# THE MORPHOLOGY OF *ANAPLASMA MARGINALE* AND RESPIRATORY ACTIVITY OF ANAPLASMA-INFECTED BLOOD

by

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# THE MORPHOLOGY OF *ANAPLASMA MARGINALE* AND RESPIRATORY ACTIVITY OF ANAPLASMA-INFECTED BLOOD

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Anaplasmosis in cattle has become an increasingly widespread disease in the United States since it was first observed here more than 65 years ago. 50 years ago it was classified as a separate disease, but there is still confusion as to the actual classification of the causative agent, *Anaplasma marginale*.

This has been largely due to the lack of evidence concerning the nature of the anaplasm so that it could not be placed in any one category. The most widely accepted theory is, however, that the agent is a protozoan parasite, and it will be referred to as such in this paper for ease of reference.

This study was undertaken to add some additional evidence concerning the actual nature of the causative agent by (1) making Warburg respirometer measurements on anaplasma-infected blood in an attempt to establish the respiratory status of the parasite, and (2) by using the phase contrast microscope for making an intensive study of the morphology of *A. marginale* during its appearance in the blood stream.

# REVIEW OF LITERATURE

Anaplasmosis is characterized in the animal by anemia, depression, anorexia, weakness, constipation, muscle tremors, high temperatures, and, occasionally, icterus (8, p. 497). The parasites are first observed as small stained dots on the margin of the erythrocytes when stained with any Romanowsky stain. During a severe attack they are found in large numbers in infected red cells, while on recovery of the animal they usually disappear, but may periodically reappear at regular intervals (18, p. 215-216). Singly infected red cells are the most common, although two, and occasionally three or four anaplasms per red cell may be found.

Anaplasma bodies have an average diameter of 0.5 to 0.6 microns and are generally circular or oval, but irregular shapes may be found (24, p. 327-328). Boynton (3, p. 10-12), for example, was able to observe ring forms, and in highly infected blood, see small protuberances on the marginal bodies, giving the appearance of buds. Dikmans (6, p. 203-204), on the other hand, noted diads, triads, and multiple points, in addition to the classical forms, in a stained smear made during relapse following splenectomy. He interpreted these as possibly being forms that were rapidly dividing due to the absence of the spleen which ordinarily inhibits and restrains the infection.

Tails or projections were found in stained preparations of A. marginale by Franklin and Redmond (10, p. 252-253), and were described as being of various lengths, but always longer than the typically shaped head was wide. These workers felt that their observations represented one stage of development of the parasite; however, Boynton (3, p. 10-12) frequently noted that the substances of the host cell are drawn to the anaplasms and could give the latter the appearance of having a tail.

Lotze and Yiengst (14, p. 312-320), in a comprehensive study of the causative agent, classified the marginal bodies into two morphological types: (1) smooth anaplasms, which were 0.2 to 0.5 microns in diameter, spherical, and of smooth contour; and (2) rough anaplasms, which were 0.6 to 0.9 microns in diameter, roughly spherical, or, occasionally, cuboidal.

The smooth type appeared to be homogenous in nature, while the rough type seemed to consist of a number of small spheroid bodies of equal size. The spheroid bodies, for the most part, appeared to be in a box-like arrangement with a clear central space. Bodies, in these instances, measured 0.8 to 0.9 microns in diameter. Some erythrocytes from acute cases showed large numbers of small bodies, similar in morphology to the spheroids of the rough anaplasms, scattered throughout the stroma of the red cells. These

measured 0.2 microns in diameter and were similar to the smooth type anaplasms, except these latter forms occurred near the cell margin. It appeared probable, then, that these bodies resulted from ruptured rough anaplasms.

Stained erythrocytic forms about 1 micron in length were observed free in the plasma or attached to the outer margin of the red cell by these same workers, appearing as small rod-like structures with a knob-like head at one end measuring 0.2 to 0.3 microns in diameter. These forms were first observed the day before detection of the intracellular forms, and then found throughout the infection. In the greatest number of cases the free forms were found on non-infected erythrocytes, with as many as two being seen on the same cell.

Body size increases were found, especially at the start of the infection when the parasites first became noticeable; then a "size rhythm" developed as the infection progressed where the average size of the bodies increased, then decreased, then increased again, and so on.

From the various forms observed by these two workers, they reasoned that the extra-erythrocytic forms enter the red cell, develop into anaplasms, then undergo growth and eventual multiple division with the production of spheroids.

