OBSERVATIONS ON SARCOCYSTIS RILEYI (STILES).

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On March 22, 1911, there was received at the laboratory of the Zoological Division of the Bureau of Animal Industry, Washington, D. C., a piece of the breast of a Mallard duck, in which were Sarcosporidian cysts. A preparation of the spores of this parasite, made for purposes of identification, showed it to be *Sarcocystis rileyi* (Stiles). This form is by no means rare in Mallards, Shoveller ducks and domestic ducks. The preparation, however, showed certain morphological details in the so-called spores which do not appear to have been described. In consequence, a number of preparations were made, the study of which brought to light the facts set forth below, which are believed to be of considerable interest and of some theoretical importance.

The duck from which the material was obtained had been shot near the mouth of the Illinois River, probably very shortly before March 20. The specimen was handed to one of the veterinary inspectors of the Bureau of Animal Industry, at Chicago, and by him forwarded to Washington. The meat had merely been sprinkled with borax, and reached Washington in good condition. It was quite liberally parasitized. The cysts were confined mainly to the more superficial portions of the breast of the duck, a good many lying immediately beneath the connective tissue covering the muscle.

It is believed that the parasites were still alive, or, if not, had been dead for so short a time that they had not suffered any degeneration. Examined fresh, they presented a picture characteristic of living organisms. They fixed well, and gave good stained preparations. On the other hand, attempts to stimulate them to display movement failed. Mounts were kept for several hours in the incubator, and brought at once under the microscope. Others were kept for a considerable period over the dark field illumination, since this procedure heats the preparation. But in no case were any of the spores[1] seen to move.

[1] The propagative bodies found in the cysts of Sarcosporidia are conventionally designated spores, and hence that term is used here. It is much more likely, however, that they are the homologues of the sporozoites of Gregarines, Coccidia and Hæmosporidia.

This does not prove that the spores were no longer living, as it is by no means easy to get Sarcosporidia spores to display movements. Some years ago, at the Pathological Laboratory of the Medical School of the University of Pennsylvania, some experiments were made with the spores of *Sarcocystis muris*. It was found that if these were kept in the incubator, in some appropriate medium, and then examined at a temperature of 32° C., very lively movements were displayed. These movements, however, were not maintained for any length of time, even at this temperature, and at ordinary room temperature quickly ceased. It is evident that the organisms are sensitive to any fall of temperature below the so-called blood heat.

Methods.

Both fresh and prepared material were studied. The latter was either smears or paraffin sections. The smears were prepared as follows: A cyst was removed from the muscle, placed on a slide with a small drop of salt solution, and broken into several pieces with needles. These pieces were then smeared over the slide, thus scattering the spores, and the preparation fixed before it had dried. Three methods were used, the best results being obtained with fixation in vapor of osmic acid. For this a wide-mouthed, glass-stoppered bottle was used, in which was placed a small quantity of a 4 per cent. solution of osmic acid. The wet smear was left in this bottle ten to thirty seconds, sometimes more, and then transferred at once to absolute alcohol. The air in such a bottle is saturated not only with osmic acid vapor, but with water vapor as well and the smears do not dry. It may be noted that any osmic acid solution which adheres to the slide after removal from the fixing bottle must be wiped off before immersion in alcohol.

Other smears were fixed by dropping on them, while still wet, a quantity of fixative. Two of these were used: Hermann's fluid and an alcoholic corrosive acetic mixture made up as follows:

95% alcohol	50 parts.
Sat. sol. of $HgCl_2$ in water	50 parts.
Glacial acetic acid	5 parts.

Both of these fluids caused the parasite to swell and to become relatively very much broader than it was in the fresh state. Nor were the structural details preserved anything like so well as with fixation in osmic acid vapor.

Three stains were used:

1. Iron hæmatoxylin and acid fuchsin.

2. Thionin and acid fuchsin.
3. Wright's stain.

The two former were used in the usual way. With Wright's stain, the conventional procedure was slightly varied. The smear, kept in absolute ethyl alcohol, was first washed in absolute methyl alcohol. It was then stained in the usual way, but after being washed in water, it was dehydrated with alcohol and transferred to oil. It could therefore be brought into balsam without ever becoming dry.

