	1·5	I.P. + subcut.	3	35	Anaphylaxis	±
G.4	C.patellae	Suspension of formalin-preserved rediae	1·5	I.P. + subcut.	3	35	Anaphylaxis	-
G.5. G.6	C.tenue	Antiserum from R.24	(1 cc)	I.P.	1	2	Anaphylaxis	-
G.2 G.7 G.8	C.lasium	Antiserum from R.23	(1 cc)	I.P.	1	2	Anaphylaxis	- - -
G.9 G.10	C.lasium	Antiserum from R.23	(0·5 cc)	I.P.	1	2	Anaphylaxis	- -
F.1	Cercaria quissetensis	Suspension freeze-thawed cercariae Suspension of living cercariae	1·1 1·5	I.V. + subcut. subcut.	4 5	80	Ring test	✓
F.2	C.lasium	Suspension freezepthawed cercariae Suspension of living cercariae	0·4 4·5	subcut. I.V. + subcut	2 7	80	Ring test	✓
F.3 F.4	C.patellae	Suspension of living rediae Homogenised suspension of rediae	30	I.V. + subcut.	7	34	Ring test	✓ ✓
F.5 F.6 F.7	C.patellae	Suspension of living rediae Homogenised suspension of rediae	30	I.V.	7	34	Ring test	✓ ✓ ✓
R.543 R.544 R.545	C.patellae	Ultrasonically treated material	6·5	subcut.	10	59	Ring test Photo-electric	✓ ✓ ✓

were also used on five occasions.

Of the different physical states, soluble substances are most likely to stimulate antibody production and the insoluble tissues are perhaps least likely to be effective. Suspensions of whole material, however, were used for injection, partly in the expectation that the more rapid preparation would lead to less denaturation of labile substances and partly so that no possible antigenic material should be lost. This is of great importance where minute quantities of material are available.

The details of the injections and the results with antisera produced are summarised in Table XIII. Unfortunately, the early trials cannot be used to assess the different injection antigens since the method of test used - nephelometric measurement - was found to be unsuitable. In general it was found that suspensions of ground material were most successful in stimulating the production of antibodies.

Rediae, sporocysts and cercariae, if not used in the living state were usually preserved by keeping them frozen until required, or freezing and thawing successively to break up the tissues

(organisms treated in this way are referred to as "freeze-thawed"material). Rediae of C.patellae were also preserved occasionally with 0.5% formalin, 0.1% merthiolate or with toluene, when material was being sent to the U.S.A.

A series of tests was carried out upon rediae of C.patellae to find the most efficient method of extraction. Numerous ways of breaking up the cells were employed and these included the use of fine and coarse sand, bursting of the material by evacuation and grinding in the frozen state. A variety of extractants was also used, including sodium and potassium chloride in both buffered and unbuffered solutions; the buffer used, adjusted to a pH of 7.0, was a phosphate mixture prepared according to Evans (1922). Potassium chloride was used in a 0.9% solution and the sodium salt in 0.9% and 10% solutions. The nitrogen content of each extract was determined by the modified micro-Kjeldahl technique, but no significant differences were apparent. The amount present in a centrifuged extract varied from 2.0 to 4.8 mg/cc, but the deviation appeared to be due to the difficulty of measuring exactly a known volume of rediae and it could not be correlated

with any of the methods of extraction used. The antigens, used during the earlier experiments and those used for the nitrogen determination, were made by grinding in an agate or specially constructed steel mortar; those prepared during the later experiments were pulverised in a small glass homogeniser which was found to be greatly superior in its action.

The lipoid-free extracts of C.patellae for use as injection antigens were prepared according to the directions of Wilhelmi (1940). In this method, the rediae were vacuum-dried in the frozen state, pulverised and extracted to constant weight with a mixture of alcohol and ether. The lipoid-free material was then agitated for twenty-four hours in a mechanical shaker with 0.9% saline and filtered through a sterile Seitz filter.

Two other methods of preparing lipoid-free extracts were developed but only used for test antigens. In the first, Wilhelmi's method was followed for the preparation of dry, lipoid-free material but the extract was made by stirring briskly for five minutes with ice cold buffered saline and centrifuging till reasonably clear, (an hour was required for C.patellae, whereas

fifteen minutes sufficed for Cercaria B.) It was found that 97.5% of the soluble material was extracted during the five minutes stirring, and it is obvious that a prolonged period of extraction is unnecessary. In the second method, the fresh organisms were homogenised with buffered salt solution, the lipoids being extracted from the aqueous solution by shaking with ether and separating the two layers; traces of dissolved ether were removed from the aqueous layer by blowing a current of air over it. Either method could be used with very much smaller quantities of material than are required for Wilhelmi's method (extractions using as little as 0.25cc of saline were carried out). Except for the presence of a small amount of colloidal material, the product should be generally similar to that of Wilhelmi, but, with the shortened time of extraction, the chance of denaturation of the labile material is greatly reduced. The antigenicity of an extract is a factor which cannot be measured in vitro (except in a test against a known antiserum), but it is bound to vary with the degree of denaturation which has occurred. Precautions against such a process are essential, of which shortening the time taken for an

extraction is the most obvious.

The results of ring tests comparing the antigenicity of these three lipoid-free extracts of C.patellae are given in Table XIV. The titres are quoted as percentage values of the highest titre, which was obtained with the extract of fresh material, from which lipoids were removed from the aqueous solution. It must be remembered that a difference of one dilution of antigen will appear as a deviation of 50%, when compared with the highest dilution, or highest titre obtained, and this is the smallest variation which can be estimated by the ring test.

Table XIV

Estimation of various extracts tested against an anti-C.patellae-serum, (F3 - F7).

C.patellae, 1 (lipoids extracted from aqueous solution)	C.patellae, 2 (lipoids extracted from freeze-dried material)	C.patellae, 3 (antigen prepared according to Wilhelmi's method)
Anti-C.patellae-serum		
100%	55%	4%

In this table, the highest titre is given as a reaction of 100% (with extract 1 of C.patellae).

A closely similar extract (C.patellae 2) shows a reaction which appears to be only half that of C.patellae 1, but in reality varies by only one tube dilution; (the deviation from the value of 50% is due to the extracts being of slightly different strengths). The 4% reaction of the third antigen, however, differs from the highest titre by approximately five tubes; it is obvious that a significant variation between the two extracts does exist in this case. In a similar test with an antiserum produced in a rabbit (R28), the Wilhelmi preparation showed no reaction in the strength used, indicating a titre of less than 0.4% of that given by extract 1 in the above table. It can be seen from these results that the rapid extraction of material during the preparation of two of the extracts provided antigens of adequate strength, whereas the antigenicity of the extract prepared according to the method of Wilhelmi is very much reduced, apparently by the long saline extraction of the material. Decomposition or denaturation of proteins and other antigenic substances will destroy the antigenicity of these

compounds. A change in the structure of the compound caused, however, by chemical or certain other processes may not destroy the antigenic activity, but may cause sufficient alteration to affect the serological specificity of the substances involved. Treatment of this sort can be employed, however, if used in moderation, and preservatives may be used if in comparatively high dilutions. Where slight alteration does occur without complete loss of the ability to react with native material, care must be exercised in the interpretation of the tests. The ideal procedure is one in which the antiserum is tested with an antigen prepared in a manner identical to that used on the antigen for injection. Valuable results may be obtained, however, if the antiserum is tested with a modified homologous antigen, providing that this, and all heterologous antigens used have undergone comparable treatment.

A short investigation along similar lines was carried out jointly with Dr. Boyden[†] on the effect

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[†] I am grateful to Dr. Boyden for suggesting this experiment and for the enthusiasm with which he carried out the tests at Rutgers University; I am also indebted to him for permission to include results of recent work with Cancer borealis.

of subjecting the material to ultrasonic vibrations prior to extraction with saline. This method has been used frequently in making extracts of bacteriological material and is considered to be highly efficient (see Kabat and Mayer, 1948). Its main action is probably to break up the cell structure of the material but prolonged exposure, at least, may lead to chemical change and an alteration in the serological specificity, (Grabar and Kaminski, 1950; Grabar, 1951).

Rediae of C.patellae in fluid suspension (divided into three lots and preserved respectively in 0.5% formalin, 'Merthiolate' - 0.1% - and toluene, for transmission to the United States) were subjected to ultrasonic vibrations. The ultrasonic treatment was supplied by a Magnetostriction Oscillator operating at 10 kilocycles, with an output of 200 watts, each sample being given a ten minute exposure. The extracts were then injected into rabbits, each being given in two series of injections; (for

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Footnote: The ultrasonic treatment was carried out at the laboratories of Sharpe and Dohme, Pennsylvania, by kind permission of Dr. J. Munoz.

details, see Table XIII, page 106). The animals were bled ten days after the final injection and the resulting antisera were tested at Rutgers University by Dr. Boyden, and by myself at St Andrews. The antigens used for the tests at Rutgers University differed from those used for injection in that the material had not been submitted to ultrasonic treatment but had been 'freeze-dried' prior to extraction; dilutions of the test antigen were made in 0.2% sodium chloride. Two homologous antigens and one heterologous antigen were used at St Andrews. One of the homologous antigens was similar to that used by Dr. Boyden except that the preservative had been omitted; the other two antigens were lipoid-free extracts in 0.9% saline, similar to those used in tests with other antisera carried out at St Andrews.

