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EDITED BY

GEORGE H. F. NUTTALL, F.R.S.
Quick Professor of Biology in the University of Cambridge

ASSISTED BY

EDWARD HINDLE, PH.D.

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THE LIFE HISTORY OF *NEMATODIRUS FILICOLLIS* RUD., A NEMATODE PARASITE OF THE SHEEP'S INTESTINE.

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(*From the Research Laboratory in Agricultural Zoology, University of Birmingham.*)

(With Plates VIII and IX and 5 Text-figures.)

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Introduction.

SHEEP are probably more frequently affected by diseases due to parasitic worms than any other of our domestic animals, and of Nematodes alone at least twenty-eight species have been recorded from the alimentary tract, chiefly from the fourth stomach (abomasum) and the intestines.

These round-worms have in recent years formed the subject of careful study by a number of investigators, the latter, however, have confined themselves largely to the systematic and anatomical features of the adult individuals, the larval forms and life histories generally receiving only brief and occasional notice. Thus of the Strongylidae inhabiting the sheep's alimentary canal and to which family the majority of the most injurious Nematodes belong, there is only a single species, *Haemonchus contortus*, whose life history has received adequate attention during the last few years. Our knowledge of the development of the other forms is very incomplete and in most cases confined to scanty descriptions of isolated larval stages.

The presence of *Haemonchus contortus*, the well-known "Twisted Wireworm," in the fourth stomach of sheep has been proved to be responsible for very serious losses among flocks, both in this country and abroad; opinions vary, however, as to the amount of damage caused by the other common Strongylid round-worms of the alimentary tract, although most agree that when occurring in large numbers the majority are capable of inflicting very serious, if not fatal injuries, whilst lighter infections may lead to weakness and loss of condition, especially among lambs and yearlings.

Vermifuges and other medicinal remedies have not proved entirely satisfactory, and of late years the efforts of agriculturists have been directed towards preventing or reducing the infection by these parasites. Effective prophylactic measures against Nematodes cannot however be devised until a thorough knowledge of their life histories is obtained and this should include the biology as well as the anatomy of the various larval forms.

It was with the intention of filling up one of the numerous gaps in our knowledge of sheep Nematodes that the observations recorded here were undertaken. *Nematodirus filicollis* was selected for study for a number of reasons: (*a*) in spite of the few records of its occurrence it proved to be a very common species in different parts of England, (*b*) our knowledge of its life history is confined to short and incomplete descriptions of the newly-hatched larva, and (*c*) the ease with which material could be obtained and the large size of the eggs in this species make it a very convenient form for study.

My observations on the parasite were commenced in August, 1913, at the South-Eastern Agricultural College at Wye in Kent[1], the greater

[1] I wish to take this opportunity of expressing my indebtedness to Mr H. E. Hornby of this institution for placing a quantity of *Nematodirus* material at my disposal;

part of the work was however carried out in the new Agricultural Zoology Laboratory in the University of Birmingham. *Nematodirus filicollis* has generally been considered to be a somewhat uncommon parasite in Europe, but both in Kent and in the Birmingham district this form proved extremely abundant at all seasons and was found in a large percentage of lambs and yearlings suffering from gastro-intestinal troubles, as well as in a number of apparently healthy animals.

The worms usually occur in the duodenum but in the case of heavy infection are also found in other parts of the small intestine; they have been recorded from the fourth stomach as well[1], I have however never come across them in this position.

Whilst in the majority of cases the parasite occurs in relatively small numbers, I have occasionally observed thousands in the duodenum alone, the contents of the latter seeming nothing else than a writhing mass of worms. Lambs thus heavily infected always exhibited symptoms of helminthiasis, to what extent these were due to the presence of *Nematodirus filicollis* is difficult to say as this worm was never found by itself but was always associated with other parasitic worms, both in the small intestine and in other parts of the alimentary tract. Among the worms found associated with *Nematodirus filicollis* in the small intestine were the tape-worm, *Moniezia expansa*, and the following Nematodes: *Bunostomum trigonocephalum* Rud., *Ostertagia circumcincta* Stad., *Cooperia oncophora* Raill., *Trichostrongylus vitrinus* Looss, and *Strongyloides papillosus* Wedl.

Historical.

The worm now under consideration was first described by Rudolphi in 1802 under the name of *Ascaris filicollis*, the type specimens having been obtained from sheep in Germany. The same investigator removed the species to the genus *Strongylus* in 1803, and under the name of *Strongylus filicollis* we find descriptions of the parasite in many helminthological publications, including those of Schneider (1866), Curtice (1890), Railliet (1893), and Stödter (1901).

Ransom in 1907 took the species as type of a new genus *Nematodirus*, and in his monograph on the Nematodes parasitic in the alimentary

I desire, also, to express my thanks to the Birmingham Natural History and Philosophical Society for assistance, by means of a grant from the Endowment of Research Fund, in defraying the cost of the illustrations of this paper.

[1] Cf. Neumann (1905), p. 361.

tract of Ruminants, published in 1911, we find the following generic diagnosis:

> Metastrongylinae: Head not over 50μ in diameter; circumoral papillae inconspicuous. Cuticle of head may be slightly inflated and in cervical region striated transversely. Cervical papillae apparently absent. Bursa without unpaired dorsal median ray. Dorsal lobe of bursa reduced to two small short lobules, each supported by a dorsal ray. Ventro-ventral and latero-ventral rays of each lateral lobe of bursa close together, parallel. Six supporting rays in each lateral lobe. Medio-lateral and postero-lateral rays close together, parallel. Externo-lateral ray distally diverges widely from the other lateral rays. Spicules more than 0·5 mm. in length, slender, tubular, filiform, united by a membrane throughout their length, or only in their distal portion. Gubernaculum absent. Vulva of female behind the middle of the body. Ovijectors well developed. Eggs large, generally over 150μ long.

In this monograph Ransom mentions three species of *Nematodirus* as occurring in Ruminants: *N. filicollis* Rud. in cattle, sheep, goats, prong-horned antelope, roe-deer and fallow-deer, *N. spathiger* Railliet in the dromedary and *N. digitatus* Linstow in the zebu. To these must be added *N. roscidus* Brumpt (1911) from deer, *N. mauritanicus* Maupas and Seurat (1912) from the dromedary, and *N. fordi* Daniels (1908) from cattle as well as from man and the pig. Railliet and Henry in a recent paper (1912) have shown that *N. digitatus* and *N. fordi* differ in many respects from the other species and have divided the genus into two sub-genera *Nematodirus* and *Mecistocirrus*[1], the latter to include these two species. The sub-genus *Nematodirus* is defined as follows:

> Corps capillaire, longuement effilé dans sa partie antérieure; extrémité céphalique munie d'un léger renflement vésiculeux souvent strié en travers; tégument rayé par 18 arêtes longitudinales assez nettes; pas de papilles cervicales apparentes. Bourse caudale bilobée, à côtes d'égale importance; les postérieures séparées, sans tronc commun; les antérieures dédoublées; la pointe des antérieures externes à égale distance des antérieures et des moyennes. Spicules grêles, longs d'au moins 500μ (et au plus du $\frac{1}{12}$ du corps). Queue de la femelle tronquée et mucronée; vulve vers le $\frac{1}{3}$ ou le $\frac{1}{4}$ postérieur du corps; vagin très court. Oeufs ellipsoïdes, grands, à coque plutôt épaisse, segmentés au moment

[1] The differences between *Nematodirus* and *Mecistocirrus* have recently been emphasised by Neveu-Lemaire (1914) who suggests raising these to generic rank.

de la ponte; l'embryon se développe à l'intérieur de la coque et y subit deux mues, après quoi il est apte à rentrer directement dans l'organisme sans phase de liberté dans le milieu extérieur. Habitat: ordinairement le duodénum des Ruminants.

Railliet and Henry also show that there has been in the past considerable confusion between the two species *N. filicollis* and *N. spathiger*, and point out that whilst Schneider (1860) correctly figured the true *N. filicollis* Rud., the specimens described under that name by Curtice (1890), Stödter (1901), and Ransom (1911) are to be referred to *N. spathiger* Railliet.

Maupas and Seurat recently (1912) described *N. filicollis* from the dromedary and sheep in Algeria; from the descriptions given by the authors there can be little doubt that these worms are also to be included in Railliet's *N. spathiger*[1].

On account of this confusion between the two species I have considered it advisable to give a short description of the adult individuals observed by me in England before proceeding to the account of their development; it will be seen that these worms belong to the true *Nematodirus filicollis* Rud. as defined by Railliet and Henry.

Specific Diagnosis.

In the adults the body is slender and considerably attenuated anteriorly; the living worms are semi-transparent and either colourless or, more frequently, tinged with a blood-red colouring matter, the latter often most intense at the anterior and posterior extremities and apparently lying in the body-cavity and not in the alimentary canal. The cuticle is nearly always inflated at the anterior extremity, and in the region of the neck is marked with transverse striations, absent from the rest of the body, which is provided with eighteen longitudinal cuticular lines. The head has a breadth of 30 to 60μ and bears six small papillae surrounding the circular mouth. Cervical papillae are absent. The oesophagus measures 450–600μ in length and is broadest posteriorly (30–40μ). The nerve-ring is situated about 300μ from the anterior extremity, the excretory pore 50–70μ in front of the junction of oesophagus and intestine.

Male. The average length of the male was found to be 11·5 mm., the smallest mature specimen measured 7·5 mm., the largest 13·5 mm.

[1] Seurat has recently (1913) described the Rodent *Ctenodactylus gundi* as a host of *Nematodirus filicollis* in Tunisia. The figures of the worms given by the author show that these also do not belong to this species.

The maximum diameter of the body varies between 90 and 130μ. The lateral lobes of the bursa are without distinct dorsal lobules such as occur in *N. spathiger*. The postero-lateral ray is the thickest; the externo-dorsal is extremely slender. It was noticed that the posterior branch of the dorsal ray is frequently bifurcated at the tip, a point not previously recorded. The long slender spicules have an average length of 810μ, varying between 750 and 925μ. The terminal membrane of the spicules is lanceolate, ending in a fine point.

Female. The average length of the adult female is 18 mm., the specimens observed varying from 13 mm. to 21 mm. The maximum breadth of the body just anterior to the vulva measures 150–225μ. The tail is truncated posteriorly and from the tip projects a very slender rod-shaped process 12–18μ in length. The truncate tip of the tail has a breadth of 20–25μ and the cuticle surrounding it is slightly inflated and frequently presents faint transverse striations similar to those in the neck region. The anus is situated 65–80μ from the posterior extremity, the body at this level having a breadth of 45–60μ. The vulva is a little less than a third of the length of the body from the posterior end. The vagina is very short, the muscular portions of the two ovijectors measure 400–500μ. The average size of the egg is 160μ × 80.

The Egg.

The eggs of *Nematodirus filicollis* are considerably larger than those of most Nematodes which inhabit the alimentary tract of Ruminants in this country. I have measured a large number both from the sexual organs of adult females and from washings of faeces; as mentioned in the specific diagnosis it was found that they have an average length of 160μ by an average breadth of 80μ. The smallest egg observed had a length of 130μ, the largest 210μ, the breadth varying similarly between 65 and 95μ. It is worth noting that the eggs from the faeces were always found to be a little larger than those taken from the maternal uteri.

The shape of the eggs is fairly constant, appearing elliptical in side view, circular in cross section. The egg-shell is thick but quite transparent and colourless; the thickness, approximately 3μ, is uniform and not increased at the two poles as in *N. spathiger* (according to Railliet and Henry). The surface of the shell is quite smooth. The eggs commence their development within the uterus and when laid usually contain seven or eight cells; more advanced eggs were never

found in fresh faeces, although earlier stages were occasionally met with. The egg contents are dark in colour, opaque and filled with coarse yolk granules; the vitelline membrane is usually conspicuous.

Development of the Egg outside the Host.

The development of *Nematodirus* has received little attention from previous investigators. Ransom (1911) seems the first to point out that the embryo within the egg-shell develops into a larva with the filariform type of oesophagus before hatching, thus differing from other Strongyles, such as *Haemonchus contortus*, in which the newly-hatched embryo possesses a rhabditiform oesophagus with a posterior bulb, not developing into the larval stage with filariform oesophagus until later. Railliet and Henry (1912) confirm this statement and add the information that the embryo undergoes two moults within the egg-shell before hatching. Maupas and Seurat (1913) describe the early development in more detail and figure the newly-hatched larva; they show that the latter is provided with a sheath formed by the second ecdysis and that the skin cast during the first moult can also be seen surrounding the mature larva within the egg-shell.

In order to study the development outside the host it was necessary to obtain eggs in large numbers and, as far as possible, in pure cultures, i.e. free from the eggs of other parasitic or free-living Nematodes. Such cultures could be obtained from two sources: (*a*) from the sexual organs of the adult worms, (*b*) from the fresh faeces of infected sheep. A certain number of cultures were made by teasing up the bodies of mature females; by this method, however, the eggs obtained were not numerous, moreover they included a very large percentage which failed to continue their development; it was, therefore, found more convenient to obtain the material from the second of the two sources just mentioned.

From the fresh faeces of heavily infected sheep eggs could be obtained in very large numbers by the usual methods of sieving and sedimenting[1]: by the use of very fine sieves it was found possible to separate the large *Nematodirus* eggs from the smaller ones of other genera.

The eggs of *Nematodirus filicollis* were found to develop equally well in tap-water and in moist faeces, in the case of the faecal cultures it was found necessary to provide sufficient aeration in order to prevent the "poisoning" of the eggs by decomposition products. Eggs in water

[1] For a comparative account of the methods of examining faeces cf. Hall (1911).

seemed not to require much air and developed well in solid watch-glasses with sealed covers or in small corked tubes.

The presence or absence of daylight has little or no effect on the development, eggs developing equally well whether exposed to light or kept in a dark chamber.

A certain amount of moisture was found to be essential, in the earlier stages of development the eggs were invariably killed by desiccation; excess of moisture is not harmful to the cultures, for, as already mentioned, development proceeds perfectly well in pure water.

The rate of development of the eggs, always slow as compared with other Strongylids, is very strongly influenced by temperature conditions which are certainly of the greatest importance. Extensive series of experiments concerning this factor could unfortunately not be made, cultures were however kept at a number of different temperatures and the general effects of these conditions ascertained.

The best results were obtained at temperatures between 19° C. and 27° C., i.e. warm summer temperatures, under these conditions eggs were found to contain mature larvae ready to hatch 24–28 days after leaving the body of the host. At ordinary laboratory temperatures (13°–15° C.) development proceeded more slowly and with less regularity, eggs often taking 5–6 weeks to produce mature larvae. At low temperatures development is suspended, freezing kills the eggs in their early stages.

Several cultures were kept in an incubator at a temperature of 38° C., under these conditions the eggs developed rapidly as far as the morula stage, after which the majority died; no mature embryos were formed, thus indicating that complete development cannot take place at the blood temperature of the host and that eggs swallowed by sheep during the earlier stages would not survive.

As already mentioned the eggs usually contain 7–8 cells when laid, they do not develop further until they leave the body of the host. In water or moist faeces the development of the embryo continues and is of quite the normal type, resembling that of other Nematode worms. Under favourable temperature conditions the morula stage is reached in 3–4 days and is soon followed by the characteristic "tadpole" stage, in which the embryo is flexed and considerably thickened at the anterior extremity. The first movements of the embryo now become apparent, the latter continues elongating and by the end of the first fortnight has reached a length of approximately five times that of the egg in which it lies coiled; the thickening at the anterior extremity has disappeared

and the embryo is approximately cylindrical in shape, except posteriorly where the body tapers to a long slender tail.

The embryo at this stage performs continuous, although somewhat sluggish, movements within the egg-shell; its internal organisation is not visible, the whole of the body with the exception of a small spot at the cephalic extremity being filled with dark, yolk-like granules.

During the second fortnight important changes take place, the opaque granules of reserve substances are slowly absorbed and the internal organisation of the embryo gradually revealed, the latter moreover undergoes two ecdyses before appearing in its final condition ready for liberation from the egg-shell. The mature embryo is quite transparent and appears highly refractive, it is enclosed in a tightly fitting sheath, the uncast skin derived from the second ecdysis, and is still surrounded by the shed skin from the first moult. The mature embryo is extremely lively at ordinary laboratory temperatures and is in continual motion within the egg-shell.

The Hatching of the Embryos.

For some time great difficulty was experienced in getting the embryos to hatch from the eggs, many mature eggs were kept for weeks in the laboratory without a single larva being freed, whilst in some cultures even after seven months more than 90 per cent. of the embryos were still unhatched although apparently healthy and moving actively within the egg-shells.

Mr H. E. Hornby, who had made some observations on this species at the South-Eastern Agricultural College at Wye, informed me that he had had the same difficulty in obtaining larvae from *Nematodirus* eggs kept in moist faecal cultures, but had been more successful with some cultures which had been dried for a short period. I therefore tried the effect of submitting some of my cultures to alternate desiccation and remoistening; this yielded somewhat better results, a few larvae hatching at each remoistening but still forming a very small percentage.

It is, of course, well known that the larvae of many Nematodes hatch only when the eggs are taken into the body of their host, this led me to try the effect of temperatures approximating to that of the blood on the mature eggs of *Nematodirus filicollis*. In an oven at a temperature of 38° C. the embryos with only few exceptions hatched

within a very short period[1], thus suggesting the possibility that in this species also the liberation of the larvae normally takes place within the alimentary canal of the host. Although a quite conceivable solution of the difficulty it struck me as a highly improbable one, since the study of some liberated larvae had shown that these are admirably adapted for leading a free existence and, moreover, that their structure and habits are precisely similar to those of other Nematode worms known to have free-living stages in their life histories.

I only accidentally became aware of the true facts of the case; during a particularly warm, sunny day in the spring one of my cultures was left exposed to the sun's rays for several hours and examination of a small sample showed that a considerable number of larvae had been liberated. The temperature in the sun was approximately 25° C.

Other cultures were then subjected to various temperatures between 24° C. and 32° C., both in ovens and in the open, and these experiments showed that the larvae are quite capable of hatching so long as the temperature is sufficiently high. During a warm spell in the early summer (24°–28° C. in the shade during the middle of the day) nearly all the larvae in my cultures were released.

Description of the "Ensheathed" Larva.

The newly-hatched larvae of *Nematodirus* are easily distinguished from those of other Nematode parasites of the sheep by their comparatively large size and by the great length and tenuity of the tail-region.

It must be noted that these larvae on hatching have already undergone two ecdyses, one complete and one partial, within the egg-shell; as pointed out by previous observers, they are therefore in a considerably more advanced condition than the newly-hatched larvae of the majority of Strongylid Nematodes, e.g. *Haemonchus contortus* or *Anchylostoma duodenale*, in fact they have reached a state of development comparable with that of the latter worms at the end of their period of free life. According to Looss' nomenclature (1911, p. 345) the *Nematodirus* larvae when liberated from the egg are in a state of transition between the second and third larval stages; in structure they have reached the third stage of development, but are still enclosed in the loose skin

[1] In these experiments the liberation of the larvae took place quite independently of the chemical composition of the fluid in which the eggs were kept. The media used were those suggested by Martin (1913, p. 64), namely, distilled water, ·2 per cent. HCl, ·2 per cent. $NaHCO_3$ and ·8 per cent. NaCl.

of the second stage. The general shape of the larvae is therefore determined by this outer skin, and in describing and measuring them it is necessary to distinguish carefully between this sheath and the enclosed larva which almost completely fills it.

The total length of the free larvae, including the tail, varies from ·85 to 1·15 mm., the average length being about 1 mm. The body has a maximum breadth of 25–30μ and is approximately cylindrical, tapering however anteriorly and posteriorly but only moderately towards the cephalic extremity which terminates in a rounded head about 15μ in width. Posteriorly the body passes gradually into the very slender tail which has a length of about one-third of the total length of the

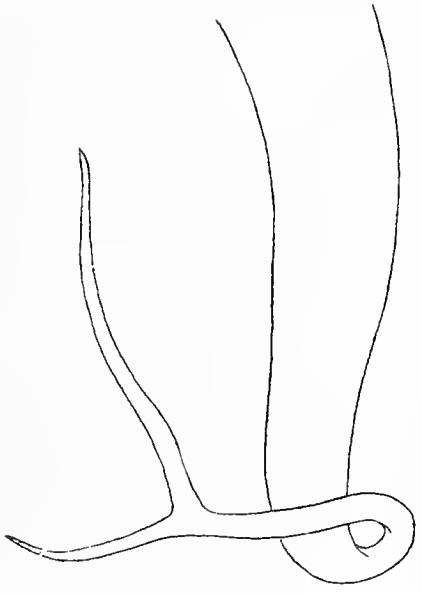

Fig. 1. Tail of larva with fork-like malformation of posterior end. × 750.

larva. In many of the larvae a slight constriction is to be seen a short distance behind the commencement of the tail, the posterior portion of which is often difficult to see except under high magnifications, having a thickness of little more than 1μ.

Abnormalities of the caudal region were frequently observed, the commonest being a fork-like malformation of the extremity, as shown in Text-fig. 1.

The skin or "sheath" is comparatively thick and provided with closely set transverse striae, only visible under high powers of the microscope; it is completely closed, the position of the mouth is however indicated by a slight thickening at the anterior extremity.

Under normal conditions and when uncontracted the enclosed larva completely fills the ensheathing skin with the exception of the slender tail, for this reason the sheath is sometimes inconspicuous, it can however be readily observed when the body is flexed, being thrown into distinct and regular folds on the concave surface; the larva has moreover considerable powers of contraction and when contracted the outer skin can be seen projecting at each extremity.

As mentioned above the enclosed larvae do not project into the tail-regions of the sheaths, they are therefore considerably shorter than the latter, measuring 5·3–7 mm. in length.

The shape of the head differs somewhat from that of the cephalic extremity of the sheath, being slightly truncated anteriorly, not rounded. There are no distinct lips, cephalic papillae are however present, in the form of six small but highly refractive spots.

The tiny mouth leads into a short buccal cavity. The oesophagus is slender, 180–220μ in length, it increases in breadth posteriorly but does not terminate in an oesophageal bulb and is not provided with a "dental" apparatus. The intestine is also narrow and consists of eight cells only, four dorsal and four ventral alternating with one another; these intestinal cells are rather dark in colour and usually filled with reserve granules and vacuoles which conceal the nuclei, the latter are however occasionally visible. From the termination of the intestine a very slender rectum leads to the anus, a tiny opening situated on the ventral surface about 50μ from the posterior extremity of the larva.

The tail of the larva is peculiar and extremely characteristic, differing markedly in shape from that of the sheath; it is divided at the posterior extremity into dorsal and ventral lobes in such a way as to appear deeply forked when seen in a lateral view. The two lobes vary somewhat in shape and size in different individuals, the dorsal is however usually somewhat larger than the ventral which it conceals in a dorsal view (Text-fig. 2). Between the dorsal and ventral lobes is an elongated rod-shaped process recalling the tail-spike of the adult *Nematodirus* female, it has a length of about 13μ and projects well beyond the lobes of the tail.

The excretory apparatus is difficult to make out in the ensheathed larva, the terminal canal can however be seen in the majority of individuals leading to the minute excretory pore, which opens on the ventral surface a little anterior to the junction of intestine and oesophagus and about 150μ from the anterior extremity of the body.

The central portion of the nervous system can be seen in many individuals and takes the form of a ring encircling the oesophagus at a point just anterior to the level of the excretory opening.

The genital rudiment is quite conspicuous, forming a lens-shaped body situated ventrally to the intestine and opposite the fifth intestinal cell. Under a high magnification it can be seen to consist of a number of cells (usually about 8–12) with conspicuous nuclei.

The Biology of the "Ensheathed" Larvae.

The free larvae are extremely active and in a liquid medium at laboratory temperatures (13°–15° C.) perform vigorous, snake-like movements similar to those described in the mature larvae of other

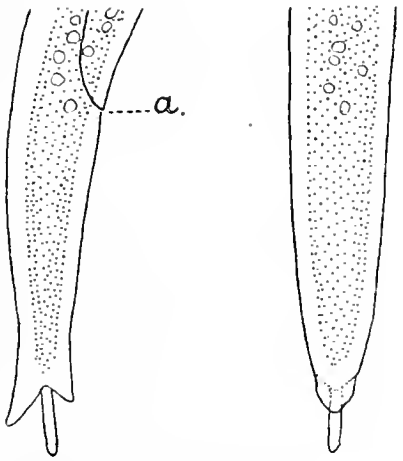

Fig. 2. Posterior end of body of "exsheathed" larva, viewed from right side and from dorsal side. *a.* anus. × 750.

Strongylid worms. When placed in a watch-glass or tube containing water they immediately sink to the bottom and are evidently incapable of keeping afloat by swimming movements.