The material for paraffin sections was fixed in Hermann's fluid.

Observations.

Fresh Material.

The Cysts.—As already stated, the cysts were mostly aggregated in that portion of the muscle lying beneath the skin. As is usual with Sarcosporidia, their long axes lay in the same direction as the long axes of the muscle fibres. Stretching of the muscle caused the cysts to lengthen and become narrower, they being in all cases very soft and flexible. They were very easily teased out from the surrounding host tissue, and could be obtained free of all such tissue and wholly intact without the least difficulty.

Both *in situ* and when free on the slide, the form was that of a cylinder with rounded ends. That is, the diameter was sensibly uniform throughout, although, when the muscle surrounding a cyst was stretched, the cyst became somewhat narrower at the ends.

The length varied from 2.75–6.5 mm.; the diameter from .6–1.0 mm. There was, however, no constant relation between length and thickness, and indeed the shorter cysts were frequently the thicker. Four, selected at random, and measured while in position, gave the following:

6.0 by .60 mm.
5.5 by .75 mm.
4.0 by 1.00 mm.
6.5 by .60 mm.

It is probable that the ratio of length and breadth depends a good deal on the pressure exerted on the cyst by the surrounding tissue. Obviously, also, in the living bird, the parasites are subjected to very violent stresses and strains.

The Spores.—Preparations made by teasing the cysts in salt solution were examined under the microscope. The spores varied a good deal in size and shape. Some were short, broad ovals, others had somewhat

the outline of a fish. The long, narrow spores (Plate XXXVI, figs. 1–3, 10–12), which were in the great majority, sometimes tapered regularly from the broad to the narrow end. In others, however, the contours were not smooth, there being little irregularities and swellings along the periphery of the body. The short, broad individuals, as will be shown later, were probably degenerate.

A vacuole near the centre could generally be seen. At times, also, a second vacuole could be made out very close to the broad end.

In a number there could be seen a dark, refractive body, rounded or rod shaped. It was sometimes near the centre; in other cases nearer the broad end.

A few of the long forms were slightly curved, but as a rule the longitudinal axis was sensibly a straight line. In a few the cytoplasm contained granules.

Measurements of several individuals, taken at random, gave:

Long.	*Short.*
13.8 x 2.6 microns.	8.3 x 4.1 microns.
13.8 x 3.1	8.3 x 4.1
14.5 x 2.1	8.3 x 3.1
14.5 x 2.6	9.4 x 2.6
15.0 x 2.6	9.4 x 2.6
14.5 x 3.1	

Hence the length varies from 8.3–15μ; the breadth from 2.6–4.1. Excluding, however, the short forms, believed to be degenerate, it is found that the spores are about 14–15μ long by 2–3 broad.

Fixed Material.

Cysts.—In cross section the cysts show a differentiation into what Stiles has termed central core and peripheral layer or zone. This differentiation, while quite evident when the cross section is viewed with low powers, has no morphological significance. The peripheral zone is that portion of the cyst in which the spores are still alive; the central core that portion wherein they have died and degenerated. This was pointed out by Stiles.

In the case of one of the mounted specimens, the cross section was oval, measuring 1.05 by .84 millimeters. The central core was roughly .71 x .57, while the peripheral shell varied in thickness from .105 to .194 mm. Presumably, in the living cyst, the central core will occupy the geometrical centre of the cyst.

Hence, in this particular case, there had been distortion, which

was also indicated by the fact that where the shell was thin, it was quite dense, and where thick, very loose and open. The distortion was probably due to exigencies of fixation, but, as already indicated, the cysts must be subjected to great distortion during the flight of the bird.

There was the mesh-work characteristic of sarcosporidian cysts, the meshes being coarser at the periphery and finer in the centre.

In the former position they measured from 10–20μ across; in the latter they were as small as 1–2μ.

In the material on which this study is based the cysts, being mature, lie between the muscle fibres and not within them.