The homologous reactions proved to be sufficiently strong to be measured photo-electrically as well as by ring test, and as the measurements extended over the whole range of the reaction, it was possible to record the extent of the reaction in terms of the areas enclosed by the curves. The results of the tests with the strongest antiserum

(that prepared against the formalin-preserved material - R 544) are given in Table XV, in which the readings on the Rutgers' photroner have been reduced to the same scale as those on the St Andrews' nephelometer. (This conversion was made possible by the fact that identical kaolin suspensions had been measured on both instruments.)

Table XV

To show the results of tests with an antiserum to ultrasonically-treated material

	Rutgers University whole extract	St Andrews University whole extract	St Andrews University lipoid-free extract	St Andrews University Cercaria B lipoid-free
Ring test (titre)				
Antiserum R 544	1:400,000		1:112,000	No reaction at 1:5,500
Photo-electric readings (area of curve)				
Antiserum R 544	2.01	0.10	0.14	
Anti-human-serum		16.5		

The most striking point in these results is the strength of the reaction measured photo-electrically at Rutgers University. Although only one eighth of the reaction recorded between an anti-human-serum and human serum, it is very much greater than any

recorded from earlier tests measured by this method. The results of a previous test (see page 87) are of the same order as those reported in Table XV, from tests carried out at St Andrews with antiserum R 544. The observed differences between results of the tests at Rutgers University and St Andrews cannot be easily explained. It is unlikely that the presence or absence of preservative in the dilutions used would cause any significant variation. An elapse of three months before the second set of tests was carried out (in St Andrews) might be considered to affect the results, but in such a case, a larger variation between the ring test titres would be expected. (During the three months' interval, the antisera, which had been Seitz-filtered, were stored at a temperature of 4° C). An alternative explanation is that some unknown, and therefore uncontrolled factor caused the difference between results.

The significant fact emerging from this experiment is the apparent increase in the number of antibodies produced in response to injections of ultrasonically-treated material. This is evidenced by the considerable increase of turbidity (which is a measure of the quantity of antibody present) compared with the amount formed

with antisera to native material.

The effects of ultrasonic treatment upon a known antigen have recently been tested at Rutgers University under the same conditions as those described above. It was found that the haemocyanins of Cancer borealis were definitely altered during a thirty minute exposure to ultrasonic radiations. This alteration was revealed by a reduction in the reactivity of the treated antigen to 67% of that of the native material; this was evident when the treated antigen was tested by the standard procedure with the photroner, against an antiserum to native haemocyanins. It is probable, therefore, that slight alteration or denaturation had also occurred during the ten minute exposure to which the samples of C.patellae were subjected.

In recent years, most of the serological work on helminths has been concerned with antisera produced in response to active infections of normal hosts, and where parasites invade the tissues of the host, the antisera are frequently of considerable strength. It is doubtful whether parasites inhabiting the gut during the whole of their existence stimulate any antibody production in

their hosts, since there is no intimate association between the host tissue and the parasite (see Culbertson, 1941; Faust, 1949; and others).

The natural definitive hosts of the cercariae used here are very different from the rabbits employed experimentally in much of the research. Domestic fowl are more nearly related to the final hosts of some of the cercariae and therefore possibly more suited to the production of antibodies to these organisms. Since, however, all these cercariae develop to maturity in the gut of their hosts, active infection of the experimental animals would have been useless, even had it been practicable. Even so, when material was available, living cercariae were injected intravenously into some of these animals. The chances of the cercariae living for more than a very short time in the blood stream of the animal are slight owing, in part, to the lethal action of most vertebrate sera, and, in part, to the extreme abnormality of the conditions. In spite of this, it was considered possible that a few metabolic products might thereby stimulate antibody production to a small degree. Attempts were therefore made to collect metabolic products

before injection, for although these products might be extremely labile it was felt to be possible that they would remain unchanged for a few hours. In one series of injections (R 12), the digestive gland and gonad of a limpet heavily infested with C.patellae were used. (In this case, however, it was realised that antibodies to limpet tissue would also be stimulated). In another series of injections (R.19) C.lasium were kept alive in inactivated rabbit serum for several hours; (it was found that although C.lasium did not live in the fresh serum of this rabbit, their activity was not impaired in this medium after complement had been destroyed). This serum and the cercariae were used for injection.

Besides the passive sensitisation of experimental animals by injection of extracts or of whole organisms, an experiment was carried out on myself involving active penetration of C.variglandis. This is one of the schistosome cercariae, normally developing in a gull, which is known to cause "sea-bather's eruption" in individuals who have come in contact with these cercariae on the shore. The experiment was conducted for the purpose of a comparison between sensitisation to an active infection by

a few organisms, and passive injection of considerable quantities of foreign material.

Approximately half a dozen active cercariae contained in a drop of sea water were left in contact with the skin (flexor surface) of the forearm for five minutes, after which the sea water was removed by pipette. A slight prickling sensation was experienced almost immediately and examination of the drop of sea water showed that only the active tails of the cercariae remained. It was apparent, somewhat unexpectedly since reports had indicated that more than one contact was required to produce a reaction, that antibodies had been produced one week later when a marked intradermal reaction occurred. First, the area which had been covered by the drop and, later, a rather larger area became reddened and oedematous, a condition which was accompanied by extreme irritation. Small pustules appeared at the points of penetration and the whole reaction was typical of "sea-bather's eruption" or "swimmer's itch" (a similar reaction caused by fresh water cercariae). Blood was taken 28 days after the initial infection, and the serum used for an *in vitro* test upon living cercariae which

showed a distinct positive reaction (see page 132); insufficient trematode material was available to allow of a ring test with this serum. A second infection was carried out one month after the first, the typical dermatitis appearing only twelve hours after penetration. This showed that the body was fully sensitised to the cercariae or their metabolic products, but unfortunately no cercariae were available for a test with the antiserum. (It is interesting to note that the area of penetration, which was still just visible nine months after the initial infection, became considerably more evident two days before an attack of chicken pox developed. From this it would appear that there may be a very slight cross reaction even between such distantly related organisms as cercariae and viruses!)

The antigens employed for testing antisera were essentially the same as those used for injection except in the case of ultrasonic treatment of the material, prior to extraction. It was impracticable, however, to use suspensions of whole worm or even undiluted paper-filtered or centrifuged extracts on account of their turbidity.

(An attempt was made to use such an extract by measuring, in the nephelometer, a reduction in turbidity; as the particles, which flocculated upon the addition of antiserum, settled out, the total amount of turbidity decreased. The reaction proved, however, to be completely unspecific, since flocculation also occurred in the control tubes containing antigen and normal serum). Sufficient clarity of the extract of C.patellae was attained either by passing through a bacteriological filter or by diluting a centrifuged extract with saline in the proportion of 1 : 10. The latter method was used successfully for ring tests which were designed to be qualitative or only semi-quantitative. In the final quantitative tests, lipoid-free extracts were employed with success. In these, the nitrogen content of the extract was found to be equal to that of an undiluted centrifuged extract, while the turbidity was equal to that of a whole extract one tenth of this strength. Tests with an antiserum prepared in a rabbit (R 24) showed clearly that the removal of lipoidal material increases the specificity of the reaction, in addition to decreasing the turbidity of the extract. Two homologous and two heterologous

ring tests were carried out, the latter with extracts of Cercaria B. In both sets of tests, a centrifuged whole extract was compared with a lipoid-free extract. The results of these are given in Table XVI, and, as in all the results of this nature quoted in the thesis, the ring test titres are based upon the total nitrogen concentration of the extracts used.

Table XVI

The effect of lipoids in homologous (C.patellae) and heterologous (Cercaria B) reactions

Extract	Homologous test		Heterologous test	
	untreated	lipoid-free	untreated	lipoid-free
Antiserum R 24 Titre at 20 mins. based on total nitrogen	1:230,000	1:290,000	1:130,000	< 1:20,000

It is seen that there is no significant difference between the titres of the homologous reactions. In the heterologous tests, the untreated extract reacted quite strongly with the antiserum, there being a difference of approximately only one dilution of antigen between this heterologous and

the corresponding homologous test. However, with the lipoid-free extract of Cercaria B, the titre was much reduced; indeed, with the antigen used in this case, the reaction was not apparent. Although the result may be negative in this particular test, it might have been positive had a stronger test antigen been available. Consequently the figure (the strength of the 'undiluted' antigen) quoted in the table indicates that the titre of the reaction must be less than (<) the dilution of the first tube in the series.

Production of antisera

Rabbits have been most generally used for the preparation of antisera, but as the first experiments with these were disappointing, domestic fowl were also used. These were chosen because of their closer relationship to the definitive hosts of some of the cercariae being used for investigations, and it seemed possible that they might be sensitised more readily to antigenic extracts of the parasites. An additional advantage which may possibly be gained from the use of domestic fowl is in respect of

the specificity of the resulting antisera. It is well known (see Boyd, 1947) that a rabbit will respond readily to the injection of Forssman and Forssman type antigens (serologically related substances, present in the cells of some animals, which are widely separated systematically), whereas the fowl, which has the Forssman antigen in its tissues and red blood corpuscles, will not. Up till now there has been no evidence of differences of this type between fowl and rabbit antisera compared at the Serological Museum, Rutgers University, but it is conceivable that in an unexplored field, such differences may occur. Although the distribution of Forssman antigens among trematodes is not known, it is possible that some may be present. If this is the case, an antiserum produced in a rabbit may contain Forssman type antibodies which will react unspecifically when tested. Injections of the animals were carried out by the subcutaneous and intravenous routes and occasionally intraperitoneally. So far as it was possible to judge on the results available, variation in method of injection had no marked effect upon the antisera produced. In general, the domestic fowl were more satisfactory as regards antibody production, there being no case

in which one failed to produce demonstrable antibodies.