The larvae of *Nematodirus filieollis* possess the same migratory instincts as the larvae of *Haemonehus eontortus* and *Anehylostoma duodenale* and are able to climb vertical surfaces if these are kept sufficiently moist. The majority of my cultures were kept in a moist chamber in small glass dishes about 40 mm. in diameter and with walls 25 mm. high, the larvae as they hatched during warm weather immediately commenced to ascend the sides of the dishes and after a few

weeks were present in such numbers in this position as to appear to the nakcd eye as a whitish deposit covering the whole of the inner surface of the vertical walls. When the dishes were removed from the moist chamber the larvae did not return to the damp faecal mass at the bottom, but dried and adhered to thc walls as the moisture evaporated. The larvae also ascended blades of grass and seedlings placed in the dishes.

Ransom has already pointed out in the case of *Haemonchus contortus* (1906) how this upward migration of the ensheathed larvae is connected with a method of infection most peculiarly adapted to the food habits of sheep and other herbivorous animals; the similar migratory instincts of the larvae of *Nematodirus filicollis* suggest that these reach the alimentary canals of their hosts in the same manner.

The ensheathed *Nematodirus* larvae are evidently able to maintain their existence in water for a considerable period, I have kept specimens in small watch-glasses for over eleven months.

Perhaps the most striking feature in the biology of the larvae is their power to withstand desiccation for long periods. This phenomenon can be studied by allowing a drop of water containing larvae to evaporate on a glass slide; as the moisture of their surroundings becomes reduced the larvae contract somewhat within the sheaths and usually coil themselves into a spiral, the fluid within the sheaths is still present for a short time after the water on the slide has completely evaporated, this however soon disappears and the larvae become much shrivelled, assume a glassy appearance and become extremely brittle. When remoistened the larvae quickly absorb water through their sheaths, regain their shape and gradually uncoil, the whole process often taking less than twenty minutes.

The larvae can in most cases endure repeated drying and remoistening and in one experiment I successfully dried and revived a number of individuals twelve times in six days.

The *Nematodirus* larvae are able to withstand complete desiccation equally well whilst still within the egg-shell, this however only applies to the mature embryos which are already provided with sheaths.

The maximum period for which the larvae can retain their vitality when subjected to complete desiccation was not ascertained, my experiments however showed that they can do so for a very long time. I will quote one interesting experiment in support of this statement. A small mass of faeces, known to contain numerous eggs enclosing mature larvae, was placed in a small open box on August 30th, 1913, and then

left untouched until September 27th, 1914. By this date, i.e. more than twelve months afterwards, the lump of faeces was as hard as stone and had to be broken with a hammer; a small piece was chipped off and soaked in water in a watch-glass, as it softened it was broken up with a needle revealing numerous eggs containing shrivelled larvae. After about fifteen minutes the latter had appreciably swollen and soon regained their normal shape, they were moving actively within the egg-shells less than forty minutes after the commencement of the experiment. Another sample from the same dried mass was treated in a similar manner on April 30th, 1915, and yielded a number of live larvae which seemed none the worse for having been dried for twenty months.

A number of experiments were also made to determine the effect of different temperatures on the ensheathed larvae, it was found that these, when perfectly dry, are able to withstand very high temperatures without losing their vitality. On one occasion seven dried larvae were kept for ten hours in an oven at a temperature of 60° C., when afterwards cooled and placed in water they all revived in less than half an hour and were still alive and active twenty-four hours after being remoistened. In the course of another experiment three dry larvae were subjected to a temperature of 70° C. for two hours, of these two revived and were swimming about actively twenty-five minutes after remoistening.

Other experiments showed that this power of resisting high temperatures was confined to larvae in the dry condition, larvae in water are quickly killed if subjected to temperatures much over 50° C.

The dried larvae of *Nematodirus filicollis* are equally resistant to cold, and several experiments showed that these could be left outdoors during sharp frosts in the winter without fatal consequences.

The Completion of the Second Ecdysis.

As already mentioned the ensheathed larvae of *Nematodirus filicollis* can live in water for many months without undergoing any change, and in this medium at laboratory temperatures make no efforts to rid themselves of the closely-fitting sheaths formed during the second ecdysis within the egg-shell. This fact together with the similarity in structure and habits of these larvae with the ensheathed larvae of other better known worms of the same family (e.g. *Haemonchus contortus* and *Anchylostoma duodenale*) leads to the conclusion that at this stage they have reached maturity and are ready to pass into the body of their definitc host.

Looss (1911) has shown that the mature larvae of *Anchylostoma* cast their protective skins as soon as they enter the body of their host, whether through the skin or through the mouth; he also showed that the completion of the second ecdysis can also take place in the open under certain artificial conditions. According to this investigator the process is always a purely mechanical one and is performed either actively by the larvae (e.g. under certain chemotactical stimuli when they unmistakably exert themselves to leave their envelopes) or passively (e.g. in certain media, such as gelatine or water containing numerous foreign particles, when the rending of the outer skin results from its mechanical retention by the medium whilst the larva itself attempts to move forwards).

The larvae of *Nematodirus* are also able to complete their moults in the open at laboratory temperatures when subjected to certain conditions. Thus it was found that ecdysis occurred simultaneously in a number of ensheathed larvae living in a culture of faeces which had been repeatedly dried and remoistened during the course of some experiments on the resistance of the larvae to drought.

A similar phenomenon was observed on two occasions in moist cultures of faeces which had been attacked by fungi, and I was able to watch several individuals in the act of moulting. In these cases the hyphae of the fungus had enveloped the larvae and arrested their progress, resulting in violent serpentine movements which finally led to the rupturing of the sheaths near the anterior extremities.

On all occasions when the completion of the moult was observed in the *Nematodirus* larvae the process took place in a purely passive manner; I never succeeded in inducing active ecdysis by means of chemical stimuli, although the effects of various stains as well as of weak solutions of neutral salts, acids and alkalis were tried.

The influence of temperature on the ensheathed larvae is, however, very marked, and repeated experiments showed that these when subjected to temperatures approximating to blood-temperature, with few exceptions, complete their ecdyses within a few hours. The experiments were performed by means of a small electric oven at a temperature of 38° C., the larvae being placed in small stoppered tubes in water and various solutions. The casting of the sheaths took place quite independently of the chemical composition of the fluid in which the larvae were kept, pure water as well as dilute neutral acid and alkaline solutions were used, always however with similar results.

The above experiments show that whilst at low temperatures the larvae of *Nematodirus filicollis* are occasionally able to complete their moults under certain somewhat abnormal conditions—they normally do so when subjected to a temperature of 38° C.—we may assume therefore that they would behave in a similar manner when taken into the body of a sheep or other host.

Structure of the "Exsheathed" Larva.

Whilst retained within the sheaths the larvae of the third stage undergo no further development, and just after the completion of the second ecdysis show practically the same structure as those described on page 143; in most individuals however the food granules and vacuoles have almost entirely disappeared from the eight intestinal cells, revealing the nuclei as spherical bodies of a rather lighter colour than the protoplasm surrounding them.

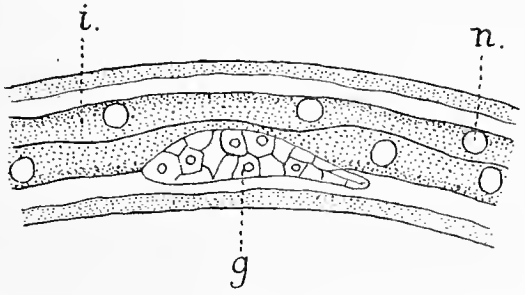

Fig. 3. Genital rudiment and adjacent parts of body of larva fifteen days after completion of the second ecdysis. *g.* genital rudiment. *i.* intestine. *n.* nucleus of intestinal cell. × 750.

In their behaviour these larvae differ markedly from those in the ensheathed stage, they have lost their agility of movement and at laboratory temperatures are exceedingly sluggish, often remaining motionless for hours. They show no tendency to climb vertical surfaces.

In water or cultures of faeces the "exsheathed" larvae usually die within a few days, I however succeeded in keeping a few individuals for fifteen days in an oven at 38° C. These larvae showed little change except in the structure of the intestine and genital rudiment.

By the fifteenth day the number of intestinal cells had increased considerably and fourteen nuclei could be counted; the cell limits were at this stage scarcely discernible and moreover the intestine now showed a distinct lumen.

The genital rudiment also showed a considerable increase not only in actual size but also in the number of its constituent cells (Text fig. 3).

Mode of Infection of Sheep.

It was unfortunately found impossible for various reasons to perform infection experiments on sheep, I am therefore not in a position to produce direct proofs that these animals become infected by swallowing the sheathed larvae of *Nematodirus filicollis*. The circumstantial evidence in favour of such direct infection is, however, very strong: in the first place, as already pointed out, in their structure, general behaviour and especially in their migratory instincts, these larvae are precisely similar to those of forms known to infect warm-blooded animals directly; the conditions under which the second ecdysis is completed also greatly favour this view; whilst lastly, as will be shown in the next paragraph, examination of the intestinal contents of sheep harbouring adult *Nematodirus* revealed practically all stages between mature larvae and adult individuals, the youngest of these intermediate stages showing but little advance in structure over the "exsheathed" larvae just described.

Whilst the larvae in the free condition are probably the more usual source of infection it must not be forgotten that these are no doubt infective whilst still within the egg-shell, being already ensheathed before hatching. That the mature eggs are a possible source of infection was indicated by Railliet and Henry (1912) when in their definition of the genus they stated "l'embryon... est apte à rentrer directement dans l'organisme sans phase de liberté dans le milieu extérieur."

Young Stages in the Sheep.

The alimentary tracts of infected sheep and lambs were carefully examined for young parasites, and a number of interesting early stages of *Nematodirus filicollis* were obtained; the youngest of these were only little more advanced than the "exsheathed" larvae described in a preceding paragraph.

The youngest stage was found in a lamb killed in March, 1914, it measured only 1·2 mm. in length and except for its greater relative breadth and for an increase in the number of intestinal and genital cells is very similar to a mature larva just after ecdysis (Text-fig. 4). The body has a maximum thickness of 40μ and is approximately cylindrical in shape, tapering however at both extremities. The truncated head measures

about 15μ in breadth and is provided with six small papillae. The oesophagus has a length of 270μ, the intestine is multicellular and the genital rudiment no longer exists as a lens-shaped body. The most interesting organ at this stage is the tail, which retains all the characteristic larval features, namely the forked extremity and the rod-like terminal process.

The first traces of external sex characters were seen in specimens 2·5 mm. in length, these very closely resemble the earlier stage just described and do not show any appreciable increase in breadth, in a few

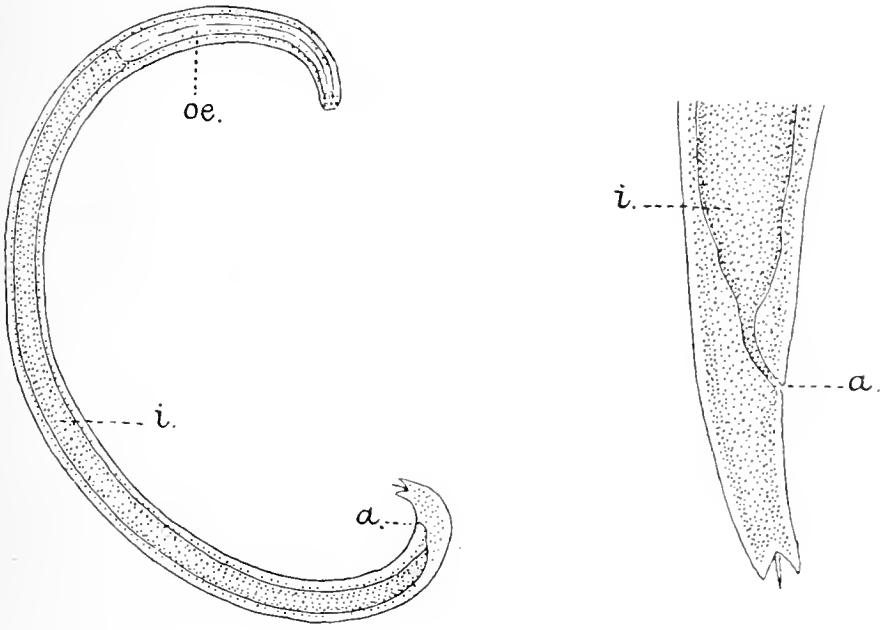

Fig. 4. Young stage from intestine of lamb. *a.* anus. *i.* intestine. *oe.* oesophagus. × 100.
Fig. 5. Posterior end of similar but slightly older stage from intestine of lamb. *a.* anus. *i.* intestine. × 300.

individuals however a distinct swelling is visible, just anterior to the tail-fork and at about the level of the anus, which probably represents the first sign of a male bursa.

The bursal rudiment is much more conspicuous in a specimen 3·3 mm. long, here it forms a hollow swelling about 60μ broad in the same position below the larval skin, the latter is very loose in the anterior part of the body and is evidently about to be cast; this is probably the final moult before the adult stage is reached. In this specimen the body has an almost uniform thickness of 50μ, tapering however at the two extremities but only slightly posteriorly, the region

just in front of the rudimentary bursa measuring 45μ. The head is 20μ broad and is provided with a circular mouth and six cephalic papillae, the latter now more conspicuous than in the earlier stages. The caudal extremity of this specimen bears a striking resemblance to that of an early stage of a Strongylid figured by Leuckart (1876) under the name of *Strongylus polygyrus*.

With the final moult the characteristic tail-fork and caudal process are lost in the male specimens, the bodies of the latter now terminating in the bursae, at first small and with very faint and inconspicuous rays. The smallest specimen in which all the rays as well as the spicules were visible measured 5 mm. in length.

In the young females the caudal fork remains for a longer period and seems to pass gradually into the truncated extremity of the adult, the rod-like process of the larva is evidently retained as the terminal spike so characteristic of the mature female.

Summary of Life History.

1. The eggs of *Nematodirus filicollis* when laid contain an embryo with seven or eight cells, they pass out of the infested host with the faeces.

2. Even under favourable conditions development takes place slowly and the embryos are not ready to hatch until 24–28 days have elapsed. In their early stages the embryos are not able to withstand desiccation and are killed if frozen or subjected to high temperatures.

3. Whilst still within the egg-shell the embryo undergoes two ecdyses and when ready to hatch is enclosed in a tightly fitting sheath formed by the incompletely cast skin of the second moult; the larvae on liberation from the egg-shell are therefore in a more advanced condition than those of most other Strongylids (e.g. *Haemonchus* or *Anchylostoma*) and are in a stage comparable with that reached by the latter at the end of their period of free existence.

4. The sheathed larvae are often retained for a long time within the egg-shells and both in this position and after hatching can resist complete desiccation for considerable periods (twenty months or even longer); when dried they are able to withstand freezing as well as temperatures much above those likely to be met with in the open.

5. The free larvae will live for a considerable time in water, they possess well-developed migratory instincts and climb vertical surfaces, such as grass stems and blades, and the glass walls of the vessels in which they are kept.

6. The sheaths are cast off by the larvae when these are subjected to temperatures approximating to the blood-temperature of the host; completion of the second moult occasionally also takes place at laboratory temperatures under certain abnormal conditions.

7. No infection experiments were made on sheep, but other evidence shows that these animals must become infected by swallowing the sheathed larvae, either when free or whilst still enclosed in the egg-shells.

8. A number of young stages of the parasite were met with in the intestines of sheep, the smallest of these being only little more advanced in structure than the larvae just after ecdysis.

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EXPLANATION OF PLATES.

PLATE VIII.

Nematodirus filicollis Rudolphi.

Fig. 1. Adult female, ×20.

Fig. 2. Posterior end of body of female, viewed from right side, ×450.

Fig. 3. Anterior end of body of female, viewed from left side, ×200.

Fig. 4. Posterior end of body of male, viewed from right side, to show bursa and spicules, ×170.

Fig. 5. Posterior end of united spicules of male, ventral view, ×950.

Fig. 6. Egg, taken from fresh faeces of lamb, ×400.

PLATE IX.

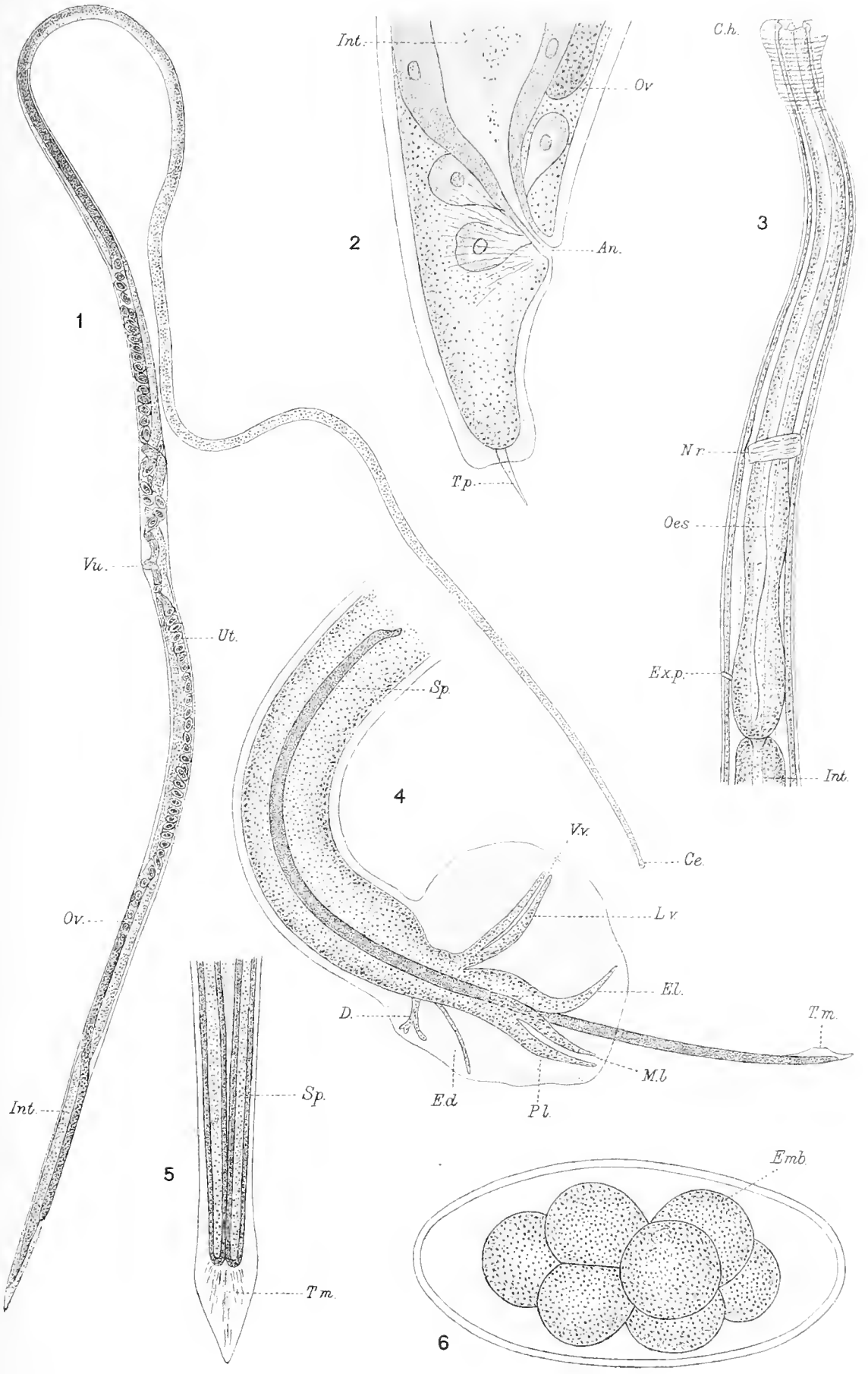
Nematodirus filicollis Rudolphi.

Fig. 1. Newly-hatched larva. ×350.

Fig. 2. Larva after completion of second ecdysis, ×350.

Fig. 3. Young stage (3·3 mm. long) from intestine of lamb, ×60.

Fig. 4. Posterior end of same specimen, showing the rudimentary bursa enclosed by the larval skin, ×400.



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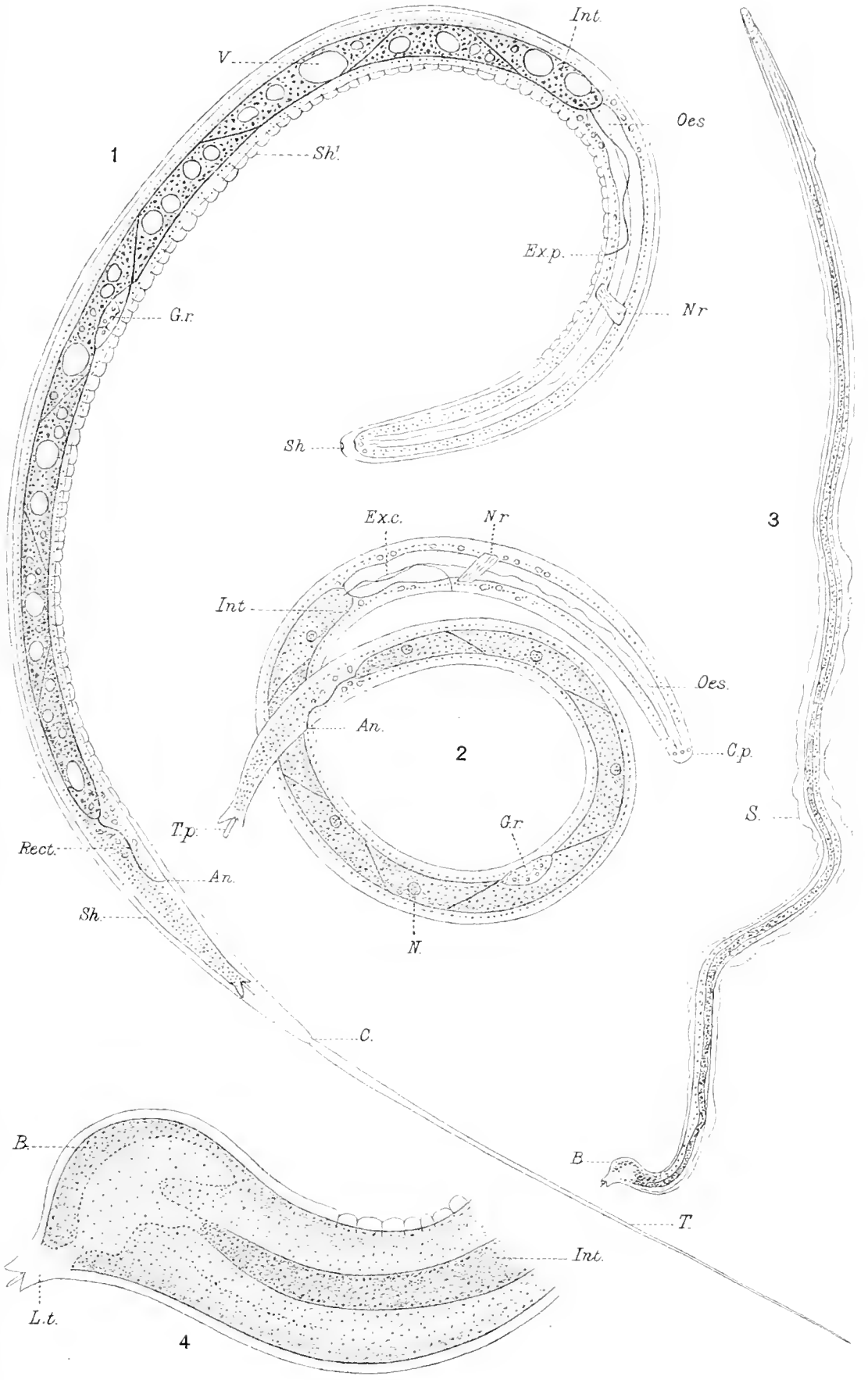
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INDEX TO LETTERING.

An.	Anus.
B.	Rudimentary bursa enclosed by larval skin.
C.	Constriction in tail of sheathed larva.
Ce.	Head.
C.h.	Inflated cuticle of head of adult.
C.p.	Head papillae.
D.	Dorsal ray of bursa.
E.d.	Externo-dorsal ray of bursa.
E.l.	Externo-lateral ray of bursa.
Emb.	Embryo within egg-shell.
Ex.c.	Excretory canal.
Ex.p.	Excretory pore.
G.r.	Genital rudiment of larva.
Int.	Intestine.
L.t.	Larval tail.
L.v.	Latero-ventral ray of bursa.
M.l.	Medio-lateral ray of bursa.
N.	Nucleus of intestinal cell.
N.r.	Nerve ring.
Oes.	Oesophagus.
Ov.	Ovary.
P.l.	Postero-lateral ray of bursa.
Rect.	Rectum.
S.	Larval skin about to be cast.
Sh.	Sheath of free larva.
Sh'.	Sheath of larva thrown into folds.
Sp.	Spicules of male.
T.	Tail of sheathed larva.
T.m.	Terminal membrane of united spicules.
T.p.	Rod-like terminal process of tail.
Ut.	Uterus.
V.	Vacuole in intestinal cell.
Vu.	Vulva of female.
V.v.	Ventro-ventral ray of bursa.