Stiles, studying the parasites of five ducks, found intermuscular cysts alone in four, but in the fifth specimen both intermuscular and intramuscular stages were seen. He states that in the latter, the cysts were thinner and showed no non-staining central core. Although Stiles suggested that they were only developmental phases of the same form, he followed Blanchard's classification, calling one *Balbiania rileyi* and placing the other in the genus *Sarcocystis*. It is now known that the distinction between inter- and intramuscular positions is only a question of development. The young stages lie within the muscle fibre, but as they grow they become too large to be contained within it. According to Minchin, the originally parasitized fibre is ruptured and the cyst escapes. It seems more probable, however, that the fibre is merely almost wholly destroyed, its remnants remaining around the parasite as an adventitious cyst. However this may be, in the present case, the parasites were inclosed within a tightly stretched membrane, showing a considerable number of long, narrow nuclei. This was derived from the host. The actual cyst membrane is very thin, homogeneous, and part and parcel of the net work.

The compartments, as we have seen, grow progressively smaller from without inwards. Within them are the spores.

These, in the peripheral compartments, are seen to be elongated elements radially arranged. That is, as seen in the cross section of the cyst, the spores are in more or less definite files directed along the radii of this cross section.

In the central portion of the central core, the meshes of the net are filled with debris, which stains only with the plasma stain. Further out, however, it is frequently possible to see that this debris is more or less well divided up into little aggregates, doubtless each such aggregate standing for a spore which has died and disintegrated. In such situations chromatin masses are also frequent, following the

well-known rule that chromatin does not break down so quickly as cytoplasm.

In general, the line of demarcation between the normal and degenerate spores was abrupt. The same compartment might be filled partly with normal spores and partly with debris.

The degenerating spores differ from the normal spores in being shorter and broader, even at times round. As the spore changes in shape, the cytoplasm becomes very loose and to a large extent loses its ability to stain, the spores frequently taking on the form of a chromatin mass lying in an empty shell. It is on account of these observations on the sectioned cyst that the short, broad spores found in the fresh preparations are believed to be degenerate.

The relative extent of central core and peripheral layer in the make up of the cyst is merely a question of its age. Thus, Stiles found the small, intramuscular cysts to be without a central core. Further, in his description of the intermuscular form, Stiles gives .48 mm. for the thickness, and in his figures the central core is relatively very much smaller than it is in my material, where the diameter is as much as 1 millimeter. It is merely a matter of evolution. The cysts herein described are older than those studied by Stiles and spore degeneration has proceeded much further.

Smears.—The description which follows is based wholly on material fixed in osmic acid vapor and absolute alcohol, the other fixations giving obviously bad results. The three different stains mentioned above were all used, and all were good. They gave, however, quite different appearances, which will be noted as the description proceeds. Yet although the appearances were quite different, it was perfectly easy to correlate them. No one of the three stains showed any structural details not shown by the other two. The distinction between them had to do primarily with the chemical composition of the different parts of the spore.

As was to be expected, the sharpest pictures were obtained with iron hæmatoxylin and acid fuchsin. This method was best for the detection of the presence and form of the figured elements. But iron hæmatoxylin does not distinguish between different grades of chromatin, staining that containing much nucleic acid the same as that which contains little. Differences of this sort were, however, brought out by thionin and Wright's stain.

The observations were made with a 2 mm. apochromatic oil immersion lens, with a No. 12 or No. 18 eye-piece. These give, respectively, 1,500 and 2,250 diameters. The light was obtained from an incan-

descent gas lamp, and no trouble was experienced in getting excellent definition.

The spore, in the fixed smears, shows distinctly a differentiation into a broad and a narrow end. As in the fresh material, the broad end is rounded; the narrow end tapering and sometimes almost pointed.

The cytoplasm is either homogeneous, staining solidly, or there can be made out a poorly defined spongioplasm. This latter is mostly confined to the broad end of the spore, where there can often be seen minute vacuoles. In this part, also, rather coarse granules are frequent, which take the chromatin stain. It was not possible, however, to say whether these belong to the cytoplasm or to the nuclear apparatus of the spore. They were usually in association with the nucleus-like body in the centre of the parasite, and may belong to the class of the so-called metachromatic granules which have been described as present in sarcosporidian spores.