Intravenous injections were administered via the lateral vein of the ear of rabbits and the wing vein of fowl. Test bleedings were made from the ear of rabbits, from either the median artery or lateral vein, and from the vein in the wings of fowl. Occasionally a test bleeding of a rabbit had to be made by means of a cardiac puncture. Final bleeding was carried out by cardiac puncture or, in some cases, from the jugular vein and carotid artery. The blood was allowed to clot and left for some hours, usually overnight, in the refrigerator before being centrifuged to provide clear serum. With either animal, the degree of haemolysis varied considerably, depending upon the ease with which the blood was extracted. If the serum was to be kept for any length of time, it was passed through a sterile Seitz filter, bottled under sterile conditions and stored at a temperature of 2°C.

Results.

The various tests performed fall into two categories - those designed to be purely qualitative, and those which were used to give quantitative results of the antigen/antibody reaction. The qualitative tests were undertaken to find out the most efficient method of producing antisera to trematode antigens, results of this nature sufficing to indicate the presence or absence of antibodies. In addition, some of the qualitative tests were carried out to investigate the possibility of adapting the methods used to a quantitative basis. Finally the most successful of the techniques investigated was used quantitatively with two selected antisera. Attempts were made to apply the ring test to the original problem of the research - the study of the ontogeny of trematodes.

Qualitative tests

a) The effects of sera upon active cercariae.

Some of the earlier qualitative tests carried out were concerned with observations on living cercariae. It was considered possible that some

in vitro effects of antisera upon active cercariae might provide evidence as to the presence of antibodies.

A test commonly used in serology involves the agglutination reaction. Liu and Bang (1950), investigating cases of schistosomiasis, have reported that the presence of antibodies cause clumping or agglutination of living but not of dead cercariae placed in antiserum. Similar tests with C.lasium were not attempted since these acaudate cercariae, incapable of swimming actively, become aggregated in large clumps even in sea water.

Tests were attempted with the actively swimming cercariae available - C.tenue, C.setiferoides and C.quissetensis, but it was found that even in normal sera, these cercariae showed a tendency to clump. It was not possible to distinguish, with any degree of certainty, whether this capacity to clump was increased by the presence of antibodies. No agglutination tests with C.patellae or C.variglandis were possible, owing to an absence of active cercariae at such times as antisera were available. It is possible, however, that

such tests might have been successful; the tendency of these particular cercariae to clump in normal sera was much reduced, possibly owing to their less vigorous swimming movements, and agglutinations in antisera might have been evident.

Other reports of *in vitro* tests on cercariae include those referring to the formation of a transparent sheath around schistosoma cercariae placed in immune sera. Several authors (Papirmeister and Bang, 1948; Vogel and Minning, 1949 a and b; Standen, 1952) in search of tests for early diagnosis in cases of schistosomiasis, have described the appearance of this sheath, the formation of which has been ascribed to a precipitin reaction. It seemed likely that a similar reaction might be obtainable with other cercariae, and tests were carried out here for the purpose of investigating this possibility.

The technique adopted was as follows:- a few active cercariae in a small quantity of sea water were placed in a well-slide: most of the sea water was removed and replaced with four to six drops of serum: the activity of the cercariae

was observed with the aid of a dissecting microscope or, when necessary, under higher magnification: observations were made initially after five minutes and thereafter at approximately ten minute intervals for a period of an hour or more if necessary (i.e., if the cercariae remained alive).

When these experiments were initiated, it was thought that normal sera from animals of the same species as those being injected would be adequate for the purpose of controls. In practice, it was found that the behaviour of any one species of cercariae varied considerably in normal sera from different individuals. In some preliminary tests with C.lasium and normal sera from six rabbits, it was possible to discern an over-all result - a tendency for cercariae to encyst. The variation among the results with each serum was, however, too great to allow of generalisations regarding the action of rabbit sera upon C.lasium. The only true control of observations of this nature is, therefore, a test with the normal serum of the rabbit or fowl to be used, prior to injections with the cercarial antigen.

Examinations on living cercariae in immune sera were made but injections of the experimental animals were carried out before the need for prior testing of the serum was realised; the results recorded had to be discarded, therefore, as being unreliable without adequate controls. The scarcity of time and of living cercariae made a repetition of the experiments impossible. Nevertheless, in spite of the lack of reliable observations, some of the results are given here, as an indication of the behaviour of some cercariae in various sera, both normal and immune. In many cases, normal serum caused encystment of previously active cercariae (e.g. C.lasium). The presence of antibodies, however, apparently caused an increase in the number of cercariae which were killed during immersion in antisera, although cercariae which encysted quickly appeared to be protected from the toxic effects of the antisera. The effects of normal sera upon other cercariae (C.tenue, C.setiferoides, and C.patellae) were much reduced. In these tests the bodies of the cercariae usually remained active, but the tails of some were lost. The effects of antisera upon some of these cercariae

(*C.patellae* was not available for such observations) appeared to be the same as upon *C.lasium* - causing an increase in the number of cercariae killed. This lethal factor, which exists, to a lesser extent, for certain species of cercariae in some normal sera, was found to be destroyed by inactivation or storage of the serum. (The toxic effects of a serum, whether normal or immune, were considerably reduced even twenty-four hours after the animal was bled, and the time of the observations had to be carefully standardised). It is possible that the lethal factor in normal sera, present in varying degrees, is associated, at least, with complement. Since the reaction attributed to the presence of antibodies was also inhibited by the destruction of complement, it is possible that complement is necessary for the completion of any antibody/antigen reaction.

In addition to the above tests with these cercariae, *C.variglandis* was used in a single test with an immune human serum. During an experimental self-infection with this species of cercaria (causing "sea bather's eruption", see p.120), antibodies were produced, as

evidenced by a typical schistosome dermatitis reaction. Serum was tested with C.variglandis twenty-eight days after the initial infection. Again no control observations were possible, but the formation of a sheath, similar to those reported by other workers (and shown to me during a visit to the U.S.Naval Research Laboratory, Washington, D.C.) was probably caused by the antibodies present in the serum.

From these few tests it would appear that antibodies may have some demonstrable effects upon living cercariae. An absolute control of each observation is impossible since, once the animal is injected with antigen, the supply of normal serum ceases. To store this normal serum is of no advantage, since the quality of sera alters very appreciably with time. The control test, therefore, must necessarily be separated in time from the observations and tests with immune sera. Conditions under which experimental animals are kept are normally reasonably constant; this being so, there is no reason to suppose that a change in the quality of a serum or in the observed effects of such a serum from injected animals is due to any factor other than the presence of antibodies.

Observations on the changes in the behaviour of living cercariae are readily adapted to a quantitative test. In a serial dilution of a serum being tested, the end point may be taken as the greatest dilution of serum which causes death, encystment etc. of the cercariae. Before tests of this nature, however, could be employed to measure the strength or sensitivity of an antiserum, it would be necessary to carry out a fuller investigation into the effects of different sera upon a number of species of cercariae.

b) Anaphylaxis.

Among the qualitative tests on antisera, a certain number of experiments were carried out utilising anaphylactic shock as a method of demonstrating the presence of antibodies.

Many workers (see Boyd, 1947) have found that animals, sensitised to a wide variety of antigens may suffer from anaphylactic shock if another injection of the appropriate antigen is administered after a certain interval of time. The sensitivity of different animals to anaphylactic reactions varies considerably. In general, guinea pigs are highly susceptible to shock of

this type, whereas rabbits are not so liable to suffer from the reaction. Sensitivity is usually induced actively, (i.e. - the animal responds to injections of foreign material, producing antibodies) by a small amount of antigen; if the dose is too large the animal may be protected from the effects of shock for a period of some weeks. Induction of anaphylactic shock depends also upon the period of time allowed to elapse after sensitisation and the mode of administration of the dose designed to produce shock - the anaphylaxis-provoking or "shocking" dose. The most effective method of introduction of this dose is by means of an intravenous injection or an injection of the antigen directly into the heart.

The symptoms of shock vary in different animals and the severity of these symptoms depends upon the degree of sensitisation produced in the animal. The guinea pig has been used and studied extensively, and a distinct sequence of events may be observed, from ruffling of the hair of the head and coughing, to the final convulsive kicks followed by death; the immediate cause of this is asphyxia. The strength of the reaction is judged, commonly, by a number of '+' signs. The complete reaction (death) is usually denoted

as a ++++ reaction, while a mere ruffling of the hair, accompanied by slight coughing, is designated ± . Death of a rabbit due to anaphylaxis is less common, but when it occurs it is usually equally rapid.

In addition to the production of sensitivity due to the injection of antigenic material, a normal animal can be rendered susceptible to anaphylactic shock by the transference to it of antiserum from some other animal in which antibodies have been actively produced. Shock can then be induced in the normal way by injection of the antigen after a short elapse of time (24 to 48 hours). Such a method is known as passive anaphylaxis.

Although an anaphylactic reaction is essentially a qualitative test, it is possible to adapt it to make quantitative estimations of the strength of an antigen. It has been shown (Schultz, 1910; Dale, 1913) that intestinal and uterine muscle from a sensitised guinea pig will show a supranormal degree of irritability towards the antigen used for sensitisation. This quality of isolated strips of uterus or intestine is made the basis of the Schultz-Dale test, which

measures degrees of contraction of pieces of tissue in contact with varying strengths of different antigens.