DOURINE AND THE COMPLEMENT FIXATION TEST.

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Introduction.

This paper is written with the purpose of drawing further attention to the value of the complement fixation reaction as a diagnostic test in dourine and of recommending a method of procedure and technique arrived at with an experience of 15,000 tests for dourine made at the Veterinary Research Laboratory, Lethbridge.

In a previous paper I have briefly described the serum reactions in dourine. Since that paper was published in 1912 (*Proceedings of the Amer. Vet.-Med. Assoc.*) diagnostic tests for dourine have been carried along continuously at this laboratory, together with exhaustive control and experimental tests and the searching out of every possible source of error. By the numbers of horses available for experiment, the prolonged trial of the test through every known phase of the disease and its widest application in naturally occurring outbreaks, coupled with observations in company with the veterinary officers in charge of the field work, the complement fixation reaction has been thoroughly established as a sure, safe and specific method of diagnosing dourine. The experience shows that the test meets every requirement with regard to specificity, uniformity and decisiveness. It has been adopted as the official test for dourine in this country by Dr F. Torrance, Veterinary Director General for Canada, who kindly permits me to publish this paper.

By the complement fixation test it has been possible—and without difficulty—not only to make a certain diagnosis of the more or less symptomatic cases, but, and of greater importance, to positively determine the existence of the non-clinical, obscure and latent forms of infection.

Only by a systematic application of the test to every animal exposed to infection—and in no other way known at present—can the healthy-looking, so-called immune carriers of dourine be detected. When it is remembered that horses may tolerate a dourine infection for periods of one to three years and remain for that time normal in general health and appearance but capable at times of transmitting the disease, the necessity of an early and definite diagnosis is evident. The complement fixation test furnishes this and thus becomes of great importance as a basis for the control and suppression of dourine. It is being applied in every known outbreak of dourine in Canada, and, as a precautionary measure, in the various studs and to stallions standing for service in the districts that have come to be considered as dourine-infected areas.

Brief Explanation.

The general principles and mechanism of the complement fixation reaction are now so widely known that it seems unnecessary for the purposes of this paper to repeat them in detail, a few remarks on the subject sufficing to make it clear and intelligible.

When an antigen is introduced into an animal either by way of natural infection or by artificial administration a group of reaction products arise in the animal's serum—known as antibodies—bearing a specific relationship to the antigen and able to combine with it outside of the animal body under certain conditions. Micro-organisms, foreign blood cells and sera, albumens and many forms of protein matter are able to act as antigens. Thus an animal infected with dourine produces antibodies resulting from and specifically related to the dourine antigen, namely *Trypanosoma equiperdum*, the actual cause of the disease.

In a similar manner, an animal which has received injections of the blood of another animal species becomes possessed of antibodies having a specific affinity for the blood of that particular species of animal. In other words, the antibody arising in response to the exciting antigen in the process of infection, sensitization, or immunization, has the specific function of acting upon that antigen to neutralize it or prepare it for destruction.

The complement fixation test applied in the diagnosis of disease consists of two sets of antigen and antibody, that is, two distinct and separable combining groups having no relationship to one another but in each of which *Complement*—a constituent of normal serum—is an essential factor. It is convenient to distinguish these groups by referring to the one comprising haemolytic serum, red cells and complement as the 'haemolytic system' and to the other—closely related to the disease—comprising dourine antibody, corresponding antigen, and complement as the 'antibody-combining group.' Thus:

Dourine or antibody combining group	Haemolytic serum Red cells — COMPLEMENT — Antigen Antibody	*Haemolytic system*

Before the test can actually be applied the exact dosage of the different elements in each group must be worked out by careful quantitative titrations—the most important step in the whole proceeding—and the operator must be absolutely assured that each group reaction

is under his perfect control and that the least disturbing factor will be known to him. In the actual test only one complement unit is employed (the minimal amount necessary for the completion of the haemolytic system) so that only one of the reaction groups can come into operation; it is according to whether the complement unit is attracted and affixed to the antibody-combining group or to the haemolytic group that we obtain a positive or a negative reaction. The former will always be effected when the antigen and antibody correspond, that is, when the serum tested contains the specific reaction products of dourine infection even though in minutest quantity, so delicate is the reaction. Neither the antigen alone nor the serum alone, when properly prepared, can take up the complement unit; to do so, all three factors must be brought into intimate contact, and when the test serum does not contain specific dourine antibodies the complement is not fixed to this group but remains free to join with and complete the two factors of the haemolytic system, so that the red cells undergo haemolysis and a negative reaction is indicated.

Technique and Procedure recommended.

Apparatus required. Heavy glass tubes without lip, 5 ins. by $\frac{5}{8}$ in., and racks to hold twenty-four tubes in a double row, one above the other. Small test tubes, 4 ins. by $\frac{3}{8}$ in., for serum inactivation. Finely graduated measuring pipettes of 0·1, 1·0, 5·0 and 10·0 c.c. capacities. Graduated cylinders of 50 and 100 c.c. capacities. Erlenmeyer flasks of heavy glass, standard sizes up to 500 c.c. capacity. Large centrifuge cups and small centrifuge tubes. Ampoules and vials. A high power centrifuge machine, large water bath, and incubator room.

All glassware is sterilized by dry heat.

Diluting, washing and preserving fluids.

(1) Normal salt solution—0·85 per cent. pure sodium chloride in freshly distilled water. A large quantity should be made up (5000 c.c.) and sterilized in flasks having a siphon attachment.

(2) Citrated salt solution:

Normal salt solution	100·0
Sodium citrate	1·5

(3) Preserving fluid for trypanosomes:

Normal salt solution	90·0
Pure neutral glycerine	10·0
Formalin (Scherings)	0·1

(4) Preserving fluid for serum:

Glycerine	95·0
Phenol	5·0

I. Preparation of Reagents.

A. *The Haemolytic System.*

(*a*) *Red Cells.* A quiet sheep may be bled in the standing position, otherwise it should be placed upon its back in a V-shaped trough and held there by the attendant, an assistant shaving the neck and preparing the site of operation. The operator draws from the jugular vein, under aseptic conditions, 50 c.c. (more or less) of blood into a flask containing glass beads and in which the blood is defibrinated. It is then run through a double layer of fine, sterilized gauze into large centrifuge cups, about 20 c.c. of blood in each, adding three to four times the amount of salt solution. The corpuscles are thrown down by centrifugal force, the upper fluid taken away and replaced with fresh salt solution, and the mixture again centrifuged. Washing in this way is repeated three times, when the red cells are carefully measured and suspended in an equal amount of salt solution, this 50 per cent. stock suspension being stored in the ice chamber until needed.

(*b*) *Haemolytic Serum.* Rabbits have a variable amount of natural haemolytic amboceptor for sheep's corpuscles—0·1 c.c. of fresh rabbit serum will usually haemolyze a like amount of 5 per cent. corpuscle suspension. For test purposes a serum with a much higher haemolytic index is required and to obtain this rabbits are hypersensitized or immunized by repeated injection of sheep's corpuscles until a serum is given showing a haemolytic index of 0·0005.

Not less than six large healthy rabbits should be selected for the immunization, for one or several are apt to die from shock during the process. The rabbits are injected intraperitoneally with a first dose of 2·5 c.c. of the 50 per cent. stock suspension of sheep's corpuscles. Every 4–5 days a further injection is given, each time increasing the dose until, after five or six injections, it has reached 10 c.c. This dose is repeated once or twice. After the sixth or seventh injection 5·0 c.c. of blood is drawn from the heart of each rabbit, using a hypodermic syringe and a fine needle. The operation can easily be performed and does no harm to the animal.

The serum of each rabbit is then heated for one half-hour at 56° C. and the haemolytic index established by titration (*vide* p. 164). It will be found, probably, that in only two or three rabbits out of six can the haemolytic index be raised to the desired degree, namely, 0·0005 or better. From such rabbits as much blood is drawn from the heart as

will not endanger the life of the animal—about 25 c.c. The rabbits are then kept in reserve and can easily be reimmunized as required.

Finally, the serum is separated from the corpuscles and stored in very small ampoules—0·2 c.c. in each ampoule for convenience and economy—in the ice chamber.

When the serum is not to be used immediately it requires neither inactivation nor carbolization, and is, in fact, better without, the index remaining constant or but very slightly lowered even after six months. But unless the serum has been collected under aseptic conditions, rather than risk it spoiling, 1·0 c.c. of the carbolized glycerine preservative is added to 9·0 c.c. of serum before measuring it into the ampoules.

The whole procedure of immunizing rabbits, drawing blood from the heart, separating and bottling serum, can and should be carried out under aseptic conditions.

(*c*) *Complement.* Normal guinea-pig serum in a fresh state furnishes a rich complement. Blood may be drawn from the heart, if desired, but as guinea-pigs are usually plentiful at a laboratory it is simpler to anaesthetize the animal in an ether jar, remove and suspend the guinea-pig over a centrifuge tube of 25 or 30 c.c. capacity, sever the arteries and veins on one side of the neck, and collect all the blood.

Centrifuge immediately, before coagulation takes place. The clear serum is taken off and placed in the ice chamber. Complement is always better used in the fresh state so the guinea-pig should not be bled until just before complement is needed for a titration or a diagnostic test.

B. *Dourine (antibody) combining group.*

(*x*). *Antigen.* A stock dourine antigen is obtained as the result of inoculating a number of white rats with *Trypanosoma equiperdum*, collecting the rat's blood when teeming with trypanosomes, and separating the trypanosomes from blood cells and serum by washing and centrifuging.

The blood of a dourine infected rat is collected in a vessel containing sufficient salt solution to prevent coagulation. Not less than ten large white rats—twenty or twenty-five rats, if a considerable amount of antigen is needed—are inoculated intraperitoneally with the diluted blood, injecting an equal amount, about 0·3 c.c., into each rat. This may be done very conveniently by taking a small sharp-pointed pipette, with rubber tubing and mouthpiece attached, drawing the blood up to a point

marked by a file or pencil, and expelling it into the abdomen, repeating the process with the same pipette for each rat. The object is to have all the rats come down together with a heavy infection. In the ordinary course a white rat dies of dourine between the end of the third and the beginning of the fifth day of infection. When twenty-five rats are inoculated at the same time about fifteen of them show a heavy trypanosome infection at the end of the third day, the remainder within the next 12–24 hours. It is necessary to make a rapid cover-glass examination of the blood of each rat forty-eight hours or so after inoculation and to sort the animals according as they show a light or a heavy infection into two or more groups. The result of the first blood examination will indicate approximately the time for a second examination and upon that the hour for bleeding may be judged. The timing of this operation is important for in the last six or eight hours of infection the trypanosomes multiply enormously, and if the rats are left until well on into this stage a very rich antigen will be furnished. Careful timing, however, is necessary, for it may easily happen that eight or ten rats will all die within one to two hours, if left too long. The bleeding should be carried out as rapidly as possible. The writer's method is simple and effective and may be worth describing in detail:

A running noose is made out of a two-foot length of thin copper wire, doubled over in the middle and twisted to the ends, the ends being passed through the ring formed at the beginning of the twist to form the noose and attached to any convenient fixture over a laboratory wash basin, six inches above an operating board resting across the basin. An ether jar, a flask of citrated salt solution, two sterile covered beakers and a razor complete the outfit.

An assistant passes the rats one at a time into the ether jar and hands them over as required. The animal is held back downwards in the left hand of the operator whose index or middle finger presses on the left front limb of the rat. The noose is slipped over the head and arranged so that the pull stretches the left side of the neck bending the head slightly to the opposite side, backwards and downwards. A beaker half filled with citrated salt solution is placed in position under the neck, the arteries and veins on that side and close to the shoulder then severed with a single sweep of the razor. Usually, the animals bleed better if one avoids severing the trachea. In this way ten rats may be bled in half-an-hour. The volume of blood and citrated salt solution should be about equal or a slight excess of the latter. The mixture is then passed through a double layer of sterile gauze to remove any small

clots and fibrin into narrow centrifuge tubes, 10 mm. diameter and 10 c.c. capacity (when wider tubes are used it is more difficult to separate the trypanosomes and the wastage is greater). Centrifuge not longer than four to five minutes at 1500 revolutions per minute so that the bulk of the corpuscles are thrown down while the trypanosomes remain in suspension. Draw off the cloudy suspension fluid into fresh tubes, then the upper layer of corpuscles—more or less mixed with trypanosomes—into another tube, and the next layer into a second tube, adding citrated salt solution and again centrifuging for 8–10 minutes. Draw off and discard as much of the upper fluid as appears clear and free from trypanosomes. Then collect from each tube into a single tube the upper pure white layer of trypanosomes, in another tube the middle layers slightly soiled with blood, and in a third and fourth tube the lower layers in contact with the blood cells. Add normal salt solution now, not citrate, shake up well and centrifuge again, repeating the washings until all the trypanosomes are obtained in a pure white mass.

Ten rats bled at the right time will furnish 5·0 c.c. of trypanosomes. Twice the volume of the glycerine-formalin preservative is added and the mixture stored in sealed amber ampoules, 1·0 c.c. in each, in a block of ice.

5·0 c.c. of trypanosomes will make 100 c.c. of antigen, sufficient for more than 500 diagnostic tests. The antigen will keep indefinitely if solidified by freezing, and for 6–8 weeks or longer when stored in liquid form, in sealed ampoules, on ice.

(*y*) *Antibody.* In the diagnostic tests the antibody, of course, is or is not present in the suspected test serum. But for purposes of control and titration and to thoroughly understand the combining action of dourine antigen and antibody it is absolutely necessary to have one or more series of known positive or specific dourine horse sera, of which the antibody content can be determined. To obtain this a horse is inoculated with *Trypanosoma equiperdum.* Ten days later and at weekly intervals thereafter, blood is drawn aseptically from the jugular vein, the serum collected and tested for antibody content (*vide* p. 173). A series of specific positive sera are thus obtained, representative of different periods and stages of the disease. Stored in the ice chamber the sera will retain their specific properties for many months, even years, if collected sterile. If not absolutely sterile the serum may be preserved by adding 1·0 c.c. of 5 per cent. carbolized glycerine, or the same amount

of iodized glycerine to 9·0 c.c. serum. At the same time one should collect and store a number of negative control sera under the same conditions.

II. Titration of Reagents.

(1) *Titration of Haemolytic Serum (Amboceptor).*

Prepare the following stock dilutions of serum and corpuscle suspension:

1.	Haemolytic serum (rabbit anti-sheep) . .	0·1 cc.
	Normal salt solution (0·85 per cent.) . .	9·9 ,,
		10·0 ,, ...1 : 100
2.	Complement:	
	Fresh guinea-pig's serum	1·0 ,,
	Normal salt solution	19·0 ,,
		20·0 ,, ...1 : 20
3.	Corpuscle suspension:	
	Washed sheep's corpuscles (50 per cent. stock suspension)	2·0 ,,
	Salt solution	23·0 ,,
		25·0 ,, ...1 : 25

Further dilutions of the haemolytic serum are made as under:

	Salt solution c.c.	Haemolytic serum c.c.			
Tube No. 1	3·0	1·0 (1 : 100)	equals	1 : 400	(0·0025 serum in 1·0 c.c.)
,, 2	5·0	1·0 ,,	,,	1 : 600	(0·0016 ,, ,,)
,, 3	7·0	1·0 ,,	,,	1 : 800	(0·0012 ,, ,,)
,, 4	9·0	1·0 ,,	,,	1 : 1000	(0·001 ,, ,,)
,, 5	0·5	1·0 (1 : 1000)	,,	1 : 1500	(0·00066 ,, ,,)
,, 6	1·0	1·0 ,,	,,	1 : 2000	(0·0005 ,, ,,)
,, 7	2·0	1·0 ,,	,,	1 : 3000	(0·00033 ,, ,,)
,, 8	3·0	1·0 ,,	,,	1 : 4000	(0·00025 ,, ,,)
,, 9	4·0	1·0 ,,	,,	1 : 5000	(0·0002 ,, ,,)

In each tube 1·0 c.c. only of the dilution is held back, the excess amount being discarded, 1·0 c.c. each of complement and red cell suspension added, which with 2·0 c.c. salt solution make a total volume of 5·0 c.c. in each tube.

The complete titration set is then:

	Serum dilutions (as above) c.c.	Salt solution c.c.	Complement 1 : 20 c.c.	Red Cell suspension c.c.	
Tubes 1–9	1·0	2·0	1·0	1·0	Titration set
Tube No. 10	1·0	3·0	—	1·0	Serum control (1 : 100 dil.)
,, 11	—	3·0	1·0	1·0	Complement control
,, 12	—	4·0	—	1·0	Red cell control

Mix well and incubate for two hours at 37° C.

The control set, tubes 10, 11, and 12, must not show any trace of haemolysis.

The *titre* of the haemolytic serum is indicated by the amount present in the *last* tube of the series 1–9 in which dissolution of all the red cells is *complete*, that is, the least amount necessary to dissolve a definite amount of red cells.

For example, if in tubes Nos. 1–6 haemolysis is complete, not quite complete in tube No. 7, and still less in Nos. 8 and 9, then the titre is the amount of serum in tube No. 6, or 1·0 c.c. of a 1 : 2000 dilution, 1 unit being expressed as 0·0005.

A serum with a unit value of between 0·0002–0·0005 is quite satisfactory, but when the value of a single unit exceeds the latter amount the results are not so good.

The relationship and combined action of haemolytic amboceptor and complement should be clearly understood. To do this a number of experimental tests should be undertaken, using in one series only one unit of amboceptor with fractional amounts of complement, in another series two units of amboceptor and lesser complement fractions, four units and so forth, progressively multiplying the number of amboceptor units while further reducing the fractions of complement. It will be found, for instance, that two units of amboceptor require a lesser amount of complement than one unit to completely haemolyse a standard amount of red cells. The lesser the amount of complement that can be safely employed in the practical tests the more delicate becomes the fixation reaction, the equilibrium of the haemolytic system being more easily upset, even by a test serum naturally weak in antibody content and which, if a relatively large complement unit was employed, might be insufficient to give a complete reaction. On the other hand the reduction of complement must not be carried to such an extreme point that any slightly inhibitive property of one of the other reagents would tend to obscure it and give a false fixation.

It is essential that for all subsequent titrations and tests a standard dose of haemolytic amboceptor be fixed and rigidly adhered to. For all practical purposes the use of two units of amboceptor permit of a sufficiently fine gradation of complement, while still allowing a margin of safety. The dose is therefore fixed constantly at two units, to which complement is always titrated as in the next procedure.

(2) *Titration of Complement.*

Prepare (1) a stock dilution of guinea-pig complement and (2) a suspension of sheep's corpuscles, as in the previous titration.

Also (3), an haemolytic serum (amboceptor) dilution, so that 1·0 c.c. of the diluted serum contains two amboceptor units. For example, if the value of one unit is 0·0005, then 0·001 will be that of two units, the dilution being accordingly 1 : 1000.

The titration of complement is of the utmost importance and requires the greatest accuracy, as already indicated. Until one has become familiar with the technique and expert in reading the reactions the titration is best carried out in a double set, the second set having one-half of the amount of each reagent used in the first set, the one serving as a check to the other.

The two sets are arranged as follows:

Titration of Complement.

	First set			Second set		
	Salt solution c.c.	Complement c.c.	Haemol. serum c.c.	Salt solution c.c.	Complement c.c.	Haemol. serum c.c.
Tube No. 1	2·0	0·3 (1 : 20)	1·0	1·0	0·15 (1 : 20)	0·5
„ 2	2·0	0·4	1·0	1·0	0·2	0·5
„ 3	2·0	0·45	1·0	1·0	0·225	0·5
„ 4	2·0	0·5	1·0	1·0	0·25	0·5
„ 5	2·0	0·55	1·0	1·0	0·275	0·5
„ 6	2·0	0·6	1·0	1·0	0·3	0·5
„ 7	2·0	0·65	1·0	1·0	0·325	0·5
„ 8	2·0	0·7	1·0	1·0	0·35	0·5
„ 9	2·0	0·8	1·0	1·0	0·4	0·5
„ 10	2·0	1·0	0·5	1·0	0·5	0·25
„ 11	3·0	—	1·0	—	—	—
„ 12	3·0	1·0	—	2·0	—	—
	Add 1 c.c. red cell suspension to each tube.			Add 0·5 c.c. red cell suspension to each tube.		

Mix well (avoiding undue frothing).

Incubate at 38–39° C.

Agitate the mixtures again by shaking the racks after ten minutes incubation.

Read the reactions one hour later.

Tube No. 10 controls the original haemolytic titration, only one unit of amboceptor being used with an excess of complement. In this tube complete haemolysis should occur.

Tube No. 1 will show only slight or partial hæmolysis; as one descends the series the reaction is seen to be increased, until, usually between Nos. 4 and 7, a tube is reached in which the reaction is absolutely complete. The first tube in the series in which *all* the red cells are completely dissolved indicates the complement titre. If this occurs in tube No. 5, for example, then 0·55 c.c. of a 1 : 20 dilution of complement is the titre, equivalent to 1·0 c.c. of a 2·75 per cent. dilution.

For the antigen titration and final tests the complement is accordingly made up so that 1·0 c.c. of the dilution contains the amount of complement indicated by the above titration.

From now on it is optional whether one employs the relatively large amounts of reagents as given in the first set of complement titration, or the one-half amounts as in the second set. The latter is the more economical, especially when a large number of tests are being performed, and is given personal preference to by the writer as it seems to provide an even more highly sensitive test reaction than when the larger amounts are employed.

(3) *Titration of Antigen.*

Dilute 1·0 c.c. of stock trypanosome antigen with 19·0 c.c. of normal salt solution.

Prepare the complement and haemolytic serum according to their titration values already determined.

Inactivate by heating for half-an-hour at 58° C. in a water bath, 2·0 c.c. of known positive dourine horse serum and 2·0 c.c. of known negative or normal horse serum.

The antigen is then titrated in a double set, the one being with the positive serum, the other with double the amount of negative serum. Thus:

	Positive set				Negative set			
Controls	Salt sol. c.c.	Known *positive* horse serum c.c.	Antigen c.c.	Complement c.c.	Salt sol. c.c.	Known *negative* horse serum c.c.	Antigen c.c.	Complement c.c.
Tube No. 1	1·0	0·1	0·02	0·5	1·0	0·2	0·05	0·5
„ 2	1·0	0·1	0·05	0·5	1·0	0·2	0·1	0·5
„ 3	1·0	0·1	0·1	0·5	1·0	0·2	0·2	0·5
„ 4	1·0	0·1	0·15	0·5	1·0	0·2	0·3	0·5
„ 5	1·0	0·1	0·2	0·5	1·0	0·2	0·4	0·5
„ 6	1·0	0·1	0·25	0·5	1·0	0·2	0·5	0·5
„ 7	1·0	0·1	0·1	0·5	1·0	0·2	0·6	0·5
„ 8	1·0	0·2	—	0·5	1·0	0·2	—	0·5
„ 9	1·0	—	0·25	0·5	1·0	—	0·5	0·5
„ 10	1·5	—	—	0·5	2·0	—	—	—

Mix well and incubate for one hour and ten minutes at 38–39° C.

Mix together equal quantities of haemolytic serum (amboceptor) and red cell suspension, then add 1·0 c.c. of the mixture to each tube.

Shake again and incubate for two hours longer.

It is usually possible to read the antigen titre in $1\frac{1}{2}$ hours and proceed with the final tests; nevertheless, the tubes should be left or replaced in the incubator for the full two hours and then put on one side for further reference and to see if any further action has taken place.

Tube No. 10 is the control for the haemolytic system and must show complete haemolysis. No. 10 in the second set contains only haemolytic amboceptor and red cells and must not show the slightest degree of haemolysis. Nos. 8 control the horse serum, Nos. 9 the antigen, the red cells being haemolysed in all.

The positive set will show more or less complete fixation of complement—no haemolysis, except perhaps in the first and second tubes, the negative set complete haemolysis. When the antigen appears very strong there may be some inhibition in the negative set in the tubes containing the larger amounts of antigen.

The amount of antigen to be selected as the titre for the final tests is that which gives complete fixation with the positive serum while double the quantity in the corresponding tube of the negative set does not prevent or inhibit haemolysis.