In the narrow end of the spore, the cytoplasm nearly always stained homogeneously, and in many cases was so solid near the extremity as to suggest a differentiation such as occurs in the anterior end of Telosporidian sporozoites. In these, the cytoplasm is greatly stiffened and forms a sort of beak, which enables the parasites to bore into the cells of the host. This is the only indication obtainable as to which is the anterior end of these spores of *Sarcocystis rileyi*.

The cytoplasm takes the usual stain, being red in acid fuchsin and blue in Wright's stain.

A distinct periplast is present, which stains the same as the rest of the cytoplasm. It could easily be seen in all of the smears, and was well demonstrated with those fixed in Hermann's fluid, where, as a consequence of the violent invasion of the cell by the fluid, it was frequently torn loose from the entoplasm.

Measurements show that there is quite a little shrinkage during fixation. The fresh spores measured 14–15 microns long by 2–3 broad. Those free in the smears range from 9–13.4 long by 1.7–2.3 broad. In a number of cases, however, there were present in the smears portions of the cysts, showing the spores still lying within the compartments. Measurements of such spores showed them to be 11.4–13.0 long by 1.6–2.3 microns wide, and gave a mean of 12.2 by 2 microns. In these fixation is probably a trifle slower, and hence there is not so much shrinkage.

Internally, counting from the broad to the narrow end, the spores show:

(1) A vacuole.

(2) A chromatin body.
(3) A vacuole.
(4) A chromatin body.

These four elements are normal constituents of all of the spores, although it is often difficult to see them all in any given spore.

The chromatin bodies are probably nuclei, but this point will be considered later. The vacuoles are not to be confounded with the small vacuoles frequently present here, as in all other protozoa, but are clearly morphological entities.

For convenience, the vacuoles will be referred to as vacuole No. 1 and vacuole No. 2; the chromatin bodies as chromatin body No. 1 and chromatin body No. 2, the count being made from the broad to the narrow end of the spore.

Vacuole No. 1.—This, when distinct, appeared as a very narrow ring, inclosing a clear space. In other cases it was not nearly so sharply differentiated, appearing only as a vague, poorly defined region, while very often it could not be seen at all. The space within the ring was sometimes colorless, but more often faintly stained, and at times took much the same stain as the surrounding cytoplasm. Occasionally, it contained a faintly staining granule or irregularly shaped mass, staining a trifle more deeply than the ground substance. In spores still *in situ* within the compartments of the cyst the vacuole was often very conspicuous, presenting the aspect of a hole in the cytoplasm.

The vacuole was usually nearly or quite round. It was sometimes at the extreme end of the spore; in other cases some distance from the end. It varied a good deal in size, at times being large enough to fill the entire width of the cell, again quite small. The appearance is shown in figs. 1–3, 10, 12.

The different stains used made little or no difference in the appearance of this element.

It is possible that this is the so-called striated body said to be present in sarcosporidian spores, and interpreted as the homologue of the polar capsule of the spores of Myxosporidia.

Chromatin Body No. 1.—The appearance of this element is shown in figs. 1–3, 5, 6, 8 and 10–12.

Viewed under a magnification of some 700–800, it appeared as a solid, deeply stained, spherical, oval or roughly demi-lunar body, frequently broken into two equal or unequal parts. (Plate XXXVI, figs. 1–3, 5*c*, 8*d*, *e*, 10, 11). Under higher powers, however, and with an intense light, it was frequently possible to make out that it was

more or less lobulated, and apparently composed of several masses, closely compacted together (fig. 5*a*, *b*, *h*). Much more rarely it consisted of an aggregate of large granules rather than lobes (figs. 5*d*, *f*, 6*b*).

Sometimes the appearances suggested division into two (figs. 13, 8*d*, *e*).