In the present work, two guinea pigs (G3, G4) and two rabbits (R21, R22) were used for active sensitisation, preparatory to tests of anaphylaxis (see Table XIII). With the former, 0.9cc of a suspension of rediae of C.patellae (preserved in 0.5% formalin) was injected intraperitoneally and subcutaneously (three injections) over a period of eight days. Twentyeight days later, one animal (G4) received 0.5 cc, the other (G3) 1 cc of a centrifuged extract of the same material. The results were inconclusive - the guinea pig (G4) which had received 0.5 cc of antigen showed no reaction, while the other (G3) showed only a very slight reaction, designated ±. Both rabbits were injected with C.patellae. One of these (R21) received nine injections of paper-filtered extracts or suspensions of freshly ground rediae (approximately 20 mg total nitrogen) over a period of forty days. Twentyeight days later 1 cc of a freshly prepared suspension of rediae produced violent anaphylaxis, death occurring within a

quarter of an hour. (The serum from this rabbit had been tested several times previously by the nephelometric method. Only the last of these tests showed a faint indication of a positive reaction). Rabbit 22 received nineteen injections of ground suspensions of rediae of C.patellae (preserved in 0.5% formalin) - total nitrogen content approximately 50 mg - over a period of seventy days. Thirty days later 1.5 cc of a centrifuged extract of this material was injected intravenously, whereupon a very slight anaphylactic reaction was apparent. (Previously, ring tests with the serum from this rabbit had been negative). It would appear that this animal was highly refractory as an antibody-producer, since, when injected later with a known and potent antigen, it still failed to produce a satisfactory antiserum.

The remaining tests of this kind were conducted on guinea pigs (G2, G5, G6, G7, G8, G9 and G10) which were used for experiments in passive anaphylaxis. These animals were given intra-peritoneal injections of serum from rabbits sensitised by injections of cercaria. Two animals (G5, G6) each received 1cc of serum from a rabbit (R24), injected with C.tenue but which showed no evidence of antibody-production by ring test.

Forty hours later, each received intravenous injections of the homologous extract (0.25 cc). Three other guinea pigs (G2, G7, G8) received an equal amount of serum from a rabbit (R23) injected with C.lasium, serum which gave a ring test titre of 1:160,000 (based on total nitrogen). Fortyeight hours later each received 0.4 cc of extract intravenously. Two more guinea pigs (G9, G10) received half this amount, being injected with 0.5 cc anti-C.lasium-serum and 0.2 cc of the homologous extract. All results of the tests involving passive anaphylaxis were negative.

It was obvious, even from these few tests, that there is considerable variation among the animals and materials used. To be able to produce constant and reliable results, the conditions involved in anaphylaxis - amounts injected, periods of time allowed to elapse before tests, etc., - would have to be very fully explored. Since, however, the method can never be fully quantitative, this field of investigation was not further pursued.

c) Ring tests.

Ring tests were used successfully to detect antibodies to several species of cercariae. Some of the tests were carried out at Rutgers University,

and in these C.lasium, C.setiferoides, C.tenue, C.quissetensis and C.variglandis were used. In the tests at St.Andrews, C.patellae and Cercaria B. were employed as antigens.

In all these experiments, the test antigens were made by alternately freezing and thawing cercariae in sea water, allowing the insoluble material to settle and drawing off the supernatant fluid. The concentration of the extracts were similar in all cases, except those of C.patellae used in the later tests which were stronger. However, since quantitative results were not required, the nitrogen contents were not determined. They are believed to have been between 0.002 and 0.001 mg nitrogen per cc. The titres quoted here are, therefore, on an entirely different standard from those of the final tests; they are based upon the concentration of antigen in the first tube in each series of dilutions which is assumed to be "full strength".

Injections of freeze-thawed material, and of living cercariae whenever available, were given over varying periods of time (see Table XIII). The antisera from rabbits injected with C.lasium (R23) and C.setiferoides (R25) gave moderately strong reactions, the titres being 1:128 and 1:16 respectively. These rabbits had received 14 cc to

20 cc of cercarial suspension (approximately 5 to 7 mg total nitrogen, or 50 to 70 mg dry weight of material) over a period of two to three months. One of the last injections of C.lasium had been of cercariae incubated in some serum from the rabbit (inactivated, and thus containing no factor lethal to the cercariae) for several hours. It is possible that the metabolic products of the living cercariae had increased the strength of the anti-serum. Three months later, the titre of the antiserum to C.lasium had dropped to 1 : 8 (by 4 dilutions of antigen) in spite of further series of injections during this time.

Three other rabbits tested in the U.S.A. - one (R22) injected with rediae of C.patellae (preserved in 0.5% formalin), one, (R24) with considerable quantities of C.benue (approximately 7 mg total nitrogen) and the third (R26) with very dilute suspensions of C.variglandis - failed to show any evidence of antibody-production. Rabbit 26 had received several injections of living C.variglandis (the schistosome cercaria which completes its life cycle in birds) but, since it was not wished to produce an active infection in this animal, these had to be administered subcutaneously. It is probable that

the first two of these rabbits (R22 and R24) were thoroughly unresponsive as antibody-producers, whereas the third (R26) had insufficient material injected (less than 2 mg total nitrogen). In addition, the amount of material available for the ring test with this antiserum (R26) was also extremely small. The antisera from two other rabbits injected at St Andrews with freshly prepared homogenised suspensions were also tested. That from a rabbit (R28) injected with rediae of C.patellae (approximately 65 mg total nitrogen) had a titre of 1:32, whereas that from a rabbit (R29) which had received an identical amount of Cercaria B failed to show any consistent evidence of the presence of antibodies. One rabbit, (R30) received injections of an extract prepared according to the method of Wilhelmi (1940). The method of injection and test were also as recommended by Wilhelmi, a total of 40 mg dry weight of material in solution being injected. When tested, however, the serum had a very low titre showing that few antibodies had been produced.

Of seven domestic fowl used for injection, not one failed to produce antibodies which could be demonstrated by the ring test. The first (F1)

received injections (approximate concentration of total nitrogen - 2.5 mg, or 25 mg dry weight of material) of C.quissentensis and, when tested, the antiserum had a titre of 1:64. The second fowl was injected with approximately double this amount of C.lasium (F2), but the antiserum had a lower titre - 1:32.

The remaining injections into fowl were of rediae of C.patellae (F3, F4, F5, F6 and F7). These received identical amounts of whole living rediae and suspensions of homogenised rediae freshly prepared. Of these animals, two (F3 and F4) each received two intravenous injections while the subsequent injections into these and to the remaining fowl (F5 - 7) were administered subcutaneously. The total amount injected into each individual was approximately 30 mg total nitrogen. Although these fowl, of the same breed and age, had received equal amounts of material, the resulting antisera varied considerably in strength. The strongest antiserum, (F4) with a titre of 1:128, was from a fowl which had received two intravenous injections; however, the other fowl (F3) treated in the same way provided an antiserum with a much lower titre - 1:8.

The results of the homologous tests with these antisera and also with those produced in rabbits are summarised in Table XVII. All the tests reported here were carried out with whole extracts, i.e., containing lipoids.

Table XVII

A summary of the titres of the homologous reactions of a variety of antisera. (Titres are based on dilutions of the initial tube of antigen).

Antiserum	Homologous titre	Antiserum	Homologous titre
R22 C.patellae (0.5% formalin)	-	F1 C.quissetensis	1:64
R23 C.lasium	1:128	F2 C.lasium	1:32
R24 C.tenue	-	F3 C.patellae	1:8
R25 C.setiferoides	1:16	F4 C.patellae	1:128
R26 C.variglandis	-	F5 C.patellae	1:8
R28 C.patellae	1:32	F6 C.patellae	1:8
R29 Cercaria B.	-	F7 C.patellae	1:32
R30 C.patellae (Wilhelmi's method)	1:2		

Having obtained reasonably strong antisera, a few experiments were carried out with one of them (R23) to investigate the possibilities of increasing the titre *in vitro*. The use of an alkaline extract of the cercariae (in Coca's solution) as test antigen was tried. Secondly, the saline medium of the antigen dilutions was replaced

by a substitute for albumin, a plastic - polyvinylpyrrolidone (P.V.P.) - being used in concentrations of 5%. Neither method had any effect upon the titre. Concentration of the antiserum, by freezing and partial thawing, also failed to have any influence on the end point of the reaction. Some additional tests were carried out with this antiserum (R23) to investigate the possibility of measuring, by the ring test, any antibodies to metabdic products. (The rabbit concerned is the one which had received a single injection of cercariae incubated previously in inactivated serum, an attempt being made to collect metabolic products of the cercariae prior to injection). Tests were carried out with a small quantity of sea water, in which a number of living cercariae (C.lasium) had been kept for several hours. Although slight rings did appear in a few tubes, the test was discounted finally, as control tests showed that sea water from the jars containing snails also tended to produce occasional rings. It is possible that further investigations might have led to the in vitro measurement of metabolic products; these, however, were not pursued.

Quantitative Measurements

Ring Tests.

Final tests were carried out in an attempt to correlate intermediate and definitive stages of trematodes; the ring test technique was therefore used on a quantitative basis. With the limited time and material available, however, these tests could be no more than exploratory in nature.

The fluid extracts used as antigens were all prepared from fresh or freeze-thawed material, the lipoids being extracted from the aqueous solutions by shaking with ether. Since fowl sera were to be employed, the concentration of sodium chloride used for extraction, and dilution of the test antigens was 2.25% and the pH was adjusted to 6.8. All tests were incubated at 37°C, and the results given were read after twenty minutes.

Two antisera were selected for these tests. The first was that prepared in a fowl (F1) to C.quissetensis, the intermediate stage of Himasthla quissetensis, which had a homologous titre of 1:160,000 (based on the total nitrogen content of the extract). The antiserum to C.patellae finally used was a pool of five fowl

Table XVIII

The effect of pooling fowl antisera.