III. The Serum to be tested.

Collection of Serum. The chief point aimed at in collecting blood from suspected animals is sterility, especially when the specimens have to be transported over long distances and mailed to the laboratory. Absolute sterility is not essential, nevertheless as near as possible aseptic conditions are to be strongly recommended and the avoidance of adding carbolic acid or any other antiseptic fluid to the sample specimen as a preservative. The blood clot should be well formed and the serum odourless and clear or only slightly tinged with haemoglobin.

The condition of a sample of blood may vary greatly according to the size and shape of the vial or tube containing it, the slowness or rapidity with which blood is run into the vial, the partial or complete filling of the vial, the shaking of the specimen before coagulation has occurred, and in other ways irrespective of aseptic conditions and of abnormal properties of the blood itself. In square or rectangular bottles and in specimen vials without a neck the clot has a tendency

to cling firmly to the sides, the serum being separated with more or less difficulty. In small round bottles, curved into a narrow neck and mouth, for corks, filled with freely flowing blood to within a margin of the narrowest diameter but not touching the cork, and allowed to stand for at least half-an-hour for coagulation, there is usually an abundance of clear serum.

Such bottles, of one ounce capacity, one inch in diameter, three-eighths inch neck and mouth, are very suitable for field work. They must be absolutely clean and free from any trace of soap, alkali or acid. These bottles are distributed from this laboratory after being sterilized in the hot air oven, corked, labelled and well wrapped in sterile paper wrappers. Also, large bore needles attached to three inches of rubber tubing with a small glass nozzle, separately wrapped and sterilized. With this simple apparatus and observing the usual precautions during operation it is an easy matter to draw blood from the jugular vein of a horse, aseptically.

Among the last 6000 samples of blood secured in this manner less than twenty have reached the laboratory in a condition unfit for testing and these few unfit specimens have been ten days or more in transit.

On reaching the laboratory the specimens are briefly examined and where necessary the clots are detached from the sides of the bottles with a sterile wire. They are then left to stand in a cool chamber overnight for the serum to clear. The serum is then drawn off into small test tubes, about 2·0 c.c. in each, and is ready for inactivation.

Inactivation of Serum. Before any specimen of horse serum can be used in the complement fixation test it has first to be inactivated. All animal serum in a very fresh state contains complement in a varying amount. This constituent is readily destroyed by heating the serum to 55–56° C. for one half hour. No complement other than that employed in the haemolytic system may take part in the reaction. As a matter of fact horse complement very rapidly becomes inert and in specimens several days old is a practically negligible quantity. However, in normal horse serum there arise several other factors which, unless destroyed or rendered inactive, are able to act upon complement and antigen and disturb an haemolytic system. All untreated horse, donkey and mule sera possess enzymotic and proteolytic properties, potentially at least, and becoming active in sera a day or two old. They act upon most preparations of antigen, especially upon macerated

organs, such as the liver and spleen, and are all more or less anticomplementary, more so in the presence of antigen than without it. Such action, of course, is non-specific and must be eliminated, otherwise it would be difficult or impossible to distinguish a specific from a non-specific reaction. Fortunately it can be eliminated, and the equilibrium of the serum fixed, by a proper and sufficient inactivation. *It is more resistant to heat than is complement and is not wholly destroyed at* 56° C. This is an important point, and one that appears to have been overlooked. I cannot help thinking that it is the explanation and the source of error of many of the apparent failures or discrepancies, especially that of non-specific fixation, which some serologists experience. A reference to the literature on complement fixation methods shows a remarkable lack of uniformity in respect to the degree of heat and the length of time for the inactivation of suspected sera—fifteen to thirty minutes at degrees varying between 50 and 58° C.

A few experiments with sets of ten or twenty different horse, mule and donkey sera, each set being heated for thirty minutes at different degrees between 50 and 62 C. and then tested in the haemolytic system, with and without antigen, will show the importance and necessity of a very careful inactivation and the temperature required (*vide*, p. 171).

Method of inactivation recommended. A water bath, sufficiently large to hold 200 small test tubes, is heated to 60° C. The tubes, containing 2·0 c.c. serum in each (numbered for identification in waterproof india ink, labels being apt to become detached), are placed within the inner tank which is to contain sufficient water to mount to the level of the serum or to about half the height of the tubes. The cover of the tank should have two perforations for thermometer tubes which are inserted into control tubes within the tank, enabling the temperature to be read without removing the cover. Another thermometer passes directly into the outer tank. For the first few minutes the temperature will rapidly fall; it is brought up to 59° again—taking about ten minutes—and maintained at that point for a full half hour, for horse serum, and to 62° for one half hour for donkey or mule serum.

There is no danger of destroying the specific antibodies of dourine sera by heating to the points given. Dourine sera can, in fact, be heated up to 65°, or to the point of coagulation, and still retain an active antibody content to give the test reaction, but the anticomplementary and non-specific factors in horse sera are wholly destroyed at 59°, and in donkey and mule sera at 62°.

To control the inactivation, with each batch of suspected sera several known positive dourine sera as well as known (anticomplementary and non-specific) negative sera are included and all tested together in the final diagnostic test.

Experiment showing the degree of inactivation of suspected serum necessary for specific reactions.

Dose of serum, 0·2 c.c., unheated and heated at different degrees of temperature and tested with trypanosome antigen as in the diagnostic test.

Normal healthy horses	Unheated serum	Serum heated for one half hour at degrees Centigrade 50	54	56	58	60	62	64—65
No. 1	++++	++++	++	+	−	−	−	−
,, 2	++++	++++	++	+	−	−	−	−
,, 3	++++	++++	++	+	−	−	−	−
,, 4	++++	++++	+++	++	−	−	−	−
,, 5	++++	+++	+	−	−	−	−	−
,, 6	++++	++++	++	(?)	−	−	−	−
,, 7	++++	++++	+++	++	−	−	−	−
,, 8	++++	+++	+	(?)	−	−	−	−
,, 9	++++	++++	++	(?)	−	−	−	−
,, 10	++++	+++	++	+	−	−	−	−

Inhibition. Non-specific.

Dourine horses								
No. 1 (1st year of disease)	++++	++++	++++	++++				
,, 2 (,, ,,)	++++	++++	++++	++++				
,, 3 (,, ,,)	++++	++++	++++	++++				
,, 4 (,, ,,)	++++	++++	++++	++++				
,, 5 (,, ,,)	++++	++++	++++	++++				
,, 7 (,, ,,)	++++	++++	++++	+++ +				
,, 7 (2nd ,, ,,)	++++	++++	++++	++++				
,, 8 (3rd ,, ,,)	++++	++++	++++	++++				
,, 9 (4th ,, ,,)	++++	++++	++++	++++				
,, 10 (5th ,, ,,)	++++	++++	++++	++++				

Specific complement-fixation.

Normal mule	1	++++	++++	++++	+++	+	−	−	−
,,	2	++++	++++	++++	++++	++	+	−	−
Normal donkey	1	++++	++++	++++	+++	++	+	−	−
,,	2	++++	++++	++++	++	+	−	−	−

Inhibition. Non-specific.

++++ See page 173 for the meaning of these reaction expressions.

Note. In the non-specific inhibition reactions the red cells are loosely sedimented. In the specific complement fixation reactions the red cells are precipitated in a mass or agglutinated in clumps. When

the sera are tested *without antigen*, as in the serum controls, the dourine sera, of course, give no specific reactions, but the inhibition reactions are given by normal and dourine sera alike when insufficiently inactivated, though to a lesser degree than when antigen is present.

Conclusion. Suspected horse serum must be heated to at least 58° C. (59–60° for safety) and mule or donkey serum to 62° C., to eliminate non-specific reactions.

The Antibody content of Dourine Sera, and the dose of suspected Serum necessary for a Diagnostic Test.

The maximum dose of horse serum used in a diagnostic fixation test is 0·2 c.c. This amount is not exceeded for fear of any disturbance to the haemolytic system by the non-specific reactions which larger doses are apt to cause. Double the amount can actually be used with perfect safety provided the serum is correctly inactivated. But it is unnecessary to use more than 0·2 c.c., for that amount of serum of a dourine horse will contain in the case of a serum very weak in antibody content at least one unit, and in the case of a serum strong in antibody ten, twenty, forty or more units—and one unit of antibody is sufficient to give a positive reaction with the fixation test.

That this is so may be determined by taking a series of sera collected from animals in active and in latent phases of the disease and titrating out each serum for antibody content.

The experiment is carried out as follows:

The sera are first inactivated by heating for one half hour at 59° C. Three stock tubes are then taken for each serum, (1) containing the pure serum, (2) a dilution of 1 : 10, and (3) a dilution of 1 : 100, these dilutions permitting of the accurate measurement of the smaller doses.

Twelve tubes are now arranged for each serum to be tested—the first and last to contain 0·2 c.c. of undiluted serum, the largest amount used in the test, the last tube being the serum control without antigen, the intervening tubes to contain gradually decreasing doses of serum. Enough salt solution is then added to make up to 1·0 c.c. in each tube, then the antigen and complement in amounts previously determined by careful titration, and finally, after incubation for seventy minutes, haemolytic serum and red cells—as in a diagnostic test.

An experiment of this kind is given below, the titres of seven sera from different horses in different phases of the disease being determined.

Experiment for determining the Antibody content of Dourine Sera by the Complement Fixation Method.

Dose of inactivated dourine serum c.c.	Complement fixation reactions with dourine serum No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7
0·2 (undiluted,	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	**+ + + +**
0·15 standard	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +
0·1 doses)	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ +
0·075 (0·75 of 1 : 10)	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+
0·05 (0·5 „ „)	+ + + +	+ + + +	+ + + +	+ + + +	**+ + + +**	+ + + +	−
0·025 (0·25 „ „)	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	**+ + + +**	−
0·01 (0·1 „ „)	+ + + +	+ + + +	+ + + +	**+ + + +**	+ +	−	−
0·0075 (0·75 „ 1 : 100)	+ + + +	+ + + +	**+ + + +**	+ + +	+	−	−
0·005 (0·5 „ „)	**+ + + +**	**+ + + +**	+ +	+	−	−	−
0·0025 (0·25 „ „)	+ +	+ +	−	−	−	−	−
0·001 (0·1 „ „)	+	−	−	−	−	−	−
0·2 (serum control, without antigen)	−	−	−	−	−	−	−
Indicated value of one antibody unit	0·005	0·005	0·0075	0·01	0·05	0·025	0·2
Number of antibody units in 0·2 c.c. of dourine serum — maximum dose.	40	40	26½	20	4	8	1

+ + + + means complete fixation of complement—absolutely no trace of haemolysis. Red cells more or less clumped. A very strong positive reaction.

+ + + is also a strong positive reaction, with just a faint trace or tinge of haemolysis.

+ + is a rather weak positive, indicating partial fixation—about one half the red cells haemolysed.

+, a very weak or faint positive—slight fixation, with more than one half the red cells haemolysed.

−, a negative reaction. Complete haemolysis of red cells.

The smallest dose of serum which combines with antigen to cause complete fixation (+ + + +) indicates the value of one antibody unit, and from this may be calculated the number of units in the standard or maximum dose and the value of a dourine serum in antibody content.

Such values are more relative than absolute, for the titre of a dourine serum may be somewhat higher or lower according to the amount of dourine antigen present and the fineness with which the haemolytic system has been adjusted—just as the titre of the haemolytic serum itself is correlative to the amount of complement and red cells (antigen).

The seven sera, Nos. 1–7, used in this experiment are taken from dourine eontrol horses in the first, seeond, third, fourth, fifth, sixth and seventh year of the disease, respeetively. No. 1 from a mare showing elinieal symptoms; No. 2 from a stallion showing oeeasional symptoms; Nos. 3 and 4 from mares very rarely showing symptoms and progressing towards reeovery; Nos. 5, 6 and 7 from mares that have not shown any symptoms for three, four and five years respectively, and whieh have made eomplete recovery, been bred to a healthy stallion eaeh year—without transmitting infeetion—and raising healthy offspring.

In addition to the above, among our experimental horses that have recovered from dourine, there are two mares that give a positive (+ + + +) reaetion with 0·2 c.e. serum after six years, and one mare a positive (+ + +) after seven years. On the other hand, there are three mares that have entirely eeased to reaet, even with twiee or three times the amount of serum, after six to seven years of reeovery, although they reaeted positively up to the fifth year.

Conclusion. 0·2 e.e. of horse serum from a dourine infected animal eontains up to forty units of specifie antibody. In the case of horses that have eompletely reeovered from dourine and whieh are no longer able to transmit the disease, one or several units of antibody are present in the same amount of serum up to the fifth year of reeovery. After that period thay may eease to reaet—indieating that not only was an absolute reeovery made but that the immunity was lost in about five years (proof of whieh has been given by inoeulation experiments with *T. equiperdum* on reeovered horses).

For diagnostie tests it is suffieient to use three doses of serum, namely, 0·2, 0·15 and 0·1 c.c.

The first appearance of a positive serum reaction in dourine infections.

Having fixed upon a standard dosage of suspected serum, it is now necessary to know the ineubation period of dourine and when a first positive serum reaetion may be expeeted, for otherwise a negative reaetion would be valueless or even misleading.

In this eonneetion there follow the reeords of some experiments:

Experiment for determining the length of time between dourine infection and the first appearance of a positive serum reaction.

A healthy filly, $2\frac{1}{2}$ years old, was infected with dourine by smearing over the vaginal mucosa a few drops of blood containing *Trypanosoma equiperdum.*

Serum was collected from this young mare before infection and daily up to the fifteenth day after infection, and tested by the complement fixation method, with trypanosome antigen. The results were as follows:

Dose of serum c.c.	Before infection	Days after infection					
		1 to 10	11	12	13	14	15
0·2	–	–	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
0·15	–	–	+ +	+ + + +	+ + + +	+ + + +	+ + + +
0·1	–	–	+	+ + +	+ + + +	+ + + +	+ + + +
0·05	–	–	–	+ +	+ +	+ + + +	+ + + +
0·01	–	–	–	–	–	+	+ +
0·005	–	–	–	–	–	–	+

Thus, the first appearance of a positive serum reaction was eleven days after infection.

In three earlier experiments of this kind, but in which serum was not collected for testing until the twentieth day after infection, the reaction in each case was strongly positive.

The incubation period of dourine in the light of the complement fixation test is indicated, by the above experiments, as not less than eleven days and not over twenty days. However, the strain of dourine used in these experiments was of high virulence; when horses become infected with strains of low virulence—and there is much variation in dourine strains—the incubation period is probably prolonged.

A negative reaction should not be taken as final or conclusive when the interval between exposure to infection and the collection of test serum is less than two months.

IV. The Diagnostic Test.

Two methods of procedure are here recommended:

(1) When only one or several tests are to be made.

(2) For daily routine testing or when 50, 100, or more tests are to be made at one time.

In either case a necessary preliminary is the titration of complement (*vide*, p. 166). This established, sufficient complement dilution is made up—0·5 c.c. of the dilution to contain the smallest amount indicated by titration—to do for the titration of antigen and for as many serum tests and controls as are to be made. It is advisable to make up an excess of complement rather than have a deficit, so as to use one stock uniform dilution throughout and avoid having to make up fresh stocks during the testing.

The trypanosome antigen is then titrated against a known positive dourine serum and a known negative serum (*vide*, p. 167).

First method of procedure—for one or several tests.

Four tubes and one pipette of 1·0 c.c. capacity, graduated 1–100, are needed for each serum to be tested. 1·0 c.c. salt solution is measured into each tube. In each set of four tubes 0·2, 0·15, 0·1 and 0·2 c.c. of the inactivated test serum is added. Antigen in the amount already decided by titration is now added to the first three tubes in each set, omitting it from the fourth tube which serves as a serum control. Complement, 0·5 c.c. of the dilution required, is then added to all tubes. Sets of positive and negative sera are included with the above, and, in addition, controls for the various reagents. For the reagent controls five tubes are needed: (1) Antigen control, omitting the test serum, (2) haemolytic control, omitting serum and antigen, (3) haemolytic serum control, omitting test serum, antigen and complement, (4) complement control, omitting test serum, haemolytic serum and antigen, (5) red cells control, containing only red cells and salt solution. The controls are made up to a uniform volume of 2·5 c.c. by adding salt solution as required.

When the test serum, antigen and complement have been mixed together, the tubes are incubated at 38–39° C. for 70 minutes.

Equal quantities of the haemolytic serum dilution and the red cell suspension (4 per cent.) are mixed together and 1·0 c.c. of the mixture added to every tube excepting the last two controls, Nos. 4 and 5, to which 0·5 c.c. red cells only are added.

The tubes are again shaken and incubated for another two hours when the reactions may be read, a second reading being made the following morning, about twelve hours later, the racks being left at a cool room temperature meanwhile.

The above procedure is indicated in the following table:

Table showing method of procedure for a diagnostic fixation test.

	Tube No.	Salt solution c.c.	Test serum c.c.	Antigen c.c.	Complement c.c.	Mixture of haemolytic serum and red cells c.c.	Fixation	Reaction		
Diagnostic set for each suspected serum	1.	1·0	0·2	0·2	0·5	1·0	+ + + +	*Strong positive.*	+ + +	*Weak positive*
	2.	1·0	0·15	0·2	0·5	1·0	+ + + +		+ +	
	3.	1·0	0·1	0·2	0·5	1·0	+ + + +		+	
Serum control	4.	1·0	0·2	—	0·5	1·0	–	(haemolysis)	–	(haemolysis)
Test control with known positive serum	1.	1·0	0·2	0·2	0·5	1·0	+ + + +	Complete fixation.		
	2.	1·0	0·1	0·2	0·5	1·0	+ + + +	,,		,,
Serum control	3.	1·0	0·2	—	0·5	1·0	–	Complete haemolysis.		
Test control with known negative serum	1.	1·0	0·2	0·2	0·5	1·0	–	Complete haemolysis.		
	2.	1·0	0·2	—	0·5	1·0	–	,,		,,
Reagent controls:										
(*a*) Antigen	1.	1·0	—	0·2	0·5	1·0	–	Complete haemolysis.		
(*b*) Haemolytic system	2.	1·0	—	—	0·5	1·0	–	,,		,,
(*c*) Haemolytic serum	3.	1·5	—	—	—	1·0	..	No haemolysis.		
						Red cells only.				
(*d*) Complement	4.	1·5	—	—	0·5	0·5	..	,,		,,
(*e*) Red cells	5.	2·0	—	—	—	0·5	..	,,		,,

70 minutes at 38–39° C.

2 hours at 38–39° C. First reading of reactions.

12 hours at cool room temperature. Second reading of reactions.

Second method of procedure—for daily routine testing or when 50, 100, or more tests are to be made.

This is only a slight modifieation of the first method of procedure to allow of more rapid and less laborious work in testing large numbers of suspeeted sera.

Two test series are made, the first series, in whieh only one tube for eaeh serum is used (instead of four tubes as before), eontaining the maximum dose, 0·2 e.c., and antigen, eliminating all negative sera and at the same time indieating the positive sera. These latter are again tested on the following day, using the four tubes—the three standard doses of serum and serum eontrol—as in proeedure No. 1, ineluding them with the next lot of sera to undergo the first test in which the single tube is used.

If a negative serum does not give a fixation reaction with 0·2 e.c. serum it eertainly will not with the lesser doses, and as a serum eontrol is only needed in the case of a serum whieh fixes complement, the single tube is obviously all that is required to determine a negative serum. Further, the sera with which eomplement fixation takes plaee in the one series serve as additional controls when ineluded with and fully tested out in the seeond series—one day's work thus eheeking the other, continuously.

In routine testing at this laboratory, when large numbers of sera are being dealt with, it is the praetiee to make a repeat test with eaeh serum negative at the first test and to arrange the work and the different series so that each day's tests inelude: (*a*) a series not before tested (one tube for eaeh serum); (*b*) the sera tested the day before with negative result (one tube for eaeh serum); (*c*) the sera tested the day before with fixation reaetions (four tubes for eaeh serum); and, in addition, the usual series of known positive dourine sera, negative eontrols and reagent eontrols.

All suspected sera are thus tested twiee over so that if any error or omission in the teehnique has been made it will surely be indieated.

Interpretation of the reaction. Fixation of the eomplement, not in itself visible in the test tube, is indieated by the prevention of haemolysis of the red eells and eonstitutes a positive reaction, on whieh a diagnosis of dourine is given.

When no eomplement is fixed the red cells are completely haemolysed and the reaetion is then said to be negative.

The prevention or inhibition of haemolysis may be complete, partial or slight—according to the richness of the serum in specific antibodies. However, with the standard doses of serum, in the great majority of cases, the reaction is either clearly positive or clearly negative. Occasionally, complement fixation complete with 0·2 c.c. serum, partial with 0·15 c.c. and slight with 0·1 c.c. may be given. This is a positive reaction and indicates that the serum is weak in antibodies, only one unit being present in 0·2 c.c. serum.

Partial fixation with 0·2 serum and complete haemolysis with 0·1 serum is a rare reaction and of a questionable nature. In the serum controls, without antigen, haemolysis should always be complete. Very rarely indeed it happens that haemolysis in the serum controls is not complete, the mixture having a cloudy or opaque appearance and some of the red cells remaining unhaemolysed. This may be the result of insufficient inactivation or of changes in the serum due to certain bacterial growths. When such questionable reactions are given a fresh specimen of serum is asked for and a retest made.

General Remarks.

The successful practice of the complement fixation test depends mainly upon the preparation and use of powerful reagents, their specificity and the accurate determination of their relative values, the fixing of standard doses wherever possible, and a constant, uniform technique and method of procedure.

Close familiarity with the activity of the reagents is essential for the best results.

Stock reagents should be prepared in quantities calculated to meet all requirements for as long a time as the activity of the reagents remains practically constant. Thus: sufficient haemolytic serum for six months' work; antigen to suffice for one month's work; fresh red cell suspension once a week; fresh complement daily or on alternate days, or as needed. It is advisable to use the blood of two sheep for sensitizing rabbits and to use the red cells of the same sheep for the haemolytic system.

The following points of extreme importance will bear repetition:

(1) The amount of red cells in suspension must be very accurately measured and the standard amount never varied.

(2) The use of the least possible amount of complement which with two units of haemolytic serum causes complete haemolysis of red cells.

(3) The use of twice the amount of antigen which with a dourine antibody unit is necessary to fix the complement, provided the same amount of antigen alone has no inhibitory action.

(4) Careful control of the inactivation of suspected sera by known positive and known negative sera.

(5) Control of the diagnostic tests by a series of known positive sera, each having an antibody unit of different value, high to low.

Discussion.

The reliability of the complement fixation test as a certain and specific means of diagnosis has been questioned, not, I think, very seriously or on strictly scientific grounds, but more in respect to its practical application and on an unwarranted supposition that it is still very imperfectly understood, that the technique and method of procedure is so intricate and laborious, that the reactions themselves are subject to and have to be guarded against so many possible disturbing influences that the adoption of such a method of diagnosis is attended with considerable risk.

Can the test be practically applied? Yes, without doubt, and with as much ease as a mallein or tuberculin test is applied. In the one case blood is collected in the field and sent in for a laboratory test, in the other the reagents are prepared in the laboratory and sent out for a field test. Further, as many retests can be made by the complement fixation method as desired, for no toxins or immunizing substances are injected into the suspected animal to interfere with subsequent diagnostic tests. This test is no longer a new departure in veterinary diagnoses; it is successfully applied in glanders, contagious abortion and in other specific diseases and is yearly coming into more general use.

Are the test reactions and the different factors concerned in them imperfectly understood? Such a view is not held by serologists and can only be retained by those who have not the opportunity of closely studying the subject and becoming familiar with the finer points of it. Any attempt to apply the test by one who has not thoroughly mastered the technique and gained complete control of the reagents would, of course, be dangerous. But the complement fixation reaction furnishes the most perfect, biological, diagnostic test yet devised, one in which all adverse or disturbing factors can be eliminated and in which a clear knowledge of the properties and mode of action of the reagents has

been ascertained,—far more so, in fact, than that of a mallein or tuberculin reaction which, in application and interpretation, is crude in comparison. The very delicacy of the fixation reaction and the strict laws and conditions governing it, add to the exactness, value and reliability of the test.

Is the technique too intricate and laborious? Not more so than many other necessary and accepted laboratory methods, and this is essentially a laboratory test.