Generally speaking, this body took a dense chromatin stain, but frequently it did not stain homogeneously, one part taking the chromatin, the other the plasma stain. Thus, the case shown by fig. 13 shows two heavily stained demilunes, with between them some plasma-like substance. Figs. 6*c* and 8*a*, *b*, *c*, show elements which are solid, but which stain partly like chromatin and partly like plasma. It is by no means unlikely that in cases where the element appears double (fig. 8*d*, *e*) the conditions really are as in fig. 8*b*, the plasmic portion having failed to stain. Further, in the case of such a body as is shown in figs. 6*c*, 8*a*, *b*, *c*, an alteration in the chemical composition of the central portion, or overstaining, would give elements such as are shown in figs. 1 and 2.

The data just given suggest that this element is a nucleus of the massive or compact type, such as the macronuclei of the ciliate Infusoria. It has ordinarily the aspect of a solid mass, but is composed rather of several closely compacted masses. Under certain conditions a larger or smaller portion of it changes in chemical composition and stains like albumin.

In smears fixed with Hermann's fluid this body frequently lay in a vacuole, but it is believed that such an appearance is the result of a too violent fixation.

In smears stained with iron-hæmatoxylin the color is sensibly black. In thionin it is deep blue, while in Wright's stain it is a nearly black garnet, precisely like the kinetonuclei of trypanosomes.

Vacuole No. 2.—In general this is more distinct than vacuole No. 1. Usually, it showed as a distinct ring, which was frequently irregularly thickened. At times, this vacuole has the appearance of a hole in the cytoplasm, and this was always the case when the spores were *in situ;* either in sections or in cyst compartments in the smears.

But in many cases, it is inconspicuous (see figs. 1, 3). Here it is seen to be irregularly shaped and quite small. More probably, this is an accident of fixation. This vacuole, in the spore, lies between two chromatin bodies, and hence can easily be pressed upon during fixation. In the spores in the cysts it is always very conspicuous, and it was also quite evident in the fresh spores.

Chromatin Body No. 2.—This apparently consists of a nuclear membrane, nuclear sap and chromatin which is typically in the form of a rounded karyosome. The appearances, however, vary to a very great extent. The chromatin is very often in the form of a rod (figs. 4*b*, *d*, 7*e*, *f*, *g*). It may also consist of two or more separate bodies (figs. 4*c*, 7*b*, *c*, *g*, *h*). At times the chromatin mass is irregular in shape, and appears to send out strands which unite with the membrane (fig. 4*e*). In a large number of cases the membrane cannot be demonstrated at all, the chromatic mass apparently lying free in the cytoplasm (figs. 4*c*, 7*b*, *d*, *e*, *f*, *g*).

In iron hæmatoxylin the karyosome or karyosomes take a deep chromatin stain and, as a rule, stand out sharply. With thionin or Wright's stain the chromatin reaction is given, but the stain is pale. This element obviously comes within the category of vesicular nuclei.

Summarizing the above observations, it is seen that the spores of *Sarcocystis rileyi* show two chromatic elements and two vacuoles. The latter, although rather curious possessions, offer no difficulties of interpretation. The young stages of certain hæmogregarines (for examples, *Lankesterella* and *Karyolysus*) typically show two vacuoles, although it is not known what their function may be.

It is not so easy, however, to explain the significance of these two chromatic elements. The one, the chromatin body No. 1, is typically a rounded solid element, belonging to the massive type of nucleus. It takes an intense chromatin stain, and is clearly an element high in nucleic acid. The other, chromatin body No. 2, consists of a nuclear membrane, inclosing a clear space within which is one or more karyosomes. These stain like chromatin. This vesicular type of nucleus is widespread in protozoa.

With iron hæmatoxylin there is no choice between the staining reactions of these two elements. Sometimes the one is more deeply stained, sometimes the other, but in general both are black, and the spores are seen at a glance to be binucleate. On the other hand, with Wright's stain, chromatin body No. 1 is larger and more conspicuous than it is with iron hæmatoxylin, while chromatin body No. 2 may at first glance be overlooked. The appearance here is of a uninucleated element. There is here very nicely illustrated the advantage of the use of more than one staining method, and the contention of the English School of protozoologists that iron-hæmatoxylin should be used in the study of trypanosomes receives emphatic support.