More recently, the electron microscope has been utilized to throw some additional light onto the nature of the causative agent of anaplasmosis. It was found by

De Robertis and Epstein (5, p. 254-258) that by using this instrument A. marginale could be divided into (1) elementary forms belonging to a mass, and (2) those distributed throughout the erythrocyte.

In the first group, which includes parasites visible in the ordinary light microscope, bodies were undivided in the central area, with rounded forms 170 to 220 millimicrons in diameter protruding from the surface. In the second case, they were found to be similar to the smaller forms observed within a mass with as many as 40 of these bodies being observed in one erythrocyte. Diameter measurements indicated that about 69% were within the 170 to 220 millimicron range.

Anaplasma bodies in ultrathin sections of infected red cells examined in the electron microscope appeared as one to seven masses of dense particulate matter measuring 0.2 to 0.7 microns in diameter. Greater magnification demonstrated a particulate composition of the anaplasms, the particle being approximately 100 Å in diameter. Organelles, however, such as nucleus, endoplasmic reticulum, and mitochondria were not distinguished in these forms (9, p. 148).

Very little has been reported concerning the histochemical nature of the parasite, but one paper outlines the results obtained employing stains to identify the chemical

components (17, p. 377-380). It was found that desoxyribonucleic acid, ribonucleic acid, protein, and organic iron were present. Further tests of this sort, however, showed the absence of arginine, tyrosine, polysaccharide complexes, lipids, fats, peroxides, inorganic iron, calcium, dehydrogenase, peroxidase, and alkaline phosphatase.

From the foregoing review it can be seen that the classification of Anaplasma marginale is difficult. It was first proposed by Theiler (23, p. 7-64) that it be classed as a protozoan, and it is still accepted as such by many. But in Bergey's Manual of Determinative Bacteriology (4, p. 931-934 and 980-984) it is classed in the order Rickettsiales, family Anaplasmataceae. Thus far, this classification has not become generally accepted.

Others regard the agent as a virus, or a reaction product of a virus. The fact that the number of infected red cells declines rapidly at a stage before the erythrocyte count reaches its minimum, at which time only one or two bodies per smear may be found, gave one author reason to express the belief that the anaplasm is an irritation product of a virus, perhaps similar to Negri bodies of rabies (8, p. 498). He was also able to produce the disease in an animal after filtering the agent through a 3 pound Mandler filter giving him further basis for classifying the agent as a virus (7, p. 19).

Evidence against the virus theory is the activation of the latent infection by splenectomizing an infected animal, as it is known that most viral diseases produce a sterile immunity after the virus disappears. Also, if the anaplasms were a "reaction product" of a virus, it is reasonable to assume that a case would arise where this symptom would be absent, but this has never been observed (24, p. 330-331).

# METHODS AND MATERIALS

The parasite used in the following experiments was Anaplasma marginale, strain 80 originally obtained from Animal 80, Oregon State College beef herd. The permanent source of the marginal body was a bovine carrier held especially for this purpose in an isolation stall at the Veterinary Department barn. Generally, non-carriers were infected by transferring 5 ml of venous blood from the carrier to the recipient by subcutaneous injection.

Several variations in the method of infecting calves were attempted in addition to the procedure described above. One animal, for example, received 5 ml of actively infected blood rather than carrier blood; while in an attempt to reduce the incubation period, two of the animals received transfusions of 350 to 400 ml of a 1:2 dilution of carrier blood in sterile Alsever's solution. Still another animal received 200 ml of a 1:2 dilution of actively infected blood in Alsever's solution after it had been previously inoculated with a 5 ml dose and a 400 ml transfusion of diluted carrier blood, both of which had failed to produce the disease within a reasonable length of time. Again, samples of deer blood were inoculated into two animals in an attempt to transmit anaplasmosis from deer, but failing this, the calves were later reinoculated with carrier blood.

Anaplasmosis in the calves used here was complicated by the presence of at least one, and perhaps two blood diseases characterized by stainable extracellular bodies, designated here as "atypical forms." This infection apparently was present in the original carrier animal and transmitted along with the anaplasma. The atypical infection did not always appear in every newly infected animal; it also varied in degree in different animals. When it did appear it was observed before the development of anaplasmosis, and seemed to lengthen the incubation period of the latter, often necessitating a reinoculation of the animal with a fresh sample of infected blood.