Is it necessary to use a pure suspension of trypanosomes as antigen? By the employment of a pure suspension of dourine trypanosomes as antigen non-specific and false or misleading reactions are avoided. Many other ways of preparing antigen for the dourine test have been tried by different investigators but, with one exception, with little success. Mohler and Eichhorn recommend a spleen preparation of a rat dead from surra. I have used the spleens of rats dead from dourine in several thousand tests and with very good results, but, on the whole, such preparations are inferior to the trypanosome suspension and possess a number of disadvantages. Spleen preparations are often troublesome on account of a more or less anticomplementary action or owing to a weakness in specific antigenic property. They are very unstable and of inconstant value and give rise to many borderline or questionable reactions which can be eliminated or definitely decided by the trypanosome antigen. In comparative titrations of dourine sera with the two forms of antigen I have found that approximately one-tenth of the amount of serum necessary for a positive reaction with spleen antigen suffices for a clear positive reaction with trypanosome antigen. Very weak positive reactions with the former become clearly and strongly positive with the latter, which, therefore, should always be given the preference. The trypanosome suspension has also the great advantage of retaining a constant value for several weeks at least, for six to eight weeks if carefully prepared, and thus allows of the keeping of a uniform stock antigen.

What is the percentage of positive reactors in dourine outbreaks? This of course varies according to the length of time the disease has been in existence in a stud or range herd before being checked by preventive measures. In the most extensive oubreak that we have had to deal with 456 positive reactors were found in a total of 2000 animals tested; nearly 23 per cent. In an outbreak on an Indian Reservation, 127 animals gave positive reactions out of 1464 tested, or less than 9 per cent. Usually it is between 15 and 20 per cent. Our experience

indicates that 100 per cent. of dourine infected animals, whether in active or latent stages of disease, give positive serum reactions, provided that an interval of two to three months has been allowed for an incubation period in the more or less resistant animals, less than one month being sufficient in most cases.

How does the value of the dourine test compare with the Wassermann test for syphilis? The old name of horse syphilis still clings to dourine infections, especially among stock owners and the general public, and comparisons have been made both in regard to the nature of the disease and the diagnostic tests, tending to lead to mistaken conclusions.

The reaction in dourine by the method recommended in this paper is a specific one. A positive reaction in other diseases or with animals in which dourine infection could be excluded, remains unknown to us, while in every authentic case of dourine the reaction is invariably positive. In my whole experience there is only one case in dispute—a negative serum reaction being given where a symptomatic diagnosis of dourine was made. However, the symptomatic diagnosis may have been at fault; unfortunately, the animal was destroyed before any proof or disproof of dourine infection was forthcoming.

The very few cases on record where a negative dourine reaction at a first test was followed by a positive reaction at a second or later test can be accounted for by infection taking place only a few days before the serum was first collected, or by continued exposure to infection between the first and later test.

In syphilis, on the other hand, negative reactions are of more value for prognosis than for diagnosis. A positive reaction may become negative after a short course of treatment returning again to positive if a cure has not been effected. Further, it is admitted that a negative reaction is frequently given in primary syphilis and again at times in latent and tertiary syphilis. A source of error, operating in the negative direction, is, as Noguchi has pointed out, in that human serum contains a variable amount of natural anti-sheep amboceptor, which in some cases may be sufficient to hide a positive reaction. Horse serum does not contain anti-sheep amboceptor, as I have found by many experiments, so that the anti-sheep haemolytic system can be used in horse serum tests with perfect reliance.

A positive Wassermann reaction may be given in several diseases in which syphilitic infection can be excluded, in leprosy, scarlatina, certain forms of tuberculosis and carcinoma. The Wassermann reaction is not specific. Owing to the great difficulty of obtaining a

pure syphilitic antigen, the extract of a syphilitic liver was first used in Wassermann's original method. But, later on, it was found that non-specific extract of normal liver and other organs answered equally well, and such are now commonly used. The reaction in syphilis is not accordingly a true and specific antigen-antibody combination and is dependent upon more or less gross changes in the serum of syphilitic patients. It is not to be compared, therefore, and is greatly inferior to our test method for dourine either in delicacy, specificity or trustworthiness.

In conclusion, I venture to express absolute confidence in the complement fixation test for dourine as it is now presented, and to claim that apparent failures or discrepancies are due, not to the method itself, but to faulty technique on the part of the operators or of the collectors of the test serum.

MULTIPLICATION-FORMS OF *TRYPANOSOMA LEWISI* IN THE BODY OF THE RAT.

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(With Plates X and XI.)

During an investigation extending over several years of the forms of *Trypanosoma lewisi*, as met with in naturally infected *Mus decumanus*, I have met with many phases which I have not seen described.

My material consisted of dead rats, killed in the immediate neighbourhood of Bournemouth, and brought in to me during the last ten years. As many of the rats had been killed some hours before I could examine them, it was not always possible to obtain blood from the peripheral vessels, but films were made from these when practicable, and from the heart, lungs, and other organs. These were fixed whilst wet with the vapour of osmic acid or iodine and stained with Giemsa.

Coiled up Trypanosomes. In a very small percentage of these naturally infected rats I met with trypanosomes coiled up more or less in the form of a ball. Even when present they were only found in smears from the lung and then in small numbers. They were always associated with the presence of fairly numerous *T. lewisi* in their "ordinary" or "adult" form, that is trypanosomes of the same size and presumably the same stage of development.

Occasionally recurved trypanosomes were encountered, in which the body had doubled on itself, or even pear-shaped trypanosomes. More generally the parasite had rolled itself into a round or oval-shaped body with the flagellum coiled up closely. Sometimes no definite flagellum could be made out, and an apparent *Leishmania*-like structure was seen enclosing a nucleus and a centrosome, but I think even in these cases a flagellum was present, forming the boundary of the body.

The photomicrograph, Pl. X, figs. 1 to 4 and 6 to 8 (× 1500 diameters) shows the general appearance of these bodies.

Figs. 1 to 12 were found in smears of the lung of a small rat (*A*), killed 10. xii. 1913.

Fig. 14 (× 2000), was from lung smear of Rat *B*, 20. viii. 1914, and shows very clearly the size of the larger of these bodies compared with an ordinary trypanosome.

Trypanosomes do not always when coiled up assume a round form, one not infrequently meets with structures resembling a figure of ∞, or even a narrow elongated body.

Figs. 9 to 12 show structures found in lung smears of Rat *A*, as to the nature of which I am in doubt.

Fig. 9 is almost certainly a coiled up trypanosome in which a nucleus and a centrosome are seen. Figs. 10 and 12 are probably of the same nature, whilst Fig. 11 may be a diatom. I have certainly met with definite diatoms in the lung smears of rats.

Pl. XI, figs. 18 and 19 (× 2000) were found in the lung of a Rat *C*, 1914. They were associated with the presence of a few coiled up trypanosomes. They are irregularly oval bodies, the outer part of which seems to be a dense capsule which stains a deep red colour with Giemsa, and contains a faintly blue stained structure bent upon itself. This internal body contains evidences of a nucleus or nuclei and a centrosome, especially Fig. 18. I think there is little doubt that this represents one or two trypanosomes encysted. I thought at first sight that they were a trypanosome coiled up in a red blood corpuscle, but the surrounding part is stained very unlike the colour which the haemoglobin of the red cell takes on. I failed to find any more of these bodies in any of the films.

Minute Trypanosomes. In the lung smear of Rat *A*, in addition to the coiled up trypanosomes and the diatom-like bodies, I found a small group of four minute but distinct trypanosomes, each of which had a nucleus and a centrosome, but no flagella could be detected. These are shown in Fig. 5 (× 1500) and Fig. 13 (× 2000). I searched this film very thoroughly but could find no other example, nor could I detect any of Carini's Pulmonary Cysts.

What is the nature and origin of this little group? They are not at all like the merozoites of the Pneumocysts of Carini, and no examples of these bodies in any of their stages were found in this specimen. My impression is that they originated from a *Leishmania* sphere, as is shown in Fig. 16, although I could find no evidence of this in the preparation.

Pulmonary Cysts in the Rat. I have described these more fully

elsewhere (1914). These bodies, first described by Carini, are round or oval, rarely pear-shaped, and stain a pale blue colour with Giemsa. They vary in size but on an average measure 5 to 6 μ in diameter. Each cyst contains eight small bodies which stain a deep blue colour and have a round or oval nucleus. In addition to the enclosed merozoites a number of similar bodies were seen free or enclosed in a bluish matrix.

Carini regarded them as forms of schizogony of *T. lewisi* and stated that they were derived from trypanosomes which had become encysted. Delanoe (1912) concludes that they represent a new parasite of rats, which has nothing to do with *T. lewisi* and terms them *Pneumocystis carinii*, and regards them as belonging to the Coccidia.

Figs. 20 and 21 represent these bodies which were found in the lung smear of an old rat killed 23. VIII. 1912, in which no *T. lewisi* were found, whilst those shown in Fig. 22 are from the lung smear of Rat *E*, 1914, which showed numerous ordinary trypanosomes. They are depicted here merely as a contrast to the group of four trypanosomes, Figs. 5 and 13, and not as evidence of their being a stage in the development of *T. lewisi*.

Multiplication Cysts of T. lewisi. In a film made from the heart blood of the small Rat *A*, 10. XII. 1913, I found a very interesting and to me entirely new type of body which is represented by Fig. 15. This rat was a small not fully developed specimen in which fairly numerous ordinary adult trypanosomes were present in the peripheral and heart blood, and in which coiled up trypanosomes and the four minute trypanosomes were found in the blood of the lung.

This structure was nearly round and measured 14 by 15 μ in diameter. The protoplasm stained a faintly blue colour with Giemsa and was of a somewhat alveolar nature. Contained within this sphere were a number of small round bodies stained a chromatin red colour, which measured about 1 μ in diameter, whilst nearly applied to each was another smaller granule staining a little deeper in colour, sometimes round, sometimes rod-shaped.

A careful search of this preparation revealed a second similar body, Fig. 16 ($\times$ 2000), which stained in the same way, and contained the same red bodies, but different in being very vacuolated. This measured about 18 μ in diameter.

Although I searched films made from the blood of the heart and lungs of several other rats I only once again met with a similar structure and that (see Fig. 17) was found in the lung smear of a rat of average

size killed 20. viii. 1914. Trypanosomes of adult size were moderately numerous in the blood and coiled up trypanosomes were also present in the lung.

These three spheres therefore were met with only in two rats, in one case in the blood from the heart and in the other in the lung blood, but in both animals coiled up trypanosomes were found in the smears made from the lung.

As to the nature of these bodies, they seem to be small *Leishmania*-like structures consisting as they do of a number of small nuclei each with a centrosome, and apparently associated with the presence of coiled up trypanosomes. It seems to me highly probable that multiplication of *T. lewisi* does occur in the lungs and that possibly the trypanosomes become coiled up, lose their flagellum, and the nucleus and centrosome undergo repeated division until a cyst or sphere of multiplication is produced.

Apparently I have found in the lung and heart blood of naturally infected rats many of the stages which Minchin and Thomson (1915) have described and illustrated in their monumental work on the stages of *T. lewisi* in the rat flea. Thus I have seen the recurved, the pear-shaped, the coiled up trypanosomes and probably spheres, "the final stage of intracellular multiplication."

Prof. Minchin, to whom I have sent photomicrographs, but who has not actually seen my preparations yet, says: "They establish definitely, as it seems to me, what has been asserted and denied again, namely that *T. lewisi* has a stage in the lung," and "there is no doubt that your photos show true *T. lewisi* and not Pneumocysts."

Multiplication of T. lewisi. It is I believe very exceptional to find any but the ordinary fully developed *T. lewisi* in the wild naturally infected rat. Laveran and Mesnil (1912) state: "Chez la plupart des rats d'égouts infectés naturellement et, en general, depuis assez longtemps, on chercherait en vain des formes de multiplication."

In the very large number of rats whose blood I have examined I had never succeeded in finding the multiplication stages. Thinking the matter over I came to the conclusion that I must limit my attention to quite young rats, and at last I was fortunate enough to find a small young rat (18. ix. 1914) in which all the stages were evident.

Trypanosomes varying greatly in size, forms dividing by equal and unequal binary fission and trypanosomes with very long posterior end were seen.

In addition to these were the following types: Fig. 23, irregular

mass with three nuclei, three centrosomes and three flagella; Fig. 25, irregular mass with four nuclei, four centrosomes and four flagella.

The remaining figures show crithidial forms many of which are dividing, whilst Fig. 31 represents a very small trypanosome. Figs. 23 to 34 were from the heart or lung blood.

I examined very carefully numerous smears from the lung of this rat but I could find no coiled up trypanosomes or multiplication cysts such as I have previously described.

Note.

Since the above was written I have found, after very prolonged examination, another group of tiny trypanosomes in the film made from Rat *A*, and also one group in Rat *C* resembling precisely Figs. 5 and 13.

I have since June 1st examined 86 more rats, by far the greater number of which were quite young specimens. Of these 55 came from the town and only two were infected, whilst in 30 from the country 12 contained trypanosomes. In three of the latter I found *T. lewisi* in all stages of division in the blood of the peripheral vessels as well as in many of the internal organs. These were small rats measuring about five to six inches in length. In two of these coiled up trypanosomes were found sparsely in the lung, and in one measuring only five inches numerous examples were seen in the liver. In a fresh cover-glass preparation made from the liver the coiled up trypanosomes appeared to be in a perfectly colourless cyst, about the size or a little larger than a red corpuscle, and the enclosed parasite was twisting and turning about very actively in its apparent envelope, but in no case did I see one escape or unroll.

In the heart blood of a rat about seven inches long I found that many of the trypanosomes were arranged in rosettes of auto-agglutinated parasites, a condition I have once before noticed.

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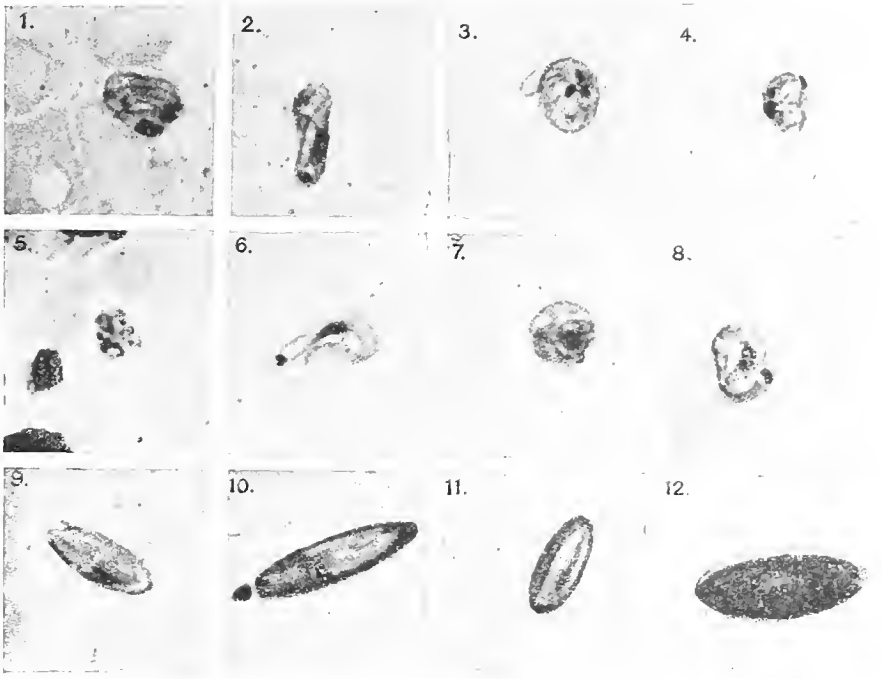
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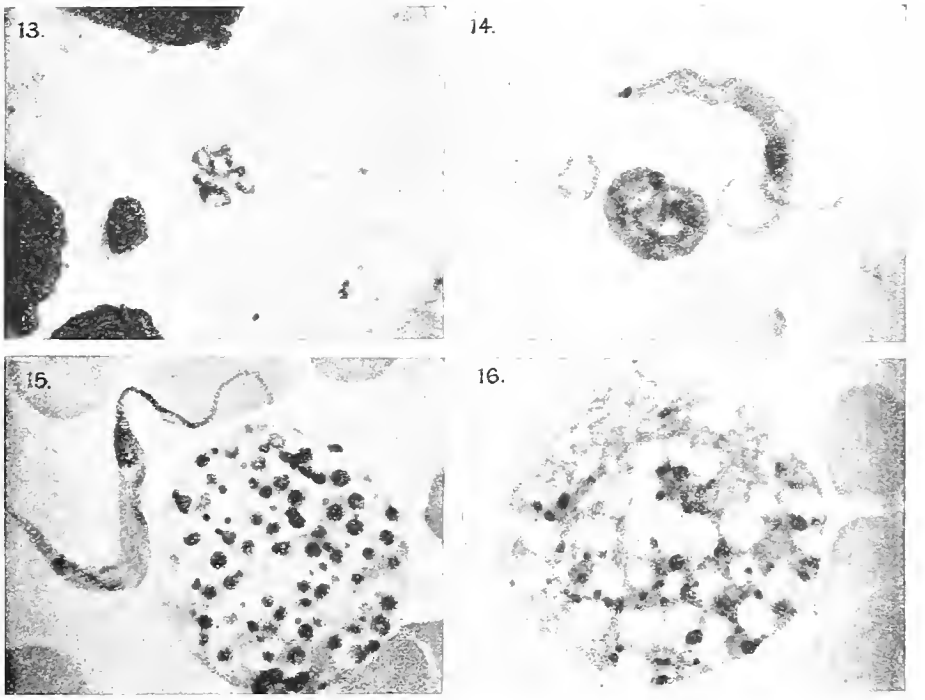
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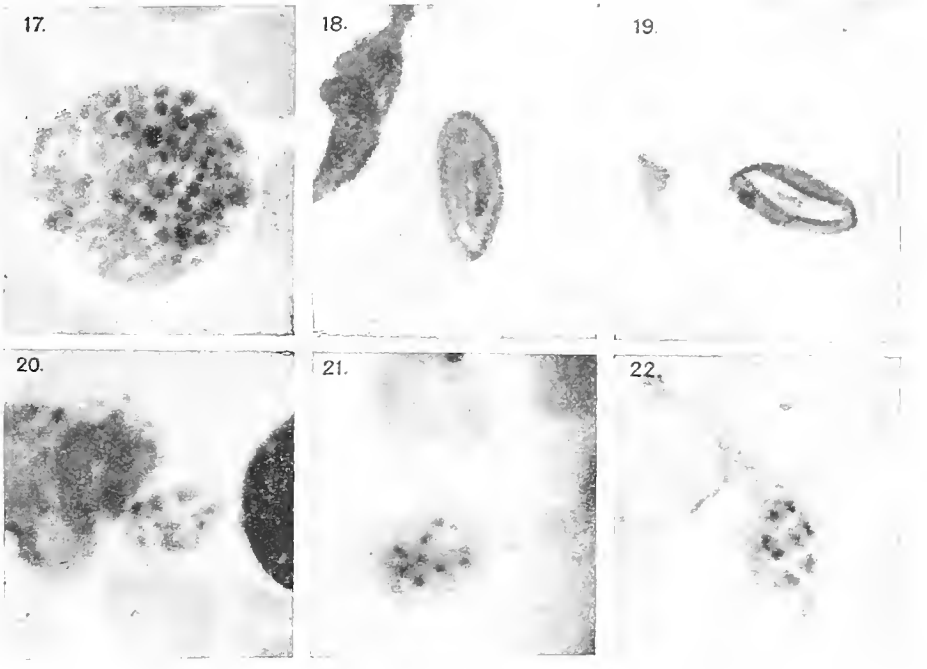
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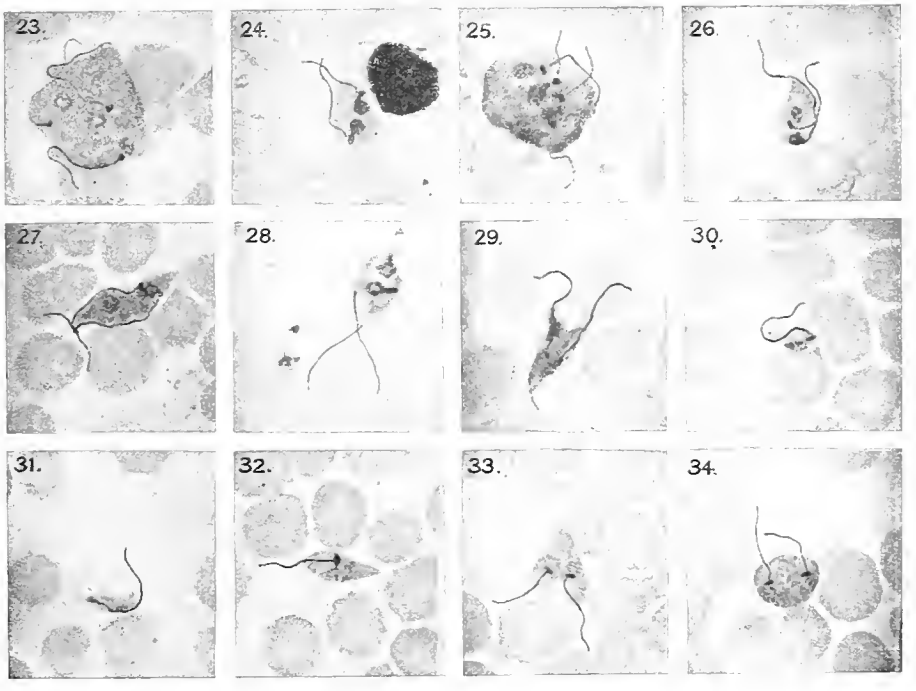
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DESCRIPTION OF PLATES X AND XI.

PHOTOMICROGRAPHS.

Figs. 1–4 and 6–8. Coiled up *T. lewisi*, × 1500.
Fig. 5. Group of four tiny trypanosomes, × 1500.
Figs. 9–12. Coiled up trypanosomes, Diatom-like, × 1500.
Fig. 13. Group of four tiny trypanosomes, × 2000.
Fig. 14. Ordinary and coiled up *T. lewisi*, × 2000.
Figs. 15, 16, 17. Cysts of multiplication of *T. lewisi*, × 2000.
Figs. 18 and 19. Possibly encysted *T. lewisi*, × 2000.
Figs. 20, 21, 22. Pneumocystis Carinii, × 2000.
Figs. 23–34. Stages in multiplication of *T. lewisi*, × 1000.

SOME OBSERVATIONS ON THE THEORY AND PRACTICE OF DIPPING.

By W. F. COOPER, B.A., and H. E. LAWS, B.Sc., F.I.C.

(With Plate XII and 2 Text-figures.)

Introduction.

That ticks are a very serious trouble in countries where stock forms the basis of the wealth of the country is too well known to require elaborate demonstration, for the fact that they are the agents of transmission of some of the most fatal and devastating diseases is now generally recognised by the stock-farmer.

As the cure of these diseases, once established, is generally impracticable, or even, in many cases, impossible, reliance must be placed mainly on preventive measures directed to the destruction and eradication of the vector—the tick.

It is true that preventive inoculation has been given extensive trials, and with some measure of success. In East Coast Fever, for instance, *inoculation* may save some 40 per cent. of infected stock, but as inoculation does nothing to reduce the numbers of ticks, young stock are as susceptible as ever to infection, and the disease is not stamped out.

Dipping, on the other hand, strikes directly at the prime cause of the trouble by destroying the tick, and, provided the operation is systematically and properly carried out, affords the most certain means of combating tick-transmitted disease of stock.

It is our experience that many of the essential points in the process of dipping are not understood or realised by those in authority, and, in consequence, dipping measures which have been impelled by the weight of official recognition have frequently failed to achieve their object. We think, therefore, that a *résumé* of the work done in the past, with deductions made from the available data, may serve a useful purpose.

We may fairly claim to have had a greater experience of the scientific aspect of the problem than many, who have had neither time nor facilities to devote to the matter. Our views on the subject have been confirmed both by experimental investigations and a large experience of practical results.

If this paper does nothing more than to invoke discussion, or to lead others to put our deductions to the test by further investigation, we shall consider that its purpose has been achieved.

In 1908, we were engaged by a commercial firm to make a study of the dipping problem at Gonubie Park, East London, S. Africa. A farm of 3000 acres, with 300 head of cattle, a swim bath and sufficient funds were placed at our disposal. A large amount of work was done, but the results obtained, being the property of the firm, were not immediately available for publication. Since then, further work has been carried out, detailed experiments have been continued, and, as permission has now been obtained to make use of such results as may be deemed to be of scientific interest, it is our intention to publish an account in the near future.

The theoretical considerations which led up to the experiments carried out at Elliotdale, British East Africa, and also our dipping experiments in connection with the Tsetse Fly and Trypanosomiasis, referred to later, were based on this investigation.

A large amount of analytical work was carried out by us at Gonubie Park, but unfortunately, the very limited quarters at our disposal soon became so thoroughly contaminated with the arsenic used in the preparation of large quantities of dipping materials that, though sufficiently good for our purpose at the time, our analytical results are not sufficiently reliable for the purpose of publication. Since then, Lieut.-Col. Watkins-Pitchford (1909, 1910 and 1911), who was working concurrently, though quite independently, on the same subject, has published results of his analyses, and as these results are readily available to the reader in reprint form (1911*a*), we have not thought it necessary to repeat this portion of the work, but shall make frequent references to this reprint.