Antiserum	Titres								
	Homologous (whole extract)			Homologous (lipoid-free)			Heterologous (lipoid-free)		
	Dilution	p(A/G)	'Age' days	Dilution	p(A/G)	'Age' days	Dilution	p(A/G)	'Age' days
F3	1:40,000	4.6	1						
F4	1:640,000	5.8	1	1:640,000	5.8	6	1:160,000	5.2	8
F5	1:40,000	4.6	1						
F6	1:40,000	4.6	1						
F7	1:160,000	5.2	1						
Pooled	1:2,560,000	6.4	1	1:5,120,000	6.7	8	1:160,000	5.2	8

sera (F3 to F7), which had a homologous titre of 1:5,120,000. (Pooling of antisera is a method frequently adopted to minimise the variations between individual animals). The effect of pooling the antisera, both on the homologous titre and on a heterologous one, is shown in Table XVIII. The titres are quoted both in the traditional manner as "dilutions" and on the logarithmic scale - (p(A/G): (see page 95). Duplicates of the homologous tests gave identical results, but the heterologous tests are from single determinations. No direct test was performed to find out if the antiserum was altering with storage (it was kept in the refrigerator at 4°C between tests) but the 'age' of the antiserum at the time of the test - the number of days which had elapsed since bleeding - is given in each case. There is no evidence of any marked change with 'age'.

It will be seen that there is little difference between the homologous titres obtained with "whole" extract and lipoid-free extract. This is in agreement with the figures quoted on p.123 . The differences between the sera of individual fowl will be noticed, as well as the increase in the homologous titre which accompanied

the pooling. This increase affects the homologous titre only, the heterologous titre remaining unchanged, so that the pooled antiserum has a much narrower range, i.e., it is more specific, than that of the one individual (F4), which was tested in this manner. The considerable increase in the homologous titre is of interest. It is possible that some of the sera contained unabsorbed antigenic substances which partially reacted with the antibodies of other sera, rendering them less soluble and effectively increasing the concentration of homologous antigen. The phenomenon may also reflect differences in the blood of the original fowl, akin to the well-known blood-groups in humans. It should be pointed out, however, that the homologous titre (whole extract) with the pooled antiserum was only two tubes beyond that with the F4 antiserum, a fact which seems to be expressed more clearly by the p(A/G) values than by the "dilutions".

Control tests were carried out with both the homologous antigens used in the final tests, against normal fowl serum. These showed that slight, indefinite, rings might appear in the first two or three tubes, due possibly to very faint traces of ether still present in the antigen.

Table XIX

Results of ring-tests carried out with anti-*C.quissetensis*-serum and anti-*C.patellae*-serum

	C.patellae (1) Lipoid extracted from aqueous solution.	*C.patellae* (2) Lipoid extracted from vacuum-dried material	Cercaria B.	*C.lasium*	*C.quissetensis*	*Himasthla leptosoma*	*Psilostomum brevicolle*
Anti-*C.quissetensis*-serum.							
Titre, based on total N	1:10,000 ?	-	-	-	1:160,000	1:160,000	-
% of homologous titre	6% ?	-	-	-	100%	100%	-
"p(A/G) value"	4.0 ?	-	-	-	5.2	5.2	-
diff. from homologous	1.2 ?	-	-	-	(0)	0.0	-
Anti-*C.patellae*-serum.							
Titre, based on total N	1:5,120,000	1:280,000	1:160,000	<1:10,000	1:320,000	1:320,000	<1:510,000
% of homologous titre	100%	55%	3%	< 0.2%	6%	6%	< 10%
"p(A/G) value"	6.7	6.45	5.2	< 4.0	5.5	5.5	< 5.7
diff. from homologous	(0)	0.25	1.5	> 2.7	1.2	1.2	> 1.0

The results in emphasis are the two homologous tests.

Consequently, when questionable rings of this sort appeared in any of the tests, the reaction was discounted.

The results of the tests, both homologous and heterologous, are given in Table XIX. In this table the titres are given according to three different systems; in the first, the dilution of total nitrogen is given; in the second, this dilution is expressed as a percentage of the homologous titre; the third is the logarithmic "p(A/G)" system. The fourth set of figures shows the difference between the p(A/G) value of the test and that of the homologous control. It has already been explained that with a series of doubling dilutions such as were used in these experiments, a difference of less than 0.3 p(A/G) units is without significance.

Except for the homologous tests with anti-C.patellae-serum, the results in Table XIX are based upon one or two tests only, since the quantity of material available was very small; (C.quissetensis and C.lasium were collected in New Jersey, U.S.A., and supplies could not be replenished; the supply of adult flukes was limited, unfortunately, to approximately twenty specimens of H.leptosoma and one P.brevicolle). In two cases, the titres are

quoted as "less than" a certain value. This indicates that no reaction was observed in any of the tubes, and the figure given is the highest strength of antigen used. It is clear that reaction might have taken place if a stronger antigen had been available, but there is, of course, no evidence for this.

With these two antisera, only one pair of reciprocal tests was possible - that between C.patellae and C.quissetensis. It is difficult to say, however, whether these correspond exactly, since the reaction between the anti-C.quissetensis-serum and C.patellae (marked by a query in Table XIX) was doubtful. The original antigen had been diluted ten-fold to eliminate any possible effect of traces of ether in the extract, with the result that the reaction appeared in the first tube only. As a matter of practical experience, it is difficult to have as much confidence in such a test as in one where rings of progressively diminishing intensity occur in a row of tubes. On the other hand, if reciprocal tests among trematodes function as they do among mammalian sera (Eisenbrandt, 1936, claimed that among cestodes and nematodes, however, they do not) any reaction

present would appear at approximately this dilution of the antigen of C.patellae.

It will be seen from the table that the titres of C.quissetensis and Himasthla leptosoma are identical, both with the anti-C.quissetensis-serum and with the anti-C.patellae-serum. These are two species of the same genus, Himasthla quissetensis having been separated from the other species of the genus by Stunkard, (1938 a). Although the tests carried out were few in number and the observations unconfirmed, it is reasonable to assume that the results obtained are sufficiently accurate to warrant an appraisal of the technique. Dinstinction between genera is apparently attainable by use of the ring test, a marked difference being evident between the reactions of the two cercariae being investigated here. It is also reasonable to expect that adult and intermediate stages of one genus could be identified by such a technique. It would seem, however, that species within a genus cannot be differentiated serologically with the technique used in this work.

Both the species of Himasthla tested - the adult H.leptosoma and the intermediate form, C.quissetensis, gave low but identical titres

(6% of the homologous reaction) in the tests against the anti-C.patellae-serum; the conclusion, therefore, to be drawn is that C.patellae is not a member of this genus.

The test with Psilostomum brevicolle cannot, unfortunately, be considered very significant. The extract from the single fluke available, though made with only 0.25 cc salt solution, was of insufficient strength to promote any ring formation, and contained too small a quantity of organic matter for analysis by dry-weight. The best estimate which could be made of the nitrogen content of the extract was 1:170,000 (based on a rough estimate of the volume of the fluke - cylindrical in form), but as it is thought that this might be in error by a factor of as much as three, the figure of 1:510,000 has been taken as the upper limit of dilution of total nitrogen. Even so, however, the titre is well below that of the homologous reaction with anti-C.patellae-serum, and the probability is that the true titre is even less than that with Himasthla leptosoma.

The question of the identity of the adult form of C.patellae has, therefore, not been solved by these tests. It is reasonable to assume,

however, that with a little more exploratory work and the use of a larger number of experiments, this test could be made competent to differentiate, with absolute certainty, between families and genera of Trematoda. In addition, it is possible that species could also be differentiated, if sufficiently specific antisera and antigens could be developed and a detailed test conducted over a narrow range of dilutions.

Discussion.

The identity of the adult of C.patellae has not been established by the work reported here; nevertheless the evidence which has been obtained is worth recapitulating if only for its negative value.

Most of the trematodes examined can be eliminated either on morphological grounds or on the grounds that the life histories are known. Two others were eliminated on the basis of their infrequent or spasmodic occurrence. The remaining species (Himasthla leptosoma and Psilostomum brevicolle) were used in serological tests but, in each case, only negative results were obtained. (The elimination of P.brevicolle on these grounds cannot, however, be considered to be final on account of the paucity of material available). It follows, therefore, either that one of the trematodes obtained has been wrongly eliminated in the above discussion or, and this is more likely, that the survey failed to provide specimens of the required parasite. The number of birds examined (seventy-seven,of which forty-two were parasitised by trematodes) was insufficient to ensure that specimens were obtained of every trematode to be

found in birds on this particular part of the shore - for example, only six individuals of the abundant Black-headed Gull were obtained. It will be realised, of course, that the steady state of infestation observed might be maintained by relatively few birds if the duration of infestation by the rediae is long.

Exploratory work into serological methods showed that these are by no means straightforward when applied to trematodes. A purely qualitative method for testing an antiserum has little practical value in the study of ontogenetic relationships; such a test is useful, however, in preliminary investigations in this field. Of the methods investigated, agglutination tests proved to be impracticable and anaphylactic shock, although not impossible as a means of test, was uncertain and difficult to reproduce. The only quantitative test involving the anaphylactic reaction is that known as the Schultz-Dale test, but the results of qualitative tests made were not sufficiently encouraging to merit a full investigation into the possibility of using this. Investigations into the behaviour and viability of cercariae in antisera were slightly more

promising. Although this method of evaluating antisera is qualitative in nature it could be adapted simply to a quantitative basis. The results obtained, however, were subject to considerable variation and the absence of adequate control observations rendered them unreliable. Before the method could be considered suitable for ontogenetic investigations, a more thorough exploration into standardisation, control and general technique is needed. Since there is no information about the action of sera upon living adult flukes, this enlarges still further the scope of necessary prior investigation, but it is reasonable to assume that such studies could be used successfully in this and cognate fields.