In Giemsa-stained blood smears two separate atypical forms were sometimes observed. Each seemed to be a pure infection, neither being found mixed with the other within the same animal. One appeared as a definite, dense body, shaped as a slightly curving rod, or as a half sphere, about the size of a large anaplasma body, attached to the periphery of the red cell. The second appeared to be finely granular forms, found either free in the plasma or attached to the periphery of the red cell. Both of these atypical forms were usually distinguishable from the anaplasma bodies in stained smears, although the rod and half-sphere forms sometimes had the appearance of anaplasms.

Differentiation of the anaplasma bodies from the atypical bodies was even more marked when the blood was observed under the phase contrast microscope. Anaplasms were distinguishable here as tailed inclusion bodies in the red cell and were not easily mistaken for other forms once they had been identified. The atypical rod and half-sphere forms failed to show up under phase contrast, while the granular forms were similar in appearance to those in stained smears.

Thus, even though these secondary infections were sometimes apparent in the animals being studied, they could readily be detected microscopically. All blood specimens used in this study contained typical anaplasma bodies in the red cells. Specimens containing the atypical forms were excluded from the data.

## Animals

All animals were held in screened quarters until the time of inoculation with infected blood. At this time they were moved into isolation stalls to separate them from other non-infected animals in the same building.

A total of nine calves were used for the production of infected blood, and were designated by numbers ranging from 402 to 413 (See Table 1). All were males, except for

TABLE 1

REVIEW OF ANIMAL INFORMATION

| Animal No. | Sex | Inoculation data | | | Record of Infection Following Inoculation | | | |
|---|---|---|---|---|---|---|---|---|
| | | Age of animal* (days) | Dose (ml) | Source of blood | C-F test reaction** | Appearance of anaplasma in the blood | Fever first observed*** | Outcome |
| 402 | F | 455 | 5 | No. 404 (carrier) | Reactor at 25 days | Before 22 days | 36 days | Expired, 38th day |
| 403 | M | 528 | ? | Deer | Negative at 36 days | None at 36 days | None | |
| | | 563 | | No. 404 (carrier) | Reactor at 6 days | 6 days | 6 days | Recovered by 54th day |
| 405 | M | 310+ | 5 | No. 404 (carrier) | Reactor at 21 days | Before 21 days | 27 days | Recovered by 35th day |
| | | (410)++ | - | ----- | Continued reactor | 20 days AS+++ | 32 days AS+++ | Recovered by 40th day AS+++ |
| 407 | M | 130 | 5 | No. 404 (carrier) | Negative | None at 26 days | None | |
| | | 156 | 5 | No. 404 (carrier) | Reactor at 1 day | 5 days | 15 days | Recovered after 15 days |
| 409 | M | 102 | 5 | No. 407 (active) | Reactor at 11 days | 27 days | 41 days | Recovered after 43 days |
| 410 | M | 127 | ? | Deer | Negative | None at 65 days | None | |
| | | 192 | 400 | No. 404 (carrier) | Reactor at 11 days | 14 days | 21 days | Recovered after 28 days |

TABLE 1 (CONTINUED)

REVIEW OF ANIMAL INFORMATION

| Animal No. | Sex | Inoculation data | | | Record of Infection Following Inoculation | | | |
|---|---|---|---|---|---|---|---|---|
| | | Age of animal* (days) | Dose (ml) | Source of blood | C-F test reaction** | Appearance of anaplasma in the blood | Fever first observed*** | Outcome |
| 411 | M | 111 | 5 | No. 404 (carrier) | Negative at 32 days | Doubtful at 18 days | None | |
| | | 144 | 350 | No. 404 (carrier) | Reactor at 1 day | 5 days | 17 days | Recovered after 61 days |
| 412 | M | 116 | 5 | No. 404 (carrier) | Suspect at 14 days | None at 38 days | None | |
| | | 154 | 400 | No. 404 (carrier) | Continued suspect | None at 17 days | None | |
| | | 171 | 200 | No. 410 (active) | Reactor at 4 days | 18 days | None | Recovering by 32nd day |
| 413 | M | 122 | 5 | No. 404 (carrier) | Negative at 31 days | None at 31 days | 7 days | Atypical infection |
| | | 155 | 400 | No. 404 (carrier) | Reactor at 19 days | 26 days | 35 days | Recovering by 40th day |

* All animals splenectomized at least 44 days prior to initial inoculation, with the exception of 405, which was splenectomized 140 days after the initial injection to produce a recrudescence of anaplasmosis in this animal.

** Reactors gave a 4+ reaction in a 1:5 serum dilution; suspects gave a 1+ to 3+ in a 1:5 serum dilution.

*** Fever temperature dependent on the average normal temperature of the particular animal, and ranged from 102.9°F to 103.7°F after the bodies had appeared in the blood.