The Process of Dipping.

For the benefit of English readers, to many of whom the method of dipping of large numbers of cattle is unfamiliar, we give the following brief account, omitting certain practical details which are, for the present purpose, unimportant·

The dipping bath (Fig. 1) is a concrete tank built in the ground into which it is sunk to such a depth that the rim of the tank is flush with the ground level. The bath measures some fifty feet in length and the side walls are inclined outwards in such a manner that while the width at the bottom is about two feet, the width at the rim is some four to five feet. The end wall at the entrance of the bath is vertical, at the exit it is sloped at an angle of about 20° to the horizontal to enable the animals to walk out of the tank. Near the entrance of the bath is an enclosure in which the stock to be dipped are collected, and from this a short entrance race leads directly to the vertical end of the tank. The exit slope leads to a draining pen. The bath is filled with the dipping-fluid to such a depth as to allow of complete submersion.

The cattle are driven through the entrance race and jump into the bath where for a moment they are completely immersed; they then swim down the bath to the exit slope, up which they walk to the draining pen where they stand while the superfluous dip drips off and drains back into the tank.

At first there may be some difficulty in persuading the cattle to enter the bath, but as they become used to the operation, the difficulty lies in checking their eagerness. The process is very rapid and economical—400–600 head of stock can be dipped in an hour at a cost of $\frac{1}{8}$–$\frac{1}{4}$ of a penny per head.

The concentration of the dipping-fluid should vary according to the interval between each successive dipping, and also the species of tick to be killed. The South African ticks may be divided into three classes in this respect, of which the Blue Tick (*Boophilus decoloratus*) forms one; the Bont Tick (*Amblyomma hebraeum*), with which may be associated the Bont-legged Tick (*Hyalomma aegyptium*), a second; and the Brown Tick (*Rhipicephalus appendiculatus*) together with all the species of *Rhipicephalus* which transmit East Coast Fever, the third.

The ticks of the first class are easily killed and are not now considered to be of serious importance.

The Bont Tick, which is important as the transmitter of Heartwater, is most difficult to kill, and the dipping-fluid must be moderately strong and an interval of a fortnight between the dippings is sufficient.

In the case of the Brown Tick (*R. appendiculatus*) the life cycle is so short that the dipping must be repeated at frequent intervals. For this reason, an interval of three days between dippings was adopted, since, as Lounsbury (1904) has shown that the minimum period of attachment of the adult tick was three days, all ticks attached to an animal must

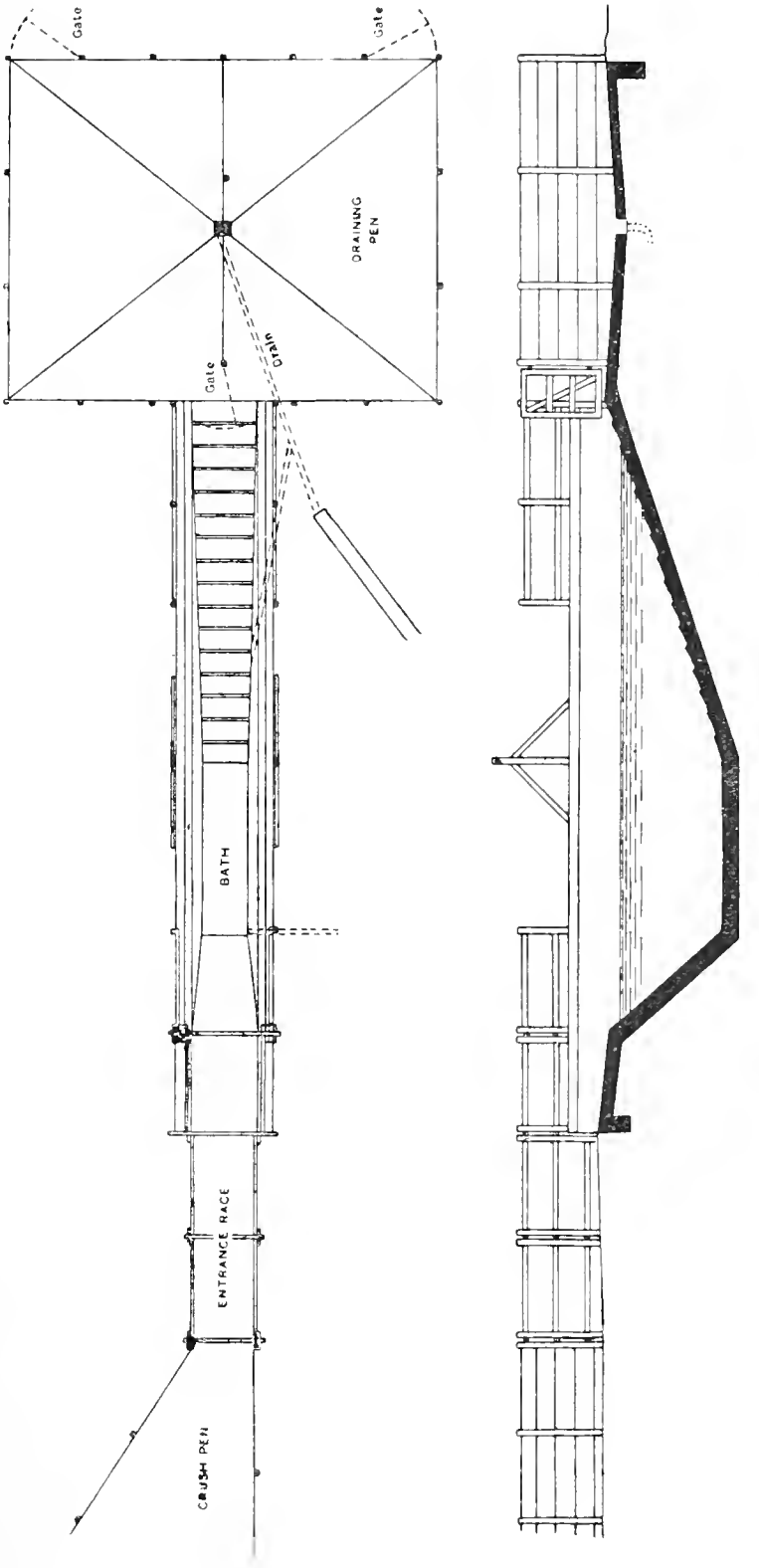

Fig. 1. Plan and elevation of Cattle Dipping Bath.

pass through the dipping-fluid and be killed. It was found later that, after dipping, the stock remain distasteful to ticks for at least two days, and, in consequence, the interval was lengthened to five days, and it would appear that an interval of seven days may suffice, though this has not yet been satisfactorily proved.

While a solution of sodium arsenite alone causes some inconvenience, owing to the fact that stock cannot be used for ploughing and transport for one or two days after dipping, the addition of an emulsion to the fluid allows the concentration of the sodium arsenite to be considerably reduced without decreasing the effectiveness of the dipping-fluid; consequently, with the use of such a dipping-wash, the stock suffer no inconvenience and the work of the farm is not unduly interfered with. At Gonubie Park, some of the oxen have been dipped regularly over a period of several years and used continually for trek work and ploughing.

Horses, mules, etc., also may be dipped regularly, but in the case of sheep, it is desirable to retain a separate bath for their use, as the loose hairs of previously dipped stock get into the wool and cause much trouble and inconvenience in the manufacture of the wool into goods.

Instead of making the cattle swim through a bath, they may be driven through a tunnel into which the dipping-fluid is sprayed by means of suitably arranged jets, the general principle being that of the ordinary 'needle' bath. The method is not so rapid as the swim bath and there is a wide-spread opinion that it is not so thorough in its effects. Nevertheless, the spray bath (Plate XII, figs. 1 and 2) is effective, in spite of the objections raised by some authorities. Practically all of Watkins-Pitchford's work was done with this apparatus and we used the method in our investigations at Elliotdale. Of those who condemn the spray bath, it is generally found that few have had any real experience in its use.

Scalding. By the use of a too concentrated dipping-fluid a more or less severe inflammation of the skin is set up, which condition is generally referred to as 'scalding.' It is not necessarily due to an excessive proportion of arsenic in the dipping-fluid; for it may be caused by the use of a faulty formula, in which the percentage of arsenic is not necessarily excessive, in the preparation of the dip, or by negligence in observing certain general principles in the actual operation of dipping. These principles are related chiefly to meteorological conditions and to the physical condition of the stock at the time of dipping, and as they are generally recognised by those who practise dipping and are not of

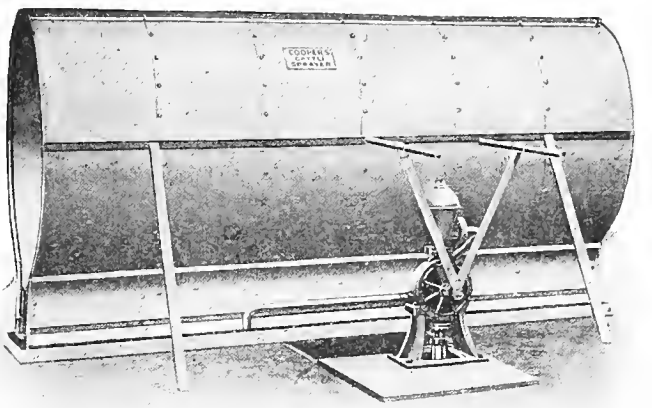

Fig. 1. Side Elevation of Cattle Spraying Machine

Fig. 2. End view showing Spraying Machine in operation

direct importance for our present purpose, we do not consider it necessary to dwell upon them.

The symptoms of *scalding* manifest themselves as follows: the skin becomes hot and tender and as the inflammation progresses, more or less induration, which in bad cases is followed by wrinkling and cracking, with oozing of blood and serum from the fissures, makes its appearance. A constitutional symptom which is almost invariably present is diarrhœa, and this tendency to 'scour,' which is often the first and only sign of scalding, is of great importance and should not escape attention. The maximum degree of soreness is naturally found in those parts where the skin is most subject to flexion, particularly the knee and the hock, and in consequence the animal walks stiffly and shows a great disinclination to movement.

As the inflammation subsides, the cuticular layers of the skin peel off and as the hair follicles are not affected a new growth of hair ultimately appears.

The Effect of Dipping on the Tick.

Concerning the precise mode of operation of the dip in killing the tick, various views have been formulated which may be summarised as follows:

(*a*) That the tick absorbs the poison through its own skin, either in the dipping bath or subsequent to the dipping.

(*b*) That the tick imbibes the poison with the blood which it sucks from the host.

In order to ascertain whether it is possible for a tick to absorb a lethal dose of the poison through its skin, various investigators have submitted ticks *removed from an undipped host* to the action of a dipping-fluid. The authors made the observation some years ago that mere immersion of free ticks in a dipping-fluid does not kill them. Ransom and Graybill (1912) found that engorged female ticks generally survived this treatment, but many failed to lay eggs, and in those cases where oviposition took place, the eggs failed to hatch. In two experiments only, did hatching occur and then only to the extent of 1 to 2 per cent. respectively of the eggs laid. In practically all cases, the untreated ticks, used as controls, oviposited normally, and from 60 to 99 per cent. of the eggs hatched, while in the few cases in which the percentage of successful hatching was low, this result was directly attributable to the unfavourable conditions of humidity and temperature which

obtained in the laboratory. Attention was first called to this infertility of the eggs of dipped females by Lounsbury (1905).

Brünnich and Smith (1914) concluded from experimental observations that the poison is in part absorbed through the skin of the tick subsequent to the time of dipping and is also imbibed with the fluid extracted from the skin of the host.

According to our own experiments with dipped animals, an effective dip kills the ticks before they can lay eggs, and this even applies to ticks removed from a host which has been subjected to periodical dipping. This is an important point when considered in relation to the results obtained by Ransom and Graybill, which showed that simple immersion of free adult ticks taken from an undipped host failed to kill.

Watkins-Pitchford (1911*a*), in his observations on the effect of the dip on adult female ticks attached to the host, writes (p. 69) as follows:

> 'How rarely after one dipping such female forms remain on their host uninjured, and go on to full distension, may be judged from the fact that out of over 10,000 adult ticks actually counted throughout these observations on cattle being subjected to the new process, only sixty-nine partially distended females have been found.
>
> 'Careful detachment of these distended ticks and observations under favourable conditions show that—in the majority of cases—the dipping arrests the process of egg-laying, while of those eggs which are laid only a small percentage are capable of subsequently hatching out.'

Again (p. 50):

> 'It can be shown that the poisonous effect, though strictly local, is not due to a simple deposition on the surface of the skin, resulting from one or more dippings. If, in an habituated animal, a patch of skin is shaved closely and then thoroughly washed so as to remove all deposited arsenic with the hair and surface epithelium before attaching the ticks, the lethal result will follow in the same degree as in the case of an habituated animal in which such precautions have not been taken.'

Watkins-Pitchford also found that ticks were killed when placed on an animal *after* dipping; and, further, that this effect persisted for several days (see Appendix I). Similar experiments carried out by us have given the same result.

Experimental inquiry therefore tends to prove that the poison is imbibed by the tick while feeding on the host.

The fact that engorged females are rendered infertile by mere immersion in the dipping-fluid can have little effect in actual practice, inasmuch as real success is only attained by killing the ticks before they can lay; if the ticks do not die, it indicates a deficiency on the part of the dipping-fluid.

The Effect of an Emulsion in a Dipping-Fluid.

It was found in the course of our investigations that, to obtain the same killing effect, a plain solution of sodium arsenite must be more concentrated than a solution to which an emulsion of soap and oil, or even soap alone, has been added.

The method followed was to record by means of diagrams the numbers and situations of the ticks on several head of cattle. The animals were then dipped in the various solutions which it was decided to test and the effect on the ticks noted. Bont (*Amblyomma hebraeum*), Blue (*Boophilus decoloratus*) and Red ticks (*Rhipicephalus evertsi*) were present.

It was found that a solution of sodium arsenite containing only 0·153 per cent. of As_2O_3, but to which sufficient emulsion had been added, was as efficient as a plain solution, i.e. a solution containing no emulsion—containing 0·225 per cent. of As_2O_3.

Watkins-Pitchford (1911*a*, p. 31) showed that sodium arsenite alone, at a strength sufficient to kill the ticks, scalded the cattle. On reducing the amount of arsenic, the dip failed to kill the ticks, but by adding a paraffin and soap emulsion to the more dilute solution, the ticks were destroyed without injury to the cattle (see Appendix II).

It may be pointed out that the Department of Agriculture of the Queensland Government attribute great importance to the presence of an emulsion in a dipping-fluid, in that they give official recognition only to such dips as contain an emulsion.

It might be supposed that the increased killing effect of emulsion dips is due solely to the presence of soap in the emulsion, but we have found that a similar increased effect is obtained by the addition of an emulsion of glue and oil. Preparations containing emulsified oil in a state of fine division are all characterised by their high wetting power, whether the emulsifying agent is soap, glue or some other compound. It is probable, therefore, that the increased killing power in both cases

is to be referred, in a very great degree, to the increased wetting power resulting from the addition of an emulsion. It was proved that by the addition to the sodium arsenite solution of a certain quantity of alcohol, which would also facilitate wetting, an increased killing power was obtained. Nevertheless, the increased killing power of an emulsion dip probably cannot be completely attributed to increased wetting power alone, it is probable that certain physical properties of the emulsion come into play.

The Action of the Emulsion.

Under the supposition that the tick is destroyed by the arsenic which it absorbs through its own cuticle, the enhanced action of a dip containing an emulsion is readily explained by the greater wetting

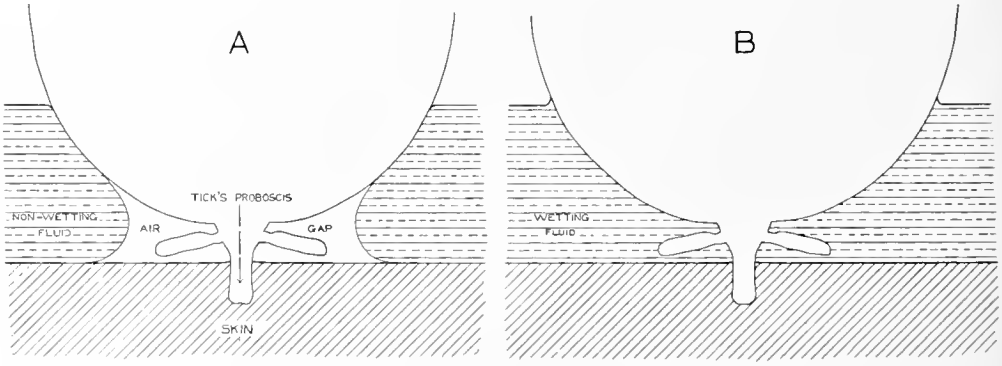

Fig. 2. Diagrammatic representation of the different results obtained by the use of *non-wetting* and *wetting* fluids respectively.

power of such a dip. With the more perfect wetting, the absorption of arsenic would be considerably facilitated. But it has been shown that the tick obtains most, if not all, of the lethal dose of poison from the skin of the host; and we explain the enhanced action of the emulsion-dip in the following manner:

A tick attached to the skin of an ox is a more or less spherical body, difficult to wet, applied to a flat surface—the skin of the ox—likewise, on account of its greasy nature, not easily wetted. It is obvious that under such conditions, a plain aqueous solution would tend to form an 'air gap' or bridge surrounding the point of contact between tick and host (Fig. 2, A).

If however the surface tension of the dipping-fluid is sufficiently small no such 'gap' would form, for the capillary attraction would

cause the solution to run between the spherical and flat surfaces so as to wet the skin of the host thoroughly (Fig. 2, B). This is precisely the object of adding an emulsion to the aqueous solution of sodium arsenite; the surface tension[1] is materially reduced thereby, and the arsenic is brought into intimate contact with the tick's proboscis and the skin of the host immediately adjacent and is thus enabled to exert its greatest effect, for as the tick then sucks up its food from the host, it is almost certain to imbibe a lethal dose of arsenic. If, on the other hand, an air-gap is formed round about the tick's proboscis, the arsenic is some distance removed from the point of insertion and the amount sucked up would be comparatively small.

Though Watkins-Pitchford showed the great value of an emulsion, he does not appear to have made any observation on the physical phenomena involved, though an examination of his results absolutely confirms the foregoing explanation.

Does the Tick take up Arsenic from the Blood or from the Skin of the Dipped Host?

For the furtherance of our inquiry, it seemed to us that the question as to whether the arsenic, imbibed by a tick while feeding on a dipped host, was derived from the blood or the skin, must be settled definitely. With this object in view, we carried out a series of experiments in which arsenic was administered, *per os*, up to the maximum possible dose, yet no ticks were killed (see Appendix III).

It may be argued that with internal dosing, no great amount of arsenic reaches the circulating blood owing to the rapid excretion from the bowel, due to purgation, arrest in the liver, etc. In order to overcome this difficulty and to ensure the presence of an active quantity of arsenic in the circulating blood we then administered the doses in the form of subcutaneous injections, again without positive result (see Appendix IV).

Finally, experiments were carried out, in which a solution of arsenic, much stronger than could be used for general application in the form of a dip or spray, was distributed over a limited area of the skin. In these experiments the ticks on the treated areas were killed, but the

[1] The term 'Surface Tension' is used here in a general sense only. It is now realised that the wetting power of a solution is dependent upon other factors in addition to the surface tension of the wetting fluid. A paper, by one of the present authors (W. F. C.) in collaboration with another, dealing with the Theory of Wetting and the Determination of Wetting Power, is in course of preparation.

action of the arsenic did not range beyond a margin of six inches surrounding these areas (see Appendix V).

The general conclusion to be drawn from all these experiments is that the tick does not obtain a lethal dose from arsenic present in the circulating blood.

Cumulative Action of Arsenic in Dipping.

Although all the ticks present on an animal at the time of dipping may be killed by a single immersion, yet this does not prevent reinfestation by ticks subsequently picked up in the grazing area. To prevent reinfestation it is necessary to repeat the dipping at short intervals and in order to do this without injury to the stock, the concentration of the dipping bath must be reduced accordingly. It is then found that, at the reduced strength, although a single dipping fails to kill all the attached ticks, repeated dippings render the host poisonous to all ticks, both those attached to the host at the time of dipping and also those ticks which may be picked up later. Now, a single immersion is ineffective, therefore, the only conclusion to be drawn is that the arsenic is cumulative in its action and that the quantity absorbed by the tissues of the skin is augmented by each subsequent dipping until, as will be shown later, a certain maximum is reached.

From Watkins-Pitchford's statements, it is obvious that he holds similar views to ourselves with regard to this cumulative effect of short interval dipping, and the affinity of the skin of an habituated animal for arsenic. Concerning this he writes (1911*a*, pp. 47–48) as follows:

> 'In fact, as will be seen from the succeeding observations, the deeper layers of the skin appear to be capable of becoming so tolerant of the presence of arsenic that the latter becomes tolerated to a considerable extent. That this accumulation is not a mere mechanical deposition or passive soaking is indicated in Schedule E, items 5 and 5*a*. This indication of the vital action of the skin is further borne out by the fact that any arsenic in excess of the maximum content is eliminated from the skin, the elimination taking place through absorption by the blood-vessels which are contained in its deeper layers, such excess of arsenic appearing shortly afterwards in the urine. When we further consider it is into this deep layer of the skin that the tick thrusts its mouth parts and obtains its nourishment, we shall partly see the significance of being able to establish and maintain a supply of arsenic at such a point of attack.'

That the arsenic accumulates by repeated applications is proved by analysis of the skin of animals dipped at short intervals. Watkins-Pitchford carried out numerous analyses, the results of which we briefly tabulate (see Appendix VI), and our own analyses absolutely confirm his findings. He goes on to state (pp. 49–50):

> 'that the value of frequent dipping lies in the sustained effect produced rather than in the mechanical destruction of ticks which follows immersion in the dip tank, and...this maximum killing efficiency of a beast is only to be maintained by the repetition of the dip or spray process with such frequency as will compensate the skin for the loss of arsenic which is continuously absorbed from it and excreted in the urine by a natural process of elimination.... The power of an habituated animal to destroy ticks placed upon it (or gaining access in the natural manner after dipping) is rapidly lost, five or six days' lapse sufficing to reduce such an animal to nearly the same condition as that which exists in the case of an unhabituated animal or beast which is dipped at the long interval of, say, ten days or a fortnight.'

Although Watkins-Pitchford expresses the opinion that a five or six days' lapse is sufficient to reduce the resistant powers to that of an unhabituated animal, we found, in our work at Elliotdale (see Appendix VIII), that, in actual practice, a five-day interval is sufficient to keep stock free from East Coast Fever.

In short, although the fact is not admitted by several eminent veterinary authorities, exclusive of those who have carried out investigations on the lines of Watkins-Pitchford and ourselves, we do not consider that this cumulative effect of arsenic applied to the skin is open to any reasonable doubt[1].

Arsenical solutions applied to the skin penetrate the actual cellular tissues by osmosis, and it is reasonable to presume, from what has already been said of the cumulative effect in the cutaneous tissues, that the arsenic enters into an actual combination with some organic constituent of the cells.

The work of Ehrlich, Nierenstein, Breinl, Levaditi[2] and others, on

[1] In order not to obscure the main points of our paper, we have collected, in the form of an appendix, some of the more cogent objections to our theories, together with our criticisms (see Appendix IX).

[2] Ehrlich (1908), *Verhandl. d. Deutschen dermatolog. Gesellsch.* x, Kongress, Juni, 1908. Nierenstein (1908), *Ann. Trop. Med. and Parasitol.* II, 249–255. Breinl and Nierenstein (1909), *Zeitschr. f. Immunitätsforsch. u. exper. Therap.* I, 620–632. Levaditi and Yamanouchi (1908), *C. R. Soc. Biol.* LXV, 23.

the mode of action of certain organic compounds of arsenic as trypanocides in the animal body, is very interesting in connection with the above mentioned subject, but a discussion of the matter would lead us away from the subject matter of this paper; it is being dealt with by one of us (W. F. C.) in collaboration with others, in another paper now in course of preparation, on the application of arsenical dipping to the prophylaxis and treatment of trypanosomiasis in stock.

In view of the success which has resulted from the detailed study of the mode of action of arsenic and other substances, the cumulative effect in the cutaneous tissues would appear to be an important and interesting problem to follow up. We may say that we hold the view that something of this sort does take place as a result of short interval dipping, and as soon as the large amount of field work in hand is completed, we intend to undertake a systematic investigation of the problem.