The most successful test employed here was the precipitin reaction measured by the ring test. However, although this, in the end, provided a workable method, it was characterised by the extreme faintness of the rings, which made a precise determination of the titre of the reaction a matter of some uncertainty. Conditions at the interfacial layers are probably particularly favourable to the formation of a precipitate, but, as an approximation, the titre may be taken as

the minimum concentration of antigen capable of forming a precipitate with the optimum concentration of antibody in that particular antiserum. The titres obtained in this work compare favourably with others reported in the literature and there is no doubt that the extracts used here contained antigen in quite high concentrations. It is of considerable importance, when comparing titres quoted in the literature, to take into account the varying standards of measurement used by different workers. These may be based upon the concentration of nitrogen, protein, the material in solution or upon a known amount of material used in the preparation of the extract. Each method is equally reliable but comparisons between the actual titres based upon different standards will lead to erroneous conclusions.

Except for some tests with antisera produced in response to injections of material which had undergone ultrasonic treatment, photo-electric measurement of the amount of precipitate formed proved to be almost useless. The relationship between ring test titres and turbidity is, however, extremely complex since each is measuring different and more or less independent qualities of the

antisera. The ring test measures the sensitivity of the antibodies to a minimum quantity of antigen, whereas the turbidity is a measure of the quantity of antibodies produced. A number of theories as to the formation of antibodies and their reaction with antigens have been suggested, but no general agreement seems to have been reached (see Boyd, 1947; also Wolfe and Bair, 1938; Pauling, 1940; and Goldberg, 1952). Although an exact comparison between ring test and measurement of turbidity is not possible, the final reading of the latter in the zone of antibody excess indicates, as does the ring test also, the minimum concentration of antigen capable of forming a precipitate. Conditions in the two tests are not, of course, identical, but the value obtained as the final reading in the measurement of turbidity, should not be significantly different from the ring test titres of the particular antiserum tested. In the experiment with human serum, which is typical of a test with mammalian sera, the end point of the reaction, based upon the nitrogen content of the antigen, is about 1:40,000 which may be compared with figures quoted in Table XVIII. This

again points to the presence of high concentration of antigen in the cercarial extracts. In sharp contrast to this, the maximum amount of precipitate encountered in the photo-electric measurement of homologous tests with anti-C.patellae-serum (other than those produced in response to ultrasonically treated extracts) was 3% of the maximum obtained with anti-human-serum x human serum. Many workers have demonstrated that, in the zone of equivalence, near the point of maximum precipitation, all the antibody present in the reaction is precipitated and the maximum precipitate obtained is a measure of the amount of antibody in the antiserum. Thus, in spite of the high concentration of antigen in the extracts injected, the formation of such slight amounts of precipitate may indicate that very few antibodies were being produced in the experimental animals. There is confirmation of this supposition in the uncertain results given by tests upon living cercariae and with anaphylactic shock. Pauling (1940) has put forward the theory that antigens with strong affinities for antibodies are likely to be poor antibody-producers. He supposes that in the experimental animal the molecules of

antigen become saturated with firmly attached molecules of antibody and are thus prevented from synthesising further antibodies. If something of this nature has occurred here, it may be that the antigens used for injection throughout this work have been too active - i.e., that the bonds between antigen and the resulting antibodies have been too strong - to allow of a high rate of production of antibodies. Greater success might therefore have been achieved by the use of extracts modified in various ways to reduce their activity. Such processes as those causing slight alteration of the antigen,or the utilisation of various fractions of the whole material might have proved to be efficacious.

The power of the extracts to produce antibodies was much improved by submitting the material to ultrasonic radiation. The primary effect of this treatment is to cause physical disintegration; colloidal particles are broken down to smaller ones thereby increasing the solubility of the material, but, in addition, some chemical changes are liable to occur, and it is the more reactive groups which would be most likely to be thus affected. Both effects are

likely to be favourable to the production of antibodies. The enhanced reaction, measured photo-electrically, was between the antiserum to a modified extract and the native test antigen. It is very probable that, in a test between this antiserum and an ultrasonically-treated test antigen, the reaction would have been considerably increased. It was not possible to carry these experiments further in this work, and there is little published information about the reproducibility and none about the specificity of the reactions with ultrasonically-treated materaal. However, it is clear that a new and promising field of investigation is open.

Attempts to utilise the metabolic products of living organisms for the purpose of injection into experimental animals were not particularly successful. That metabolic products, produced during an active infection of a host, are capable of stimulating antibody production is clearly shown by the experimental case of "sea bather's eruption". In all, not more than a dozen cercariae were allowed to penetrate the skin, and a week later a very considerable reaction was evident. The possibility of this sensitivity being

elicited by the constituent antigens of a few cercariae is slight indeed and it is most likely that it was produced in response to metabolic products secreted during the period in which some, at least, migrated within the body.

The only fractionated extracts employed for injection were those prepared according to Wilhelmi's method. None of the four animals so treated showed any sign of antibody production at all. Wilhelmi, who worked with material of known life histories, claimed that his method could be used to produce strong antisera to helminths; these antisera, with homologous titres of 1:40,000 to 1:60,000, could, he claimed, be produced with certainty against any of the trematodes or cestodes tested by him and that a very useful range of specificity was attained. The method advocated by Wilhelmi consists of removing lipoids from "freeze-dried" material and extracting the residue with saline over a period of twenty-four hours at room temperature; during this time the suspension is subjected to continual shaking before being filtered finally through a bacteriological filter. An extract prepared in this way and tested against an antiserum of known

effectiveness, showed an activity of less than one twentieth that of another preparation, similar in every way except for a marked reduction in the time of the aqueous extraction; also, the extract was centrifuged instead of being filtered. It appears, therefore, that almost complete destruction of the antigenicity of the fluid had taken place either during the prolonged extraction or, much less likely, during the process of filtration. Wilhelmi's figures for both homologous and heterologous titres accord quite well, however, with those obtained in this work (allowance being made for the difference in standards used), and it is possible that, in fact, he extracted most of his solutions for a very much shorter time and with less vigorous agitation than he finally decided to recommend. Prolonged extraction is quite unnecessary, for it was found that at least 97.5% of the soluble material was in solution after five minutes shaking. It should also be noted, in regard to Wilhelmi's results, that, in an unspecified number of cases, he concentrated his test antigens by evaporation. This would result in an increase in the concentration of salt and it has already been emphasised that, with rabbit sera, an increase of salt in a reaction tends to inhibit the formation of rings. No

reliance, therefore, can be placed on those of his results which show low titres.

Though the materials available for the final experiments in this work were very meagre, enough has been achieved to demonstrate the value of serological tests in work of this nature. It is obviously very desirable to extend these tests, using techniques of the type investigated here, to as many species of adult flukes as can be obtained. It is highly desirable, at the same time, to continue experiments designed to improve the quality of the antisera, for without a highly active antiserum, the advantage of photo-electric measurement will not be obtained; also, without specific antisera, it will not be possible to use the technique of the ring test to differentiate between closely allied species. In this respect, the success of the ultrasonic treatment of material seems to open up further possibilities.

Summary

1. This thesis describes the investigations and tests carried out; these have been directed towards solving the problem of the identity of the adult stage of *C.patellae*, a digenetic trematode parasite of the limpet, *Patella vulgata*.

2. Morphological descriptions of the trematode parasites (*Cercaria patellae* and *Cercaria B*) of the limpet are given and brief comments of those found in the American mud-snail, *Nassa obsoleta*, have been included. An ecological and statistical survey on the occurrence and distribution of the parasites of the limpet has indicated that the final host of *Cercaria patellae* is a bird; in addition, evidence has been presented to show that the metacercariae found probably develop from *Cercaria B*.

3. A brief survey is reported of flukes found in the birds examined - those likely to harbour the adult form of *Cercaria patellae*.

4. The serological methods used in this investigation are reviewed historically, and the chief technique for measuring the precipitin reaction - photo-electric estimation and the ring test - are described.

5. Antigens used for the purposes of injections and tests have been prepared variously and the rôle of lipoids in the specificity of the reaction is discussed briefly. Some of the methods, used by other workers, to estimate the strength of an antigen have been compared, and the most suitable used here for standardisation of the ring test. The methods used for preparation of antisera to a variety of cercariae are described. Rabbits and domestic fowl were used, and it appears that domestic fowl are the more efficient antibody-producers in this particular field of serology. It has also been shown, although not conclusively, that better antisera are produced during an active infection, than by a series of injections of dead material.

6. The tests carried out are divided into qualitative and quantitative techniques. The former include observations on living cercariae in normal sera and antisera, employment of the anaphylactic reaction and, finally, use of the ring test. These tests were employed for the purpose of judging the efficiency of different methods of producing antisera.

7. Quantitative tests were applied to two adult trematodes against two selected antisera to cercariae, in an attempt to apply the ring test technique to the ontogeny of C.patellae.

A P P E N D I X

The following is a paper which has been accepted for publication in Parasitology.

A Re-description of Echinostephilla virgula Lebour, 1909.

Echinostephilla virgula occupies an isolated position in the Digenea; apparently it does not belong with certainty to any of the existing families and, as the original description is incomplete, it was considered worthy of re-description. This trematode was found frequently at St Andrews between the months of September, 1949, and March, 1951, in the intestine of the Turnstone, (Arenaria interpres Linn.). There was usually a heavy parasitism, 50 to 100 worms being found in one host. However, in spite of frequent examinations of birds shot after this period, only a single immature specimen was found.