\+ Non-splenectomized. ++Splenectomized. +++After splenectomy

402, ranging in age from approximately 4 months to 18 months at the time of the first inoculation.

The animals were splenectomized at least one month prior to the injection with infected blood, except in the case of animal 405, which was inoculated before having its spleen removed. However, because of the low grade infection produced in this animal it was later splenectomized to produce a recrudescence of the disease.

Control blood was collected as needed from both splenectomized and non-splenectomized animals of various ages, breeds, and sex. Splenectomized animals used for this purpose were those being held for future inoculation with anaplasma infected blood. All animals used for control purposes gave negative results with the complement-fixation test for anaplasmosis, except for 3 animals which gave suspect reactions. Phase contrast microscope observations of wet mounts from these animals, however, failed to show any anaplasma bodies.

## Examination of Infected Animals

After the initial injection, the newly infected animals were examined for the presence of the active infection, after an incubation period of 1 to 22 days, depending on the size of the inoculum. When the anaplasma bodies began

to appear in the red cells the animals were examined at least twice weekly, and sometimes daily. As the number of bodies subsided, or after it became apparent that the infection had run its course, checking of the animal was discontinued.

Examinations consisted of (1) taking the animals rectal temperature, (2) collecting slightly over 5 ml of venous blood with a dry syringe for the preparation of blood smears and oxalated blood for hematocrits, and (3) collecting another 10 to 15 ml sample of venous blood for the complement-fixation test. The latter samples were generally not collected more than twice weekly.

Hematocrits were run according to the method of Wintrobe (13, p. 60), using blood samples collected in tubes containing 0.5 ml of dried anticoagulant solution consisting of:

| | |
|---|---|
| Ammonium oxalate | 1.2 g |
| Potassium oxalate | 0.8 g |
| Formalin (preservative) | 1.0 ml |
| Deionized water | 100 ml |

Duplicate hematocrit tubes were run for each sample by filling to the top mark "10," centrifuging for one hour in either an Adams Safeguard centrifuge at 2430 revolutions per minute (according to the manufacturer's data) or in an International Clinical centrifuge operated at a measured 1820 rpm; then reading for the packed cell volume (pcv),

volume of the buffy coat, and the color of the plasma as to whether normal or icteric.

Blood smears were processed using Giemsa stain. Smears were first fixed in absolute methanol for three to five minutes, then stained one hour with a working solution of Giemsa stain, prepared fresh each day, consisting of:

| | |
|---|---|
| Giemsa stock solution | 1 ml |
| M/15 phosphate buffer, pH 6.5 | 2 ml |
| Deionized water | 47 ml |

The smears were then briefly rinsed with a solution made up of:

| | |
|---|---|
| M/15 phosphate buffer, pH 6.5 | 2 ml |
| Deionized water | 48 ml |

The Giemsa stock solution was prepared as follows:

| | |
|---|---|
| Giemsa powder | 1.0 g |
| Glycerine (95 to 98%) | 66.0 ml |
| Methanol (C. P. grade) | 66.0 ml |

The Giemsa powder was added to the glycerine, then this placed in a 60°C oven for 1½ to 2 hours after which the methanol was added. This was filtered through a No. 1 filter paper before use.

The number of anaplasma bodies and the per cent infected red cells were determined by the use of the oil immersion objective (97X) of the ordinary light microscope. A Breed and Brew ocular micrometer was inserted into the ocular (10X) of the microscope to facilitate the counting of cells. A tally counter was used to count the 500 red cells observed for anaplasma bodies while examining the smear,

which was usually done in a lengthwise manner through the smear near its center. All cells falling within the upper quadrants of the ocular micrometer were counted, and all parasites within these cells noted. In almost all cases, if the red cells showed evidence of being infected another smear was counted, thus making a total of 1000 red cells examined per animal.

The complement-fixation test was run by the staff of the Veterinary Science Department according to the methods published by the United States Department of Agriculture Research Service (August, 1958). Any serum sample giving a 4+ reaction in a 1:5 or higher dilution was considered to be a positive reactor for anaplasmosis.

## Blood Collection

Large samples of blood for experimentation purposes were collected by the "closed bleeding method", and immediately defibrinated.