For the moment, it is sufficient to know that the arsenic is taken into the body and we have evidence, derived from the work on trypanosomiasis in dipped animals referred to above, that although the quantity of arsenic thus absorbed into the circulating blood is insufficient to poison ticks, yet it may be present in sufficient quantity to exert a toxic action on such pathogenic protozoa as trypanosomes.

The Quantity of Arsenic applied in Dipping.

The amount of arsenic left on the animal after dipping may be roughly estimated as follows: an average animal, after allowing the superfluous dip to drain off, retains about 0·75 gallon. In the case of the 'Laboratory' dip, 50 gallons of the dip contains 1 lb. of sodium arsenite (80 per cent.), giving a concentration of 0·16 per cent. of arsenious acid. Three-quarters of a gallon would contain, therefore, 5·4 gms. (84 grains) of arsenious acid. Thus, some 5·4 gms. are applied to the skin of the animal at each dipping, and at the rate of five-day intervals, the amount applied per month would be 32 gms., and under proper conditions, this process can be continued for years without causing any inconvenience or injury to the stock. The lethal dose of arsenic for cattle, according to Kaufmann[1] (see Appendix III), varies from about 15 gms. to 30 gms.: it follows, therefore, that quantities of arsenic which would almost certainly prove fatal to the animal if administered internally may be safely applied to the skin by dipping.

[1] Cited by Finlay Dun (1910), *Veterinary Medicines—their Actions and Uses*, 12th ed., Edinburgh, David Douglas, p. 275.

The Effect on Pathogenic Organisms of Arsenic in the Blood of Dipped Animals.

It has been shown that the epidermal cells possess a special affinity for arsenic (see pp. 200–202 and Appendix VI), and once this arsenophile proclivity is satisfied, the excess of arsenic is available for absorption by the blood.

It is obvious that the *total* amount of arsenic in the general blood system cannot be very great, since the general health of the animal is not interfered with in any way. But a consideration of the amount of arsenic applied every week, or even at shorter intervals, and especially the appearance exhibited by the inner surface of the skin of an animal which has been dipped in a solution only slightly too strong, would lead us to suppose that the amount of arsenic present in the blood circulating in the *most peripheral vessels of the skin* would be very considerable. It is quite conceivable that the blood circulating in the capillaries which are in close relation to the external surface of the skin to which the very strong dose of arsenic has been applied, might contain a very large amount, far greater in fact, than the amount that would be possible in the general blood system; for this peripheral blood containing a large quantity of arsenic would be immediately diluted by the general mass of the blood from the internal parts of the animal, so that the total amount in general circulation would not be excessive.

This is what we consider actually takes place, and the results attained at Elliotdale (see Appendix VIII) are sufficiently good evidence that this deduction is correct; and upon this deduction we base our anticipation of success in our experiments at present in hand.

In the case of such diseases as are transmitted by external parasites—East Coast Fever for example—the infective organisms can only be injected into the most peripheral cutaneous capillaries, where the concentration of arsenic is sufficiently great to kill them. Such being the case, regularly dipped animals running on infected pastures would remain free from the disease.

Watkins-Pitchford (1911*a*, p. 56) has given figures to show that the incubation period of East Coast Fever is prolonged in dipped animals; we give an abstract of these in Appendix VII. In connection with these results, it should be observed that the cattle were only dipped a few times before exposure to infection and that after exposure they were not dipped at all.

Had these cattle been dipped as soon as a rise in temperature was observed, we think it probable that they would have recovered. This however is a different matter to the killing of the pathogenic organisms before they can propagate. The point was tested by us at Elliotdale, on a large practical scale, and found to be correct. The details of this experiment are given in Appendix VIII, from which it is seen that 500 head of cattle remained free from disease for a period of eighteen months during which they were able to produce young and increase to 620 head.

Subsequently the Government Veterinary Department adopted a system of inoculation against East Coast Fever, in place of dipping, but in consideration of the expense and trouble involved in the system of inoculation, together with the losses occasioned thereby, we venture to express our opinion that the practice of dipping would have proved to be superior.

We hoped to be able to continue our experiments in British East Africa, but the Government officials refused to grant facilities to enable us to carry out systematic work. However, we purchased a farm in an area reported to be badly infected with East Coast Fever and placed 100 head of stock upon it. Three died in the first month after which no further deaths from East Coast Fever occurred.

These experiences, especially those at Elliotdale, showed that cattle could be kept alive and increase by dipping; but it remains to be ascertained for how long, after ceasing to dip, cattle remain immune to East Coast Fever when running on infected pasture. It is possible that they will not act as carriers of East Coast Fever for a period of several days after dipping. If this point could be proved, it would be possible to relax the present system of quarantine set up in some of the infected areas so as to allow some movement of stock. Under present conditions, if East Coast Fever breaks out, the affected farm and the surrounding area are placed in quarantine for fifteen months and all movement of stock is prohibited—a great inconvenience and the cause of considerable pecuniary loss. In a country where oxen form the chief means of transport, the prohibition of movement of stock is a very serious matter, but, until it can be definitely proved that dipping does confer such immunity, at least for a certain period, the quarantine system is absolutely necessary. We believe, however, that sufficient evidence has been obtained to indicate that in systematic dipping there lies a means of bringing about such a relaxation of these irksome regulations as would enable farmers to make a turnover on their stock.

Dipping in Relation to Trypanosomiasis.

If by dipping, the amount of arsenic which can be introduced into the peripheral cutaneous vessels is sufficient to kill ticks, then it is reasonable to imagine that it might also kill other blood-sucking pests, such as Tsetse flies; also the natural infection of stock by Tsetse flies should be preventible by the same means.

Regarding the effect of dipping on biting flies, the fact that the latter feed so rapidly, in comparison with the very slow rate of feeding of the tick, introduces a possible difficulty, but experiments are now being carried out by one of us (W. F. C.) in the Congo, to test this point.

With regard to *trypanosomiasis* it is highly probable that dipping would exert a pronounced effect. In the experimental treatment of trypanosomiasis, it has been shown repeatedly that arsenious acid, administered *per os*, is very effective, but the effect is not permanent because it is impossible to prolong the treatment without producing symptoms of arsenical poisoning and the ultimate death of the host.

But dipping furnishes a method of applying arsenic in comparatively large doses at frequent intervals without deleterious effects on the treated animal, and there is reason to believe that its use in this manner would exert a remedial as well as a prophylactic effect.

The whole point has been considered in connection with some preliminary investigations on Nagana-infected dogs carried out by one of the authors (W. F. C.) in collaboration with Dr E. Hindle and Mr L. E. Robinson, at Cambridge, which form the subject of a paper now in course of preparation for the press.

Appendix I.

A tabulation of the results of Watkins-Pitchford's experiments, to show that the killing effect of the dip on the ticks persists for some days after dipping.

No. of days since attachment of ticks	Days since dipping: 1st day No. of ticks attached	2nd day No. of ticks attached	3rd day No. of ticks attached	5th day No. of ticks attached	7th day No. of ticks attached	10th day No. of ticks attached	Controls (*not dipped*) No. of ticks attached
	12	19	22	28	21	26	8
½ day	19	18	21	22	20	21	8
1 ,,	3	9	8	15	14	15	8
1½ days	0	5	8	13	12	14	8
2 ,,	0	2	3	12	12	14	8
3 ,,	0	1	0	8	9	13	8

This table is modified and abridged from the table given by Watkins-Pitchford (1911*a*, p. 54). Two beasts were used for each experiment.

Appendix II.

The effect of the addition of various substances to solutions of sodium arsenite in averting 'scalding.'

Watkins-Pitchford (1911*a*) gives the results of his experiments with plain solutions of sodium arsenite (p. 31) and with sodium arsenite solutions to which other substances have been added (pp. 34–37). In order to facilitate a comparison of these results, the following table has been compiled from his published results.

Composition of Dip	No. of sprayings before scalding occurred	Remarks
Sodium arsenite	4	
,, + soap	4	
,, + glycerine + soap	4	
,, + ,, + ,, and paraffin emulsion	0	In 3 separate trials, no 'scalding' occurred after 23, 19 and 10 sprayings respectively
,, + soap and paraffin emulsion	11	

It will be observed that in these comparative tests, the fluid was applied as a *spray*; our own experience leads us to believe that a *dip* is less apt to scald than a *spray* and can, therefore, be used a little stronger, but, for all practical purposes, these results apply equally to either method of application.

Watkins-Pitchford tried the addition of glycerine, with the hope that it would exert an emollient action tending to reduce the irritating

effects on the skin of both arsenic and paraffin. With the same view the authors tried glycerine but, as it seemed to have little effect, and the cost prohibited its use on an extensive scale, they dropped it. It will be observed that Watkins-Pitchford omits glycerine in his final formula (1911*a*, p. 37).

Appendix III.

The Effect on Ticks of Arsenic administered per os.

The object of this experiment was to ascertain whether it was possible to destroy attached ticks by dosing the host with arsenic. By internal dosing, it was surmised that some of the arsenic would be taken up by the blood and thus conveyed to the skin. The lethal dose of arsenic for cattle is stated by Kaufmann (see footnote on p. 202) to be 4–8 drachms (= 15·5–31 gms.).

Beast No. 1200. ♂, aged $2\frac{1}{2}$ years.

Date 1909	Temperature °F.	Effect on ticks	Remarks
March 9th			0·75 gm. ($11\frac{1}{4}$ grains) of As_2O_3 administered *per os*
„ 10th	104·0	None killed	
„ 11th	103·0	„	
„ 12th	105·7	„	Diarrhoea
„ 13th	104·2	„	
„ 14th	103·4	„	
„ 15th	105·8	„	
„ 16th	106·6	„	

Up to March 16th, no ticks were killed. The animal was not showing any signs of arsenical poisoning. This beast had been continuously dipped prior to the commencement of this experiment and as it had not shown any untoward symptoms after the initial dose of 0·75 gm. we assumed that it had acquired some degree of tolerance, and it was then decided to reduce the dose and administer it at frequent intervals:

Date 1909	Temperature °F.	Effect on ticks	Remarks
March 31st	103·2	None killed	0·3 gm (5 grains) of As_2O_3 administered
April 1st	104·4	„	„ „
„ 2nd	104·2	„	„ „
„ 3rd	103·4	„	„ „
„ 4th	104·6	„	„ „
„ 5th	103·8	„	Beast emaciated
„ 6th	102·8	„	Beast breathing hard, bowels very loose
„ 7th	—	„	Beast found dead

At no time could any effect on the ticks (*Boophilus decoloratus*) be observed. The post-mortem examination showed the usual symptoms of acute arsenical poisoning.

It is evident therefore that the internal administration of arsenic is useless, whether in large or small quantities.

Appendix IV.

The Effect on Ticks of Arsenic administered subcutaneously.

The object of these experiments was to determine the effect of local subcutaneous injections of arsenical solutions on ticks attached to the host. It was anticipated that by such means it might be possible to ensure the presence of a sufficient quantity of arsenic in the circulating blood to destroy the ticks which fed upon the treated animal. The subjoined protocols show that such injections are purely local in their action, and that with the exception of those ticks which are attached immediately round the site of the needle-puncture no effect is to be observed. Unfortunately the general symptoms produced in the host made it impossible to continue these experiments, and, for the same reason, no certain conclusion can be drawn from the results obtained.

Beast No. 0102. ♂.

Date 1909	Temperature	Effect on ticks	Remarks
March 9th			0·25 gm. of As_2O_3, as sodium arsenite, dissolved in 3 c.c. of water injected subcutaneously on right shoulder; a similar dose injected on the escutcheon
„ 14th	105·8° F.	All ticks dead over an area of some 6 ins. × 3 ins., surrounding the site of the needle-puncture. Some adults had gorged. Elsewhere, ticks unaffected	Parts round sites of injection swollen and inflamed
„ 17th	104·0 „	Ticks on body generally, still unaffected	Skin over swollen areas cracked and raw surfaces infested with maggots. Wounds cleaned and disinfected with carbolic acid
„ 26th	105·2 „		All living ticks killed by application of oil; beast cleaned and disinfected
April 7th			Wounds healed and beast quite well

Beast No. 2010. ♂.

Date 1909	Temperature	Effect on ticks	Remarks
March 9th			0·5 gm. of As_2O_3, as sodium arsenite, dissolved in 15 c.c. of water injected on escutcheon
„ 15th	104·4° F.		Skin badly swollen between hind-legs, from anus to sheath
„ 19th	101·8 „	Ticks within few inches of needle-puncture all dead. Elsewhere ticks unaffected	Swelling incised and dressed with carbolic acid and subsequently attended to daily
„ 23rd	103·4 „	Ticks on body generally, unaffected	Beast almost unable to walk
„ 31st	103·6 „		All living ticks destroyed by application of oil
April 7th			Beast recovered.

Appendix V.

The Effect on Ticks of Arsenic in strong solution applied to a limited area of the skin.

The following experiment was carried out with the object of determining whether arsenic, applied in strong solution to a *limited* area of the skin, would be absorbed in sufficient quantity to destroy ticks distributed generally over the skin, or, in other words, whether such a solution is only effective in the area to which it is applied.

A beast was taken and the skin of one side of the body was divided into two approximately equal halves by a vertical red paint line situated midway between the fore and hind legs. The fore quarter of the ox having been covered to prevent contamination, the hind quarter posterior to the paint line was carefully sprayed with a 2 per cent. solution of arsenious oxide as sodium arsenite. Such a solution is, of course, far too strong for an application to the entire skin surface and would cause the death of an animal so treated in a very short time[1]. Four days later, the skin of the sprayed area showed a marked glossiness and the ticks attached thereto were dead. Later, symptoms of scalding developed, and in about three weeks the epidermis, with the hair attached, peeled off in sheets the size of a piece of note-paper. New hair ultimately appeared and recovery was apparently complete. The experiment was repeated twice, using less concentrated solutions, with identical results.

[1] The strongest solution ever advised is 0·25 per cent.; the strongest advocated by any Government is one containing 0·20 per cent. of As_2O_3.

The animal was infested with Blue ticks (*Boophilus decoloratus*) which are easy to kill, and whereas all the ticks on the sprayed area and within a margin of six inches surrounding this area, were killed, none of the ticks distributed over the unsprayed part of the animal's skin was in the least affected.

In other cases, in which we had the misfortune to cause the death of the animal, the autopsies showed that the cutaneous vessels in the scalded areas were much congested and there was some considerable extravasation of blood. This, together with the fact that the animals exhibited symptoms of acute arsenical poisoning prior to death, indicates that arsenic was absorbed, but in quantities insufficient to affect ticks attached at a distance of six inches or more from the sprayed area.

Appendix VI.

The Quantity of Arsenic retained in the Skin of Oxen after Dipping.

Watkins-Pitchford has given data (1911*a*, Schedule "E," pp. 58–61) relative to the amounts of arsenic retained by the skin (hair and hide) of dipped animals; these figures are tabulated below and some further calculations, based on these, have been added in the last three columns.

The animals under investigation were dipped for varying periods; some every five days for many months, others once only (see Column 4 of table). After the lapse of different intervals (Col. 5) the animals were killed, and from each one square foot of skin was taken. The hair was removed and the arsenic contents of both hair and hide were separately determined (Cols. 6 and 7).

It appeared to us, however, that the results would be more comprehensive if the amount of arsenic in the entire skin of the animal was estimated. Several measurements made on three animals of normal size established the fact that the mean area of the skin, inclusive of the head and legs, was about fifty square feet, and the figures given in the last three columns of the table represent Watkins-Pitchford's data multiplied fifty times.

The Quantity of Arsenic retained by the Skin of the Oxen for Various Periods after Dipping.

No.	Nature of Integument		Frequency of dipping	Period elapsing since last dipping	Amount of arsenious oxide per square foot of skin			Amount of arsenious oxide in entire skin (based on average superficies of beast of 50 sq. ft.)		
	Hair	Hide			Hair gm.	Hide gm.	Total gm.	Hair gm.	Hide gm.	Total gm.
1	Long, fine and thick	Very thin	Every 5 days, then 6 sprayings in 24 hours	1 day	0·302	0·228	0·530	15·100	11·400	26·500
2	Strong, medium length, not thick	Medium thickness	Every 5 days, then daily for 10 days	1 ,,	0·201	0·196	0·397	10·050	9·800	19·850
3	Fine, long and very thick	Medium thickness	Every 5 days for months; last sprayings every 3rd day	1 ,,	0·527	0·224	0·751	26·350	11·200	37·550
4	Strong, medium length, fairly thick	Very thick	Every 5 days for several months	5 days	0·352	0·251	0·603	17·600	12·550	30·150
5	Moderately long	Fairly thick	Dipped regularly for several months	10 ,,	0·260	0·137	0·397	13·000	6·850	19·850
				Mean	0·328	0·207	0·535	16·420	10·360	26·780
6	Fairly long, fine, medium thickness	—	1 spraying 5 days before death, not sprayed for previous eight weeks.	5 days	0·116	—	—	5·800	—	—
			Carcase sprayed immediately after death	Analysis made as soon as skin was dry	0·470	0·008	0·478	23·500	0·040	23·540
7	Fairly long, medium thickness	—	—	8 weeks	0·159	—	—	—	—	—
8	—	—	—	6 months	0·388	—	—	—	—	—
9	—	—	—	7 ,,	0·019	—	—	—	—	—

In such an investigation as this, it would be desirable to make a very large number of analyses before drawing conclusions from small variations in the figures, but the amount of labour and the cost of stock would be excessive.

By taking the mean values of Watkins-Pitchford's figures, however, the results should be sufficiently accurate for practical purposes, and

we have therefore added these values for the first five experiments (see Col. 6 *et seq.*). From these it will be seen that some 26 gms. of arsenious oxide are retained by the skin of a regularly dipped animal, of which 16 gms. are present in the hair and 10 gms. in the hide. From the figures it would appear that the quantity of arsenic remaining in the hide of a regularly dipped animal is much the same up to five days after dipping, but after a lapse of ten days the amount has been reduced by nearly one-half. From this it follows that between the fifth and the tenth day after dipping, some 3·5 gms. of arsenious acid have been removed from the skin, and as this removal must have been effected by the blood circulating in the cutaneous capillaries, the animal has received a continuous internal dose of arsenic at the rate of about 0·70 gm. per diem, extending over five days. If, then, this amount of arsenic is absorbed by the blood from the fifth to the tenth day, what happens during the five days immediately following the dipping? We believe that the absorption of arsenic from the skin proceeds continuously from the time of dipping, but that for the first five days or so, the amount thus removed from the skin is constantly replaced by further quantities absorbed by the skin from the very considerable amount which dries on the surface.

Appendix VII.

The Augmentation of the Incubation Period of East Coast Fever by Dipping.

In our own work, a direct practical test of the correctness of our deductions was carried out at Elliotdale (see Appendix VIII), but permission could not be obtained to carry on the work sufficiently to determine the details.

Watkins-Pitchford, however, has since published his data of observations, showing that the incubation period of East Coast Fever is prolonged by dipping. He took three lots of five animals each, which were exposed on infected pastures for periods ranging from half an hour to nine hours. Lot *A* was *undipped* and served as a control to Lot *B*, dipped *after* exposure, and to Lot *C*, dipped *before* and after exposure. An abridged form of his schedule of results is given below.

Lot	Treatment	Mean incubation period
A	Not dipped	9 days
B	Dipped directly *after* exposure	11 ,,
C	Dipped 24 hours *before* and immediately *after* exposure	17 ,,

It would have been interesting to see the results, had these tests been extended. For instance, it is exceedingly probable that in Lot *C*, two animals of which, out of a total of five, failed to exhibit any febrile symptoms within the period of the experiment (eighteen days), the incubation could have been prevented entirely by continuing the dipping at five-day intervals. In Lot *C* again, the earliest appearance of fever was on the fifteenth day after dipping and it is quite within the bounds of probability that, up to as much as fourteen days, the animal was non-infective and could have been used for transport purposes within that period with perfect safety. It is very desirable to establish this point in order that some system of allowing transport, under licence, to and from infected areas might be devised. Such a system would prove a great boon to farmers and graziers in certain parts of South Africa and particularly in British East Africa.

Appendix VIII.

Dipping Experiments at Elliotdale.

East Coast Fever broke out at Elliotdale (Lat. 32° S., Long. 29° E.), in the Transkeian Native Territories, South Africa, in February, 1910, as diagnosed by Veterinary Surgeon J. Spreull.

By that time, the chief deductions expounded in the body of this paper had been made and this outbreak appeared to offer an excellent opportunity of putting them to an extensive practical test. As it was necessary to get the work under way without delay, it was impossible to make elaborate preparations, and, after all, our object was to ascertain whether, by means of dipping, animals could be rendered immune to the disease.

On May 18th, 1910, one of us (H. E. L.) commenced operations with 500 head of Kaffir stock (bullocks, cows and calves—mixed) collected in the district. A dip, prepared by us at Gonubie Park, consisting of sodium arsenite with an emulsion, was used. At that time there were no cattle swim baths in the area and we had to rely on the spraying method, using the Seabury machine[1]. The spraying operations were started at the end of May and were repeated at intervals of five days.

Between the first and second sprayings one animal died, and after the second dipping another animal died, but from that time no further deaths occurred amongst the sprayed stock.

[1] The construction and mode of operation of the Seabury machine is dealt with in Lounsbury, C. P. (1908) and Cooper, W. F. and Laws, H. E. (1908).

At the end of June, the experiment was left in the charge of our agent until it could be taken over by a stock inspector at the end of July.

In April, 1911, eleven months after the commencement of the experiment, Mr A. H. Stanford, the Chief Magistrate, reported that all the stock in the district had succumbed, except those which had been sprayed. Among the latter, with the exception of the two cases mentioned above, not a single death occurred and the herd had increased to some 600 head.

The experiment was continued for a further six months, by which time the results were becoming generally known among the natives throughout this part of the Transkei. Consequently they introduced other stock, at first surreptitiously, many of which were heavily infested with ticks. It was impossible, owing to the absence of fences of any kind, to prevent the promiscuous mixing of these newly-introduced animals with the domiciled stock, and eventually such large numbers were brought into the area that it became impossible to deal with them and the work had to be abandoned.

This experiment cannot be regarded as a strictly scientific investigation, but it proves in a very conclusive manner that dipped cattle remain immune for indefinite periods although they are allowed to run on heavily infected pastures and to mix freely with undipped stock which were dying from East Coast Fever.

As a matter of fact, in such a case as this, where the 'controls' were so numerous, there was no necessity to make a definite proof that the deaths in the undipped stock were due to a *Theileria parva* infection. The disease had been diagnosed at its outbreak as East Coast Fever by a competent authority, and the subsequent deaths were clearly attributable to the same cause.

Appendix IX.

In most of the published work on the effects of dipping very little consideration has been given to the essential importance of the addition of an emulsion. The objection generally raised, *e.g.* Dixon (1911, p. 16), is that when hard or brackish water is used in the preparation of the dip, the soap used in the preparation of the emulsion is precipitated and rendered useless.

We claim that, by this precipitation, calcium and magnesium salts, which otherwise would form ineffective arsenates, are removed. The amount of soap thus rendered inert is at the most relatively small in

quantity, and the presence of the precipitate does not inhibit the action of the dip in any way.

It has been shown (pp. 197–198) that the presence of an emulsion in the dip facilitates wetting, the importance of which latter factor is demonstrated in Appendix V. Both Watkins-Pitchford and Brünnich and Smith have called attention to the ease with which certain parts of the body (as under the tail) escape wetting. Hence it is a matter of vital importance that the solution should have the maximum possible wetting power, and any addition which increases this factor is more than justified.

A point which is generally disregarded in matters concerning the theory of dipping is the cumulative effect (pp. 200–202). For instance, Theiler (1911, p. 505), writing on the subject of the eradication of East Coast Fever, states: 'When the disease has taken a firm hold on a farm, that is to say, a number of cattle sickened and died and disseminated ticks in large numbers, then no dipping will help to stop the disease.' Further, on p. 508, he warns farmers 'not to trust to it (dipping) as a panacea for the prevention or eradication of East Coast Fever....No dip has yet been found which prevents ticks from biting, and as long as infected ticks are present, so long East Coast Fever will be found.'

We claim that on account of the cumulative effect the fact that the ticks bite does not matter. Watkins-Pitchford (1911*a*) showed most conclusively that the 'Laboratory dip' kept stock clean after a few dippings at five-day intervals. On p. 18 he says: 'Such ticks as have attached themselves to the cattle have been regularly destroyed.' In Schedule "B" (p. 54) he shows that ticks placed on a recently-dipped beast may attach, but, if they do, they are killed within a period of 2–3 days after dipping. Schedule No. 21 (p. 38) shows that after ten dippings, the animals remain free from ticks, provided the dipping is repeated at five-day intervals.

On the basis of these observations of Watkins-Pitchford we are at a loss to see how such statements as those cited from Theiler's paper can be reconciled with actual experimental data.