Material and Methods.

The worms were studied in the living state and as mounted specimens. The latter were fixed in Bouin's fluid, and stained either in Weigert's haematoxylin, Gower's acetocarmine or in acetic acid alum carmine. Serial sections studied were stained, some with Mallory's Triple Stain, others with Heidenhain's iron alum haematoxylin. All measurements given in the description, except those of the cuticular spines, are mean values and are

taken from preserved specimens except where otherwise stated; a full list of measurements is appended.

External Morphology.

The body is elongate (6.5 mm. long), blunt anteriorly and widening rapidly to a maximum breadth (0.815 mm.) in the region of the ventral sucker which lies approximately one-sixth of the length of the body from the anterior end. A slight constriction is apparent immediately behind the ventral sucker, thereafter the body tapers gradually to the posterior end. Living specimens are muscular and active, convex dorsally and concave ventrally; the latter character is more marked between the suckers.

The oral sucker is sub-terminal, circular and small, with a diameter of 0.132 mm. The ventral sucker, situated one-sixth of the length of the body from the anterior end, occupying the greater width of the body, is circular and has a diameter (0.535 mm.) four times that of the oral sucker.

Spines are present as 'body spines' and as a double row of 'head spines'. 'Head spines' are present as two rows, the spines of one row alternating with those of the other, and they lie

close together in the otherwise unarmed tip of the body. The posterior row of 'head spines' lies 0.026 mm. in front of the most anterior row of 'body spines'. The 'head spines' on the dorsal surface are similar in size (0.006 mm. long and 0.008 mm. broad) to those of the eighth row of 'body spines' but are closer together. On the ventral surface the 'head spines' are very minute, appearing to extend almost to the lateral border of the oral sucker. The 'body spines' are absent from the extreme anterior and posterior ends, but elsewhere are arranged in transverse rows, the rows being close together anteriorly and far apart posteriorly, and anteriorly show a definite quincunx order. The spines, triangular in shape, and broader at the base, are small in the anterior rows and larger posteriorly; they increase in size from 0.005 mm. long by 0.006 mm. broad in the most anterior rows, to 0.013 mm. long by 0.011 mm. broad immediately anterior to the ventral sucker. The largest spines are in rows 0.016 mm. apart, the space between the bases of these spines in one row being 0.016 mm.

Internal Morphology.

Alimentary Canal. A short prepharynx (0.04 mm.

long) is followed by a fairly large muscular pharynx (0.107 mm. long and 0.082 mm. broad). The oesophagus, 0.241 mm. long, extends to its bifurcation at a point three quarters of the distance from the anterior end to the anterior border of the ventral sucker. The caeca are unbranched; they lie laterally and extend through the body to terminate a short distance (0.307 mm.) from the posterior end.

Excretory system. The bladder opens to the exterior by a terminal pore. It is elongate but irregular in shape owing to the number of ducts which enter it. The two main longitudinal ducts enter the bladder anteriorly, each formed by the union of a branched network of canals, but in addition smaller canals enter the bladder directly on each side.

Reproductive system.

Male: The two sub-spherical testes, with their transverse axes slightly greater, are approximately equal in size: (anterior testis 0.229 mm. by 0.251 mm.; posterior testis 0.232 mm. by 0.256 mm.). They lie close together, and when mature touch each other, the adjacent surfaces being slightly flattened. They are situated one

behind the other and separated from the hind end of the body by a distance of 0.807 mm., measured from the posterior border of the posterior testis. The vas deferens enlarges to form an extra-cirral seminal vesicle (0.53 mm. long) situated posterior to the ventral sucker, the posterior margin of the seminal vesicle being 1.14 mm. behind the ventral sucker. The vas deferens is continued as a short narrow duct, which enters the cirrus sac and expands to form an intra-cirral seminal vesicle which occupies the posterior third of the cirrus sac. The cirrus sac, slightly sinuous in shape, extends from 0.595 mm. behind the ventral sucker to the genital atrium. It contains a spiny cirrus.

The small genital atrium lies in front of the ventral sucker, immediately behind the bifurcation of the gut, and within the atrium the male opening lies anterior to that of the uterus.

Female: The ovary is sub-spherical, with its longitudinal axis greater (0.218 mm. by 0.213 mm.), and is situated in the mid-line in front of the testes and is separated by a distance of 0.191 mm. from the anterior testis. The vitellaria, consisting of from forty to sixty follicles on each side, are mainly situated laterally, underlying the

caeca. They extend posteriorly from the region of the external seminal vesicle to the middle of the ovary. Ducts, passing posteriorly from the vitellaria, unite in a small reservoir which lies between the ovary and the anterior testis and is close to the junction of Laurer's canal and the oviduct. The uterus has a short descending limb which underlies part of the anterior testis, while the main part is much convoluted, with about twenty loops. These convolutions, obscuring the ovary and occupying the greater part of the body between the testes and the extra-cirral seminal vesicle, are mainly confined to the region between the caeca; occasionally they overlap the caeca laterally and may extend as far forward as the posterior border of the ventral sucker. The final section passes close to the seminal vesicle to open into the genital atrium.

The eggs are fairly large (0.118 mm. by 0.06 mm. in the living state) and are very numerous, showing stages in development, up to completely formed and hatched miracidia. The latter, each with a conspicuous eye-spot, are found in the anterior part of the uterus. The shell is colourless and oval, and has a small operculum at the broader pole. In

the fixed state the eggs appear smaller (0.075 mm. by 0.033 mm.) and are boat-shaped.

Diagnosis.

Body elongate, (5.8 to 7.2 mm.); maximum breadth (0.65 to 0.93 mm.) in region of ventral sucker. Muscular and active, convex dorsally, concave ventrally. Oral sucker sub-terminal, circular, small (0.11 to 0.145 mm.). Ventral sucker one sixth of length of body from anterior end, occupying greater part of width of body; diameter (0.437 to 0.6 mm.) four times that of oral sucker.

'Head spines': Two incomplete rows of alternating spines; on dorsal surface similar in size (0.006 mm. long by 0.008 mm. broad) to eighth row of 'body spines' but closer together; on ventral surface very minute extending almost to lateral borders of ventral sucker. 'Body spines' absent from extreme anterior and posterior ends, more numerous anteriorly and there arranged in quincunx order. Spines triangular, broader at base; small (0.005 mm. long by 0.006 mm. broad) in anterior rows, larger (0.013 mm. long by 0.011 mm. broad) in region of ventral sucker. Prepharynx short (0.033 to 0.051 mm. long),

pharynx muscular, fairly large (0.088 to 0.121 mm. long by 0.055 to 0.101 mm. broad); oesophagus (0.196 to 0.311 mm. long) extending nearly to anterior border of ventral sucker. Two unbranched caeca terminating short distance (0.24 to 0.383 mm.) from posterior end. Much branched excretory system. Testes two, sub-spherical, approximately equal in size (anterior 0.196 to 0.284 mm. long by 0.207 to 0.295 mm. broad; posterior 0.186 to 0.284 mm. long by 0.207 to 0.295 mm. broad), touching when mature; one behind other in posterior region of body. Extra- and intra-cirral seminal vesicles present, both situated posterior to ventral sucker. Cirrus sac, with intra-cirral seminal vesicle and spiny cirrus, opening into genital atrium immediately posterior to bifurcation of gut. Ovary sub-spherical, in mid-line anterior and similar in size (0.218 long by 0.196 to 0.23 mm.) to testes. Vitellaria, of 40 to 60 follicles, lateral in position and ventral to caeca, in region between external seminal vesicle and middle of ovary. Convoluted uterus with short descending limb underlying part of anterior testis; ascending limb obscuring ovary and occupying most of body space as far as seminal vesicles. Eggs fairly large (0.109 to 0.119 mm. by 0.058 to 0.068 mm. in

living state); hatched miracidia each with conspicuous eye-spot, in anterior part of uterus. Shell colourless and oval in living state, with operculum at broader pole.

Discussion.

Although there are certain points of difference between Echinostephilla virgula and the trematode described above, there appears to be insufficient evidence to support the creation of a new species.

The present description confirms the presence of two rows of minute 'head spines' in the region of the oral sucker, but these rows are incomplete ventrally. In size, these spines differ from those immediately behind, but, except for the spacial arrangement, are identical with those of the eighth row of 'body spines'; i.e., a distance of 0.1 mm. behind the 'head spines'. Although the spines are called 'head spines' and 'body spines', the 'head spines' resemble in no way those of members of the Echinostomatidae.

In addition to the structures identified by Lebour, (1909), the presence of an extra-cirral seminal vesicle is noted, but in mature specimens this is much obscured by the convoluted uterus.

The discrepancy between the sizes of eggs given in the original description and those given in this paper is so small as to be insignificant. However, the measurements taken from the paratype specimen at the British Museum, are considerably smaller than either. This difference can apparently be accounted for by the fact that fixed eggs are very noticeably smaller than living ones, as was found by comparing measurements before and after fixation.