It is impossible to establish a correlation between the spores of *Sarcocystis rileyi* and those of other Sarcosporidia. Concerning the

latter, the most noteworthy feature of published figures is a lack of clear-cut details. In one of the most elaborate studies, that of Erdmann, the figured spores show vague differentiations, but, to the present writer, no exact correlation can be established. The granules figured by Erdmann may be the same as those sometimes seen in *Sarcocystis rileyi*, or they may stand for the ordinary sarcosporidian nucleus.

There is a general consensus of opinion that sarcosporidian spores show a vesicular nucleus, and this probably corresponds with the vesicular nucleus of *S. rileyi*. As already suggested, vacuole No. 1 may correspond to the supposed homologue of the polar capsule of myxosporidian spores.

But neither chromatin body No. 1 nor vacuole No. 2 appears to be represented in any sarcosporidian spores other than *S. rileyi* which have hitherto been described.

Conclusions.

(1) The spores of *Sarcocystis rileyi* are 14–15μ long and 2–3μ wide.

(2) One end is broad and rounded, the other narrow and tapering.

(3) The cytoplasm is spongy in the central portion, nearly or quite homogeneous in the narrow end.

(4) Within, counting from the broad end, the spore shows a vacuole, a chromatin body, a vacuole, a chromatin body.

(5) Vacuole No. 1 lies in or near the broad end. It is not usually very conspicuous.

(6) Chromatin body No. 1 is next in order to vacuole No. 1. It is apparently a nucleus of the massive or compact type and stains like the kinetonuclei of trypanosomes.

(7) Vacuole No. 2 lies roughly in the middle of the spore. It is usually more conspicuous than vacuole No. 1.

(8) Chromatin body No. 2 is usually nearer the narrow end than the broad end. It is apparently a nucleus of the vesicular type, the chromatin of which is typically aggregated into a single large karyosome.

(9) The spores of *Sarcocystis rileyi*, being apparently binucleate, are very different from any other sarcosporidian spores hitherto described.

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EXPLANATION OF PLATE XXXVI.

Figs. 1–3, 10–12 are made from camera outlines, drawn on the table with a 2 mm. objective and No. 18 eye-piece. The balance are free-hand sketches of the two chromatin bodies. The published figures are copies made by Mr. Haines, artist of the Bureau of Animal Industry, from pencil drawings made by the author.

Figs. 1–3, 6–12 are from smears stained with hæmatoxylin and acid fuchsin.

Figs. 4 and 13, from smears stained with Wright's stain.

Fig. 1.—Spore. All the elements large except vacuole No. 2. All further from the broad end of the spore than is usual.

Fig. 2.—Spore. Vacuole No. 1 small; other elements large. This spore is typical for the positions occupied by the vacuoles and chromatin bodies.

Fig. 3.—Spore. Vacuole No. 1 small; vacuole No. 2 almost obliterated. Karyosome of chromatin body No. 2 sending out strands toward the nuclear membrane.

Fig. 4.—Chromatin body No. 2. *a* is taken to be the typical appearance, but *b* and *d* are very frequent; *e* is not so often seen.

Fig. 5.—Chromatin body No. 2. *a*, *b*, *c*, *g* and *h* are frequent appearances; *d*, *e* and *f* are not so often seen.

Fig. 6.—Chromatin body No. 2. Compare *a* with *h* of fig. 5. In *c* the element stains partly like chromatin and partly like plasma.

Fig. 7.—Chromatin body No. 2. *a*, *c* and *h* show the entire nucleus; in *b*, *d*, *e*, *f* and *g* only the karyosomes can be seen. This condition occurs frequently.

Fig. 8.—Chromatin body No. 2. In *a*, *b* and *c* the bodies stain partly like chromatin and partly like plasma. In *d* and *e* it is apparently double.

Fig. 9.—Chromatin body No. 2. Appearances frequently presented by the karyosome.

Fig. 10.—Spore. Vacuole No. 1 contains a granule. Chromatin body No. 2 has here the typical appearance of a vesicular nucleus with one karyosome.

Fig. 11.—Spore. The vacuoles are obliterated.

Fig. 12.—Spore. Chromatin body No. 2 shows three karyosomes.

Fig. 13.—Chromatin body No. 1. The element is here cleft into equal parts.