Further, these remarks have been shown to be invalid in actual practice, in a paper published by Manning and During (1912). They give details of a case (p. 451) in which East Coast Fever broke out in a herd of 805 cattle in April, 1911. By the end of September, up to which time very desultory dipping had been practised, 221 head had died of the disease. Five-day dipping was then established and in the following

two months ninety-six deaths occurred. From December to the end of June, 1912, only ten deaths occurred! The authors do not state whether an emulsion-dip or a plain solution of sodium arsenite was used—a very important point in a case where it was necessary to be so very particular about wetting the whole surface of the animal. It may be pointed out that the stock was native-owned, a fact that would considerably reduce the chances of efficient control. Manning and During estimate the cost of dipping as $\frac{1}{2}d.$ per head, and on this basis the cost of dipping, in the case cited above, would be approximately £52. If, by inoculation, the mortality was 30 per cent., the total loss on a valuation of £5 per head would have been £750. Moreover, if inoculation had been practised, the stock would still have remained infective, whereas with continued dipping, the disease would have been stamped out. As a matter of fact, instructions for inoculation were issued, and to obtain a supply of the necessary virus twenty head were placed on the most heavily infected ground, but as they were regularly dipped, 'none of them became infected.' In their concluding remarks, the authors state 'practically no more cases of East Coast Fever have since occurred on these combined farms.'

Surely this is proof enough!

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The papers cited below form a small part only of the numerous papers dealing with the subject of dipping, but they are the most important from our point of view and are those to which reference has been made in our paper.

Since 1905 practically the only detailed scientific work on the subject published in South Africa, is that of Lieut.-Col. H. Watkins-Pitchford (1909, 1910 and 1911). These separate papers have since been published collectively in the form of a pamphlet (1911*a*), and it is from this reprint that our references are cited.

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EXPERIMENTS IN THE "TRYPOSAFROL" TREATMENT OF TRYPANOSOMIASIS (*T. BRUCEI*) IN GUINEA-PIGS AND OF PIROPLASMOSIS IN DOGS.

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In 1912, Bricger and Krause reported upon experiments with a dye to which they gave the name of trypasafrol (since called tryposafrol). They stated that it cured Nagana in rats and guinea-pigs. The authors experimented with four laboratory strains of *Trypanosoma brucci*. The animals were treated immediately after inoculation or after the lapse of a day or two, the dye being given every other day in quantities of 0·05 to 0·1 g. well mixed with the moistened food which consisted of bread and bran for rats and guinea-pigs respectively. On the alternate days the animals received "Kraftfutter," *i.e.* non-medicated food.

On the occasion of the International Congress of Hygiene and Demography, held at Washington in September 1912, Geheimrat Brieger approached one of us (G. H. F. N.) with the suggestion that experiments with tryposafrol should be tried in Cambridge on some of the protozoal diseases upon the study of which we were engaged. Geheimrat Brieger, on his return to Berlin, sent a supply of the dye, and, in a letter dated 28. xi. 1912, wrote that the remedy had also given uniformly good results in the treatment of bovine piroplasmosis. In the treatment of cattle he recommended an initial dose of 0·25 g., followed on the next day by 0·5 g. and then 1·0 g. on each succeeding day, the whole amount being dissolved in 5 litres of water to which sugar or molasses was added.

Initial experiments in the treatment of canine piroplasmosis and East Coast Fever of cattle were conducted in Cambridge but they yielded negative results. The experiments on East Coast Fever have recently been published by Nuttall (25. vi. 1915).

The glowing accounts given by Brieger and Krause of the cures they had effected with the dye in the treatment of experimental Nagana, soon led to its trial by other investigators. Rietz and Leupold (VII. 1913) reported adversely upon its efficacy in experimental Nagana and stated that tryposafrol exerts a toxic action. Brieger and Krause (1914) claim, however, that the experiments reported by Rietz did not fulfil the conditions required. Whereas the authors used guinea-pigs and rats, Rietz and Leupold experimented with mice coupled with highly virulent laboratory strains of *T. brucei* known as "Ehrlich ferox" and "Morgenroth." The authors deny that tryposafrol is toxic for they report having given 1 g. a day for 30 days to a man and for about 50 days to a dog. Whereas tryposafrol may produce diarrhoea they find that *novo-tryposafrol* (an improved preparation) does not do so.

The dye was next tried by others in the treatment of *sleeping sickness* in man. Lurz (IX. 1913) reported upon 22 cases treated by Wittrock and himself: *Group I* consisted of six patients in a relatively good condition; the patients received 0·25 g. tryposafrol *per os* as directed. When given on an empty stomach the dye produced nausea, and diarrhoea on the day following. The dye was best supported when given dry after meals. The urine and faeces were stained red from the second day onwards. The dye exerted no beneficial effect. *Group II* consisted of six patients. Acting on the advice of Brieger and Krause the dose was reduced to 0·1 g. daily for a week, after which the dosage was further decreased. Result negative. *Group III* consisted of ten patients who received 0·1 g. per day for 10 days, then 0·2 and 0·3 g. up to the 42nd day. Result negative. Lurz therefore concluded that tryposafrol, both in large and small doses, is useless in the treatment of sleeping sickness.

Werner (IV. 1914) treated two cases of sleeping sickness in Europeans with tryposafrol supplied by Brieger and Krause. The dose ranged from 0·1 to 0·39 g. The dye caused diarrhoea and did not cure the patients. Similarly Mouchet and Dubois (1914), working in the Congo, state that the dye is useless in the treatment of human trypanosomiasis.

Furthermore, in the paper by Brieger and Krause (1914), already quoted, the somewhat surprising statement is made that tryposafrol (or novo-tryposafrol) is capable of curing the most diverse diseases besides trypanosomiasis: a report reached the authors from Brazil of the successful treatment of a case of *Leishmaniasis* in man and good results have been obtained in the treatment of *Foot and Mouth Disease* in cattle, *Swine Fever*, and *Distemper* in dogs. *Piroplasmosis in cattle*, as it occurs in Europe, has been treated with brilliant results ("glänzende

Erfolge") and success has followed the treatment of mules and donkeys suffering from *trypanosomiasis* in German East Africa and Cameroon. These claims are bewildering and the manner in which they are presented compels criticism.

That the authors are inaccurate in some of their statements is clear. Referring to piroplasmosis of cattle in Europe they write "die wohl identisch ist mit dem Texasfieber in Amerika und dem Küstenfieber in Ostafrika" and in a footnote we read that "Nuttal und andere haben Trypanrot und Tryganblau (*sic*) bei Piroplasmose der Rinder erfolglos angewendet." It would take some ingenuity to compress more errors into as few words. European Redwater or Piroplasmosis is not identical with Texas Fever and East Coast Fever is in no way related to either of these diseases. Nuttall and Hadwen and others have demonstrated in the most convincing manner that Trypanblue does cure piroplasmosis in cattle as well as in dogs and horses.

The statements regarding the curative effects of tryposafrol in some of the diseases above enumerated are based on insufficient data. A single case of *Leishmaniasis* successfully "treated" cannot be accepted as evidence, it may have been a case of spontaneous recovery. The reports on the curative effects in *bovine piroplasmosis* possess no value since we know how frequently cattle recover anyhow from the European disease (due to *Piroplasma divergens*) and there is no evidence that the veterinarians who tried the dye were competent to form an opinion or that they made any observations of an accurate character following the methods laid down by Nuttall and Hadwen in 1909 (*Parasitology*, II, 236–266). The methods used by the latter authors are essential when it is desired to test the efficacy of a purported remedy for piroplasmosis and it is well known how fallacious may be the conclusions of the ordinary veterinary clinician. When the results obtained with tryposafrol on piroplasmosis dogs in Cambridge proved negative, it was assumed that the like would hold for bovine piroplasmosis because the *only* drug which cures both of these diseases is trypanblue as demonstrated by ample clinical and experimental evidence collected before and after the discovery of the curative effects of the dye by Nuttall and Hadwen.

In view of the wide claims made by Brieger and Krause and the contrary results obtained by other authors it became necessary to carry out further experiments with the dye, especially on guinea-pigs infected with Nagana, so as to determine the effects of tryposafrol under similar experimental conditions to those reported by Brieger and Krause. In March 1914, one of us (G. H. F. N.) visited Berlin and had occasion to consult with representatives of the Aktien-Gesellschaft für Anilin-

Fabrikation who manufacture tryposafrol. These gentlemen desired that the claims advanced by the discoverers of the dye (Brieger and Krause) should be put to an independent trial and it was agreed that preliminary tests should be carried out in Cambridge, and, if positive results were obtained, that field experiments with animals suffering from Nagana should be carried out in Africa under the auspices of the Quick Laboratory Expedition then contemplated. The Aktien-Gesellschaft subsequently sent us various samples of tryposafrol and novo-tryposafrol for experimental purposes, the last samples being posted about 27. vi. 1914. We asked for the latter samples because we thought that the first negative results obtained might be due to some difference in the composition of the samples which we had received and tested.

In a letter from the Aktien-Gesellschaft dated as late as 7. vii. 1915 we were given further information regarding tryposafrol and about the samples we had used with negative results and returned to Berlin for chemical examination. The writers assured us that all the samples received by us were of the same chemical constitution and similar to those used by Brieger and Krause. They gave us the following particulars:

Tryposafrol readily dissolves in boiling water in the proportion of 11 : 00; even more concentrated solutions can be prepared in this manner. In a 1 % solution hardly any of the dye separates on cooling. (We had found that scarcely any of the dye went into solution in cold water, a fact which Brieger and Krause do not state in their publications.) Novo-tryposafrol differs from tryposafrol through being a tannin compound; it is barely soluble in boiling water and is only administered in suspension. Brieger and Krause recommend the following doses: for cattle and other large animals 1 g. thrice a day dissolved in much water; for pigs (swine fever) and dogs (distemper) 0·1 g. thrice daily. Dr Curschmann had tested the dye and found it non-toxic in the dose of 0·3 per kilo. of animal in the case of dogs, guinea-pigs and rabbits.

We shall now turn to our own experiments with tryposafrol and novo-tryposafrol on guinea-pigs infected with Nagana and upon dogs infected with piroplasmosis.

Experiments in the treatment of Nagana in Guinea-pigs.

1*st series*.

Three guinea-pigs (Nos. 1, 2 and 3) were inoculated subcutaneously on 4. vi. 1914 (day 1) with infected guinea-pig blood containing 15 *T. brucei* per field. *Treatment* began on the day of inoculation, 0·05 g. of *novo-tryposafrol* being given daily in bran and water. The faeces were

coloured red by the dye from the third day[1]. Two guinea-pigs (Nos. 4 and 5) were inoculated at the same time as the foregoing and received the same treatment except that it began on day 5 and, in the case of guinea-pig 5, the dose was increased to 0·1 g. beginning with day 28.

(*Novo-tryposafrol.*)

Guinea-pig No.	Weight	Occurrence of trypanosomes in the blood on succeeding days, etc.
1	220 g.	1 tryp. per field on day 5: 20–30 tryps. per field on day 6. Death on day 6. At autopsy the intestines were found inflamed and distended.
2	215 g.	Showed no tryps. in its blood but died on day 5. At autopsy the intestines were found ulcerated.
3	220 g.	1 tryp. per blood-film on day 5; 0 on 6th; 2 per field on 7th; 6 and 2 per film on 8th and 9th; 0 on 10th and 11th; 4 per film on 12th and 13th; 1 per 4 fields on 14th; 1 per 2 fields on 15th; 15, 3, 4, 1, 1 and 6 per field on 16th–21st; 1 per 2 fields on 22nd; 1, 2 and 3 per film on 23rd–25th; 0 on 26th and 27th. Death on 27th day.
4	195 g.	1 tryp. per blood-film on day 5; 1 per 2 fields on the 6th. Death on 7th day.
5		1 tryp. per 10 fields on day 5; 5, 40, 3 and 1 per field on 6th–9th respectively; 6 per blood-film on 10th; 0 on 11th; 1 per film on 12th; 0 on 13th–16th; 1 per 2 fields on 17th; 1, 2 and 3 per field on 18th–20th; 1 per 3 fields on 21st; 2 per film on 22nd; 1 per 4 fields on 23rd; 6, 1, 2, 6 and 1 per film on 24th–28th; 1, 2, 3 and 10 per field on 29th–32nd. Death on the 33rd day.

Six guinea-pigs (Nos. 6–11) were inoculated on 9. VI. 1914 (day 1) with heart blood of guinea-pig containing 25 *T. brucei* per field. *Treatment* began on the 3rd day, 0·1 g. of *Tryposafrol* being given daily in bran and water. The faeces appeared coloured from the 4th day.

(*Tryposafrol.*)

Guinea-pig No.	Weight	Occurrence of trypanosomes in the blood on succeeding days, etc.
6	220 g.	4 tryps. per blood-film on days 4 and 5; 1, 45, 50 per field on 6th–8th; numerous tryps. on 9th–11th. Death on 11th day.
7	260 g.	6 tryps. per film on day 4; 1 and 6 per field on 5th and 6th respectively. Death on 7th day.
8	225 g.	4 tryps. per film on day 4; 1 and 6 per field on 5th and 6th. Death on 7th day.
9	250 g.	1 tryp. per film on day 4; 1 and 12 per field on 5th and 6th. Death on 7th day.
10	230 g.	2 tryps. per film on day 4; 8 per film on 5th; 5 per field on 6th; numerous tryps. on 7th. Death on 7th day.
11	215 g.	3 tryps. per film on days 4 and 5; 3 per field on 6th; numerous tryps. on 7th and 8th. Death on 8th day.

[1] At autopsy, in all our experimental animals, the dye was found confined to the intestine; it does not cause diffuse staining like trypanblue.

Control Experiments.

Three guinea-pigs (A, B and C) served as controls to the treated guinea-pigs Nos. 1–11. They were inoculated on the same day as guinea-pigs 1–5 with the same amount of infected blood; the animals received no treatment.

Guinea-pig	Weight	Occurrence of trypanosomes in the blood on succeeding days, etc.
A	190 g.	1 tryp. per field on day 5; 10 on 6th; many on 10th–11th; 15–20 on 12th–14th; 1 per 2 fields on 15th; 8, 30, 25, 50 on 16th–19th; many on 21st–23rd. Death on 23rd day.
B	235 g.	2 tryps. per field on day 5; 30 on 6th; many on 10th; 1 on 11th; 2 and 6 per film on 9th and 10th; on the 11th–23rd the number of tryps. per field varied as follows: 6, 8, 3, 30, 10, 1, 10, 4, 1, 2, 4, 40, 6; 1 tryp. per 4 fields on 24th; on the 25th–35th their number per field numbered each day 1, 2, 2, 1, 1, 3, 3, 6, 6, 4 and 30 respectively; numerous on the 36th; on the 37th–40th they numbered 10, 1, 6, 50 per field; numerous on 41st–44th. Death on 44th day.
C	210 g.	1 tryp. per field on day 5; 12 on 6th; numerous on 7th–8th; on the 9th–16th they numbered 20, 1, 2, 45, 1, 1, 12 and 50 per field respectively; 6 per film on 17th; on the 18th–23rd there were 6, 10, ¼, 4, 20 and 6 per field respectively; 4 per film on 24th; on the 25th–27th there were 1, 4 and 6 per field respectively; numerous on 28th–29th; on the 30th–32nd there were 20, 40 and 40 per field respectively; numerous on 23rd; 1 and 4 per film on 34th–35th; on the 36th–39th there were 1, 20, 40 and 30 per field respectively; numerous on 40th–45th. Death on 45th day.

2nd Series.

Four guinea-pigs (Nos. 12–15) were inoculated subcutaneously on 10. VII. 1914 (day 1) with guinea-pig blood containing 10 *T. brucei* per field. *Treatment* began on the day of inoculation; 0·1 g. of *novo-tryposafrol* was given daily in bran and water. The faeces were coloured with the dye from the 4th day.

Four guinea-pigs (Nos. 16–19) inoculated at the same time and in the same manner as Nos. 12–15. *Treatment* began on the day of inoculation; 0·1 g. of *tryposafrol* was given daily in bran and water. The faeces became coloured as in the others.

(*Novo-tryposafrol.*)

Guinea-pig No.	Weight	Occurrence of trypanosomes in the blood on succeeding days, etc.
12	250 g.	1 tryp. per film on 6th day; 2 on 7th; 1 per 4 fields on 9th; 0 on 13th and 15th; 1 per 10 fields on 18th; on the 19th–23rd there were 2, 5, 20, 10 and 15 per field respectively; numerous on 26th. Death on 26th day.
13	245 g.	1 tryp. per film on 6th day; on the 7th, 9th, 13th and 15th there were 1, 2, 1 and 2 per field respectively; 25 per field on 18th. Death on 18th day.
14	235 g.	1 tryp. per film on 6th day; 0 on 7th; 1 per 10 fields on 9th; 0 on 13th–14th; 1 per 2 fields on 18th; 1 per 10 fields on 19th. Death on 19th day.
15	225 g.	1 tryp. per film on 6th day; 2 on 7th; 1 per 3 fields on 8th; the animal showed paralysis in the hind quarters and acute diarrhoea before. Death on 8th day.

(*Tryposafrol.*)

16	320 g.	1 tryp. per film on 6th day; 1 per field on 7th; 0 on 9th; 1 per 10 fields on 13th; 5 on 15th; 15 on 18th; many on 19th. Death on 19th day.
17	355 g.	2 tryps. per film on 6th day; 1 per 10 fields on 7th; 1 per 2 fields on 8th. Death on 8th day.
18	245 g.	1 tryp. per film on 6th day; 1 per 10 fields on 7th, the animal shows paralysis of hind quarters. Death on 7th day.
19	255 g.	2 tryps. per film on 5th day; 1 per field on 6th; 5 on 7th. Death on 8th day.

Control Experiments.

Three guinea-pigs (D, E and F) served as controls to the treated guinea-pigs Nos. 12–19, being inoculated on the same day and with the same blood; the animals received no treatment.

Guinea-pig	Weight	Occurrence of trypanosomes in the blood on succeeding days, etc.
D	360 g.	1 tryp. per film on 5th day; 1 per 4 fields on 6th; 1 per 5 fields on 7th; 15 per field on 12th; on 14th–19th there were 1, 10, 5, 20, 15, 10 per field respectively; numerous on 25th. Death on 25th day.
E	230 g.	1 tryp. per film on 5th day; 6 on 6th; on the 7th, 12th, 14th, 17th–20th days there were 5, 20, 15, 2, 15, 40 and 25 per field respectively; numerous on 22nd, 25th and 27th. Death on 27th day.
F	225 g.	1 tryp. per film on 5th day; 1 per 6 fields on 6th; on the 7th, 12th, 14th, 17th and 18th there were 5, 15, 15, 35 and 20 per field respectively; numerous on 19th, 20th and 22nd. Death on 25th day.

Experiments in the treatment of Piroplasmosis in Dogs.

Five dogs, on 14. v. 1914 (day 1), were inoculated subcutaneously with 5 c.c. of defibrinated blood containing *Piroplasma canis*. One of these dogs (dog A) served as a control, and four dogs (dogs Nos. 1–4) were subjected to treatment with *novo-tryposafrol*; the first dose was mixed with the food, the subsequent doses were suspended in water and given directly *per os*. Treatment began on the day of inoculation. The faeces showed the presence of the dye on the third or fourth day.

Dog 1. Fox terrier, weight 10·5 lbs.

Day	Temp. °F. a.m.	p.m.		Treatment
1			The dog was inoculated	0·05 g. novo-tryposafrol
2				0·1 „ „
6	103·4		Fever began; parasites found in dog's blood	
		102·6		
7	105			0·2 „ „
		104·6		
8	104·8			0·4 „ „
		105	Weight 9·5 lbs.	
9	103·5			0·4 „ „
		104·6		
10	103·2			0·4 „ „
		102·2	Dog not feeding	
11	101		Temperature falling; anaemie	0·4 „ „
		98·2		
12			Weight 8·5 lbs. Dog died of piroplasmosis	

Dog 2. Terrier, weight 14 lbs.

Day	Temp. °F. a.m.	p.m.		Treatment
1			The dog was inoculated	0·05 g. novo-tryposafrol
2				0·1 „ „
6	102·6		Fever began; parasites found in dog's blood	
		103		
7	102·2			0·2 „ „
		103·8		
8	103·4		Dog not feeding	0·4 „ „
		103·8		
9	103		Dog very weak, anaemie. Died from piroplasmosis	0·4 „ „

Dog 3. Collie, weight 38·5 lbs.

Day	Temp. °F. a.m.	Temp. °F. p.m.		Treatment
1			The dog was inoculated	0·05 g. novo-tryposafrol
2				0·1 ,, ,,
3				0·1 ,, ,,
4				0·1 ,, ,,
5				0·2 ,, ,,
6	101·2			0·2 ,, ,,
		103·8	Fever began	
7	105			0·2 ,, ,,
		104·8		
8	106·2		Weight 37 lbs.	0·4 ,, ,,
		106·4		
9	105·6			0·4 ,, ,,
		105·2		
10	103		Dog not feeding	0·4 ,, ,,
		103·3		
11	104·4		,, ,,	0·4 ,, ,,
		105·4		
12	105·6		,, ,,	0·4 ,, ,,
		105·8		
13	104·3		,, ,,	0·4 ,, ,,
		104		
14	103·8		Dog feeding a little	0·4 ,, ,,
		103·7		
15	104·5		,, ,,	0·4 ,, ,,
		104·6		
16	104·6		Dog not feeding, weak, anaemic	0·4 ,, ,,
		103·9		
17	103·4		Weight 31 lbs. Haemoglobinuria. Died of piroplasmosis	0·4 ,, ,,

Dog 4. Sheep dog, weight 37·3 lbs.

Day	Temp. °F. a.m.	Temp. °F. p.m.		Treatment
1			The dog was inoculated	0·05 g. novo-tryposafrol
2				0·1 ,, ,,
3				0·1 ,, ,,
4				0·1 ,, ,,
5				0·2 ,, ,,
6			Parasites appear in dog's blood	0·2 ,, ,,
7	103·4		Fever began	0·2 ,, ,,
		103·2		
8	103·2			0·4 ,, ,,
		102·8		
9	104			0·4 ,, ,,
		104·6		
10	103·1			0·8 ,, ,,
		102·8		
11	104·2			0·8 ,, ,,
		105·3		
12	104·6		Weight 38 lbs. Weak, anaemic, diarrhoea, dyspnoea. Died of piroplasmosis	

Dog A (eontrol). Collie, weight 38 lbs. Not treated.

Day	Temp. °F. a.m.	p.m.	
1			The dog was inoculated
2			
3			
4			
5			
6	101·5		Parasites appear in dog's blood
		102·6	
7	105		Parasites inereasing. Fever began
		104·2	
8	104·2		Weight 37 lbs.
		104·6	
9	105·4		
		104·8	
10	105		Dog not feeding
		104·4	
11	105·4		,, ,,
		105·1	
12	105		,, ,, anaemie, haemoglobinuria
		105·2	
13	105·2		,, ,, Dog died of piroplasmosis

Summary and Conclusions.

All of our guinea-pigs infeeted with *Trypanosoma brucei* (strain "ferox") died whether they were treated or not. It is evident that both tryposafrol and novo-tryposafrol exerted a direetly injurious effeet upon the guinea-pigs. Reekoning the day on which the guinea-pigs were inoeulated as day 1, the 19 treated guinea-pigs died respeetively on days 5, 6, 7, 7, 7, 7, 7, 7, 8, 8, 8, 8, 11, 18, 19, 19, 26, 27 and 33. The six untreated guinea-pigs died respectively on days 23, 25, 25, 27, 44 and 45. The two preparations of the dye are therefore worse than useless as remedies for Nagana in guinea-pigs.

Five dogs were infected with *Piroplasma canis* (Cambridge strain) of whieh four were treated and one not treated with novo-tryposafrol. All of the dogs died although treatment was given under the most favourable conditions, starting on the day of inoculation. The four treated dogs died on days 12, 9, 17 and 12 after inoeulation respeetively; the untreated (eontrol) dog died on the 13th day. The drug exerted no influenee upon the eourse of the disease, nor upon the appearanee of the parasites and their progressive increase in the blood. Novo-tryposafrol may therefore be regarded as useless in the treatment of eanine piroplasmosis, and, judged from these results on dogs, it will no doubt prove

to be equally useless in the treatment of bovine piroplasmosis when it has received a scientific trial in competent hands.

In view of the negative results obtained by ourselves and other independent investigators, working especially with trypanosomiasis, we conclude that the value of tryposafrol or novo-tryposafrol as a remedy for any of the diseases enumerated by the authors is open to grave doubt since the chief claims as to its efficacy were based on experimental results which the authors state that they obtained with Nagana.

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