The characters in which this trematode differs from genera of the Echinostomatinae in which Lebour placed it, appear to be of sufficient importance to warrant an alteration in the classification. As noted by Lebour, it differs from other members of the Echinostomatinae in being of a much thicker build, in the presence of miracidia in the uterus and having a poorly developed vitellaria. The 'head spines' are of a form very different from those typical of Echinostomes, and there is no characteristic difference between them and the cuticular 'body spines'. Accordingly its presence in this family is unwarranted. In general structure and in the presence of miracidia in the uterus, it is similar to the genus Parorchis. Although

Dawes (1946) includes both Parorchis and Echinostephilla among the isolated genera of the Echinostomatidae, the majority of workers regard Parorchis as a member of the Philophthalmidae. The classification of both these genera is uncertain and it is probable that serological investigation into their relationships would be of value. However, the morphological relationship of Parorchis to Cloacitrema Yamaguti and Pygorchis Looss, both members of the Philophthalmidae is close and on such evidence, it is considered that Echinostephilla should be included, provisionally, within this family.

Measurements of Echinostephilla virgula
(in millimetres)

Length	... 5.8 - 7.2,	Mean 6.5 mm.
Maximum breadth	... 0.65 - 0.93,	Mean 0.815 mm.
'Head spines' - length	... 0.006 mm.	
- breadth	... 0.008 mm.	
'Body spines' - length	... 0.005 - 0.013 mm.	
- breadth	... 0.006 - 0.011 mm.	
Distance between 'head' and 'body spines'	... 0.026 mm.	
Oral sucker - diameter	... 0.111 - 0.145,	Mean 0.132 mm.
Ventral " - "	... 0.437 - 0.6,	Mean 0.535 mm.

Measurements of Echinostephilla virgula (cont'd)

Prepharynx - length	... 0.033 - 0.051,	Mean 0.04	mm.
Pharynx - length	... 0.088 - 0.121,	Mean 0.107	mm.
- breadth	... 0.055 - 0.101,	Mean 0.082	mm.
Oesophagus - length	... 0.196 - 0.311,	Mean 0.241	mm.
Caeca - distance from posterior end	... 0.24 - 0.383,	Mean 0.307	mm.
Testes - longitudinal axis.	0.186 - 0.284,	Mean 0.23	mm.
- transverse axis...	0.207 - 0.295,	Mean 0.253	mm.
Length of body behind posterior testis	... 0.568 - 0.98,	Mean 0.807	mm.
Extra-cirral seminal vesicle - length	... 0.459 - 0.6,	Mean 0.53	mm.
extent behind ventral sucker	... 1.012 - 1.29,	Mean 1.14	mm.
Cirrus sac - extent behind ventral sucker	... 0.502 - 0.678,	Mean 0.595	mm.
Ovary - longitudinal axis..	0.198 - 0.254,	Mean 0.218	mm.
- transverse axis ...	0.196 - 0.23,	Mean 0.213	mm.
- distance from anterior testis ...	0.142 - 0.24,	Mean 0.191	mm.
Vitellaria - number of follicles ...	40 - 60		
Eggs, living - length	... 0.109 - 0.119,	Mean 0.118	mm.
- breadth	... 0.058 - 0.068,	Mean 0.06	mm.
fixed - length	... 0.066 - 0.081,	Mean 0.075	mm.
- breadth	... 0.026 - 0.044,	Mean 0.033	mm.

Appendix

Abbreviations to Figures (1) and (2).

c.s. - cirrus sac

exc. v. - excretory vesicle

ext. sem. ves. - extra-cirral seminal vesicle

g. ap. - genital aperture

int. c. - intestinal caecum

int. sem. ves. - intra-cirral seminal vesicle

L. c. - Laurer's canal

Oes. - oesophagus

o.s. - oral sucker

ovd. - oviduct

ovy. - ovary

ph. - pharynx

t. - testis

ut. - uterus

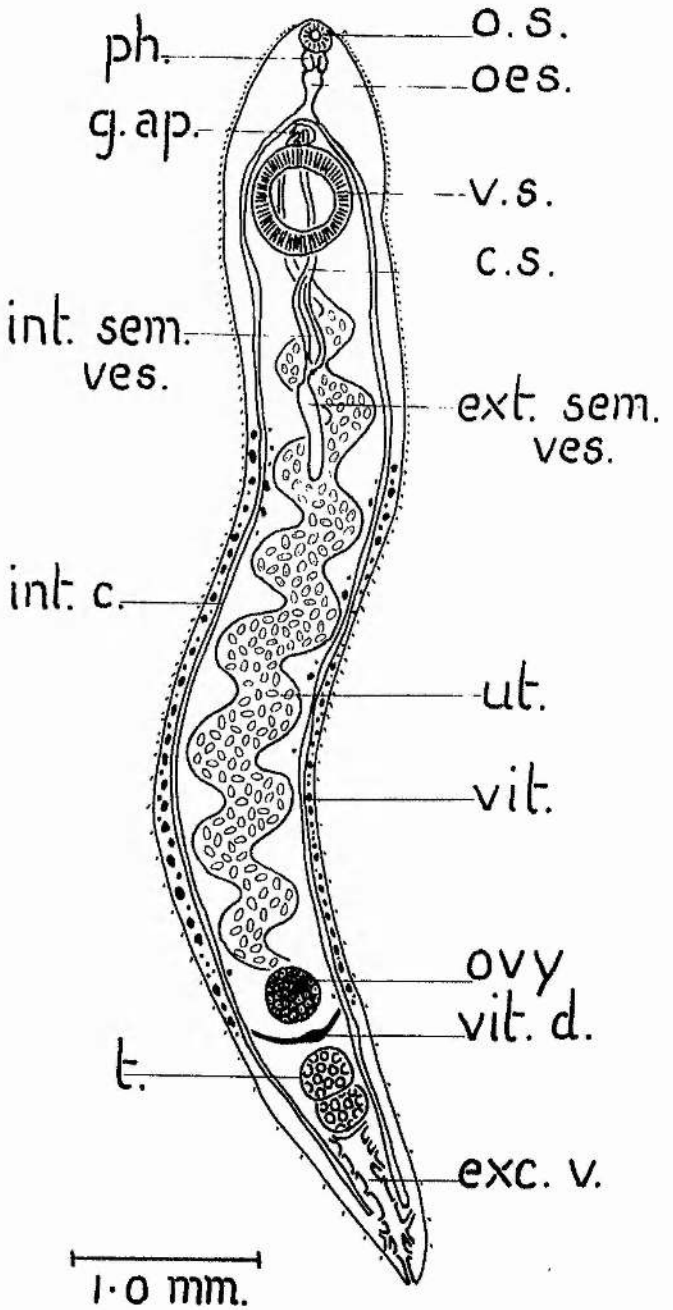
vit. - vitellaria

vit. d. - vitelline duct.

v. s. - ventral sucker

Appendix.

Figure (1). The general morphology of Echinostephilla virgula.



Appendix.

Figure (2).

The anterior extremity of Echinostephilla virgula, showing the size and arrangement of 'head' spines.

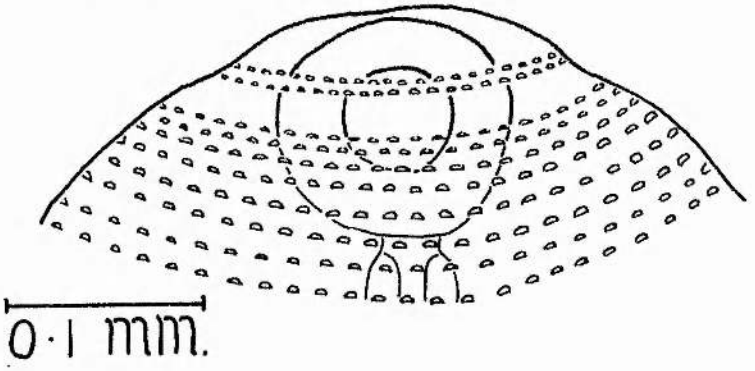
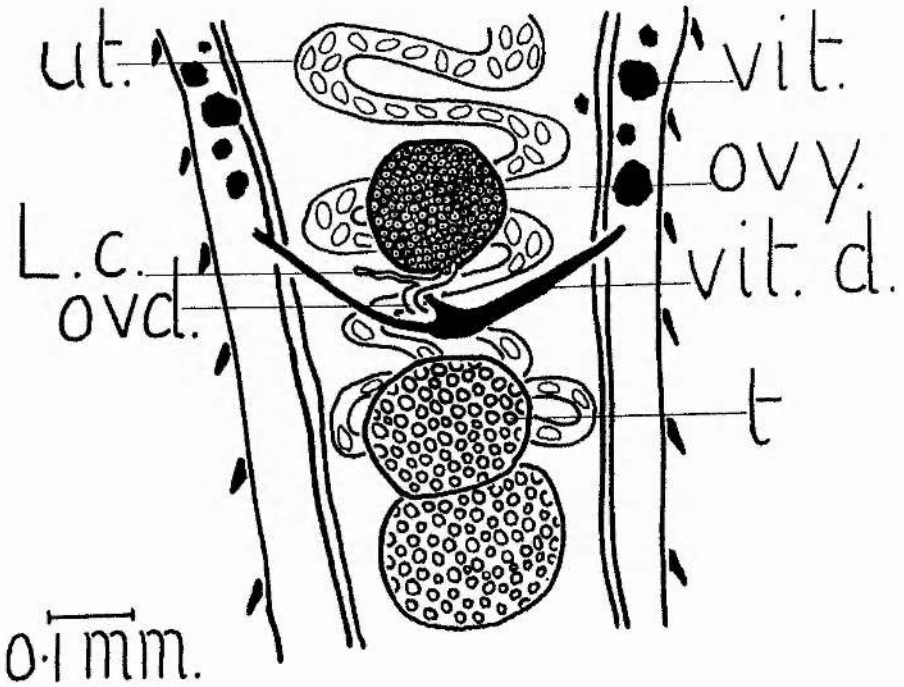


Figure (3).

The posterior region of Echinostephilla virgula.



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