The apparatus for performing this consisted of an Erlenmeyer flask into which a stainless steel wire coil had been inserted such that a spring was formed around the inside of the flask bottom. Into the top of the flask was inserted a 3 to 4 inch length of glass tubing about 3 mm in diameter, wrapped in cotton to form a plug. Next, a 2 to 2½ foot length of gum amber rubber tubing was, at one

end, attached to the glass tubing, and into the other end was inserted a hypodermic needle adapter. The apparatus was wrapped so as to prevent contaminants from entering, and sterilized by autoclaving at 121°C for at least ½ hour. A 12 gauge needle was sterilized at the same time.

At the time of bleeding, the area about the jugular vein on the animal's neck was thoroughly swabbed with tincture of iodine, after which the needle was inserted into the vein. Usually, at this point, a separate sample was collected for the complement-fixation test. Otherwise a small amount of blood was allowed to drain to get rid of any contaminants that may have been present, and the flask tubing was connected to the needle by inserting the adapter. As the bleeding proceeded the flask was slowly swirled to defibrinate the blood. When the required volume had been collected, which amounted to about 100 ml after defibrination, the needle was removed from the vein, and a pinch clamp put on the connecting tube to eliminate contaminants from the flask. Swirling was continued until the blood was completely defibrinated.

At the time of collection of the infected blood by this method a 5 ml syringe was also generally used to take a small sample from the animal for blood smear and hematocrit purposes.

A control, or normal blood sample, was always collected when a large infected sample was taken. The same procedures

as outlined above were employed, except the 5 ml sample was omitted.

## Phase Contrast Microscope Observations

A Leitz binocular Ortholux microscope equipped with a Heine phase contrast substage condensor, built-in illuminating system, and 90X oil immersion objective was used for examining blood specimens. Routine examination of the mounts was made using the oil immersion objective and 10X or 20X oculars giving a total magnification of 900 or 1800 diameters respectively. This microscope was further outfitted with a 35 mm Leica camera for taking photomicrographs.

In an attempt to correlate forms observed in wet mounts of infected blood with the typical anaplasma bodies seen in stained smears, a comparative count was made on frozen hemolyzed infected blood from several animals, and compared to counts made on smears prepared the same day. Wet mounts of non-infected blood served as control observations.

One of the prerequisites found for the examination of blood for internal parasites using the phase contrast microscope was that the erythrocytes be either partially or almost completely lacking in hemoglobin. Because of this fact, counts were made only on frozen hemolyzed blood.

Usually, at least one, and often two or three weekly samples were selected for comparative counts. Those most commonly used were the 5 ml oxalated samples originally used for hematocrits, the remainder of which had been stored for observation purposes. Occasionally, a small sample of defibrinated blood was also used in place of the oxalated sample. All samples were quick frozen by immersing them for one to two minutes in ethanol at -60°C; they were then stored at -60°C until observations could be made. At this time the samples were thawed and the mounts prepared.

Wet mounts were prepared by placing a small loop of the blood sample on a slide and covering it with a No. 1 cover slip. This was paraffined about the edge to exclude air from the mount, then examined, using much the same procedure as was used for stained smears described previously.

Counting was again facilitated by inserting Breed and Brew ocular micrometer disks into each of the 10X oculars. Several fields were observed, using the oil immersion objective, horizontally and vertically across the middle of the mount until 500 red cells had been tallied, all those falling within the area of the quadrants of the ocular micrometer being counted. The number of parasitized cells per field was noted, and the per cent infected erythrocytes recorded.

Hemolyzed control blood was also examined under phase contrast, although in these specimens, as well as those of infected blood that had not yet shown the presence of anaplasma, actual cell counts were not made. Rather than this, a "scan" was made of the mount by observing several fields vertically and horizontally across the middle of the wet mounts, thus insuring the absence of any anaplasms in at least 500 erythrocytes.

## Special Treatment of Red Cells

Other methods of hemolysis were tried in addition to freezing so that the anaplasma bodies could be observed. The addition of water to the red cells was one method used. Here, infected and normal blood samples that had been stored in Alsever's solution were first centrifuged at about 2000 revolutions per minute for 10 minutes, after which the supernatant fluid was removed. The cells were then usually washed in one volume of sterile Alsever's solution, again centrifuged, and the supernatant fluid removed. Next, sterile deionized or distilled water was added in the ratio of 15 volumes of water to one volume of packed cells, and this mixture placed in the 4° to 6°C refrigerator for 90 minutes to allow hemolysis to take place. Wet mounts were then prepared as previously described and examined under the phase contrast microscope.

A sterile saponin solution was also used to hemolyze cells by adding 0.1 ml of a 10% saponin solution to 0.9 ml of defibrinated blood or defibrinated blood stored in Alsever's solution giving a final saponin concentration of 1%. The mixture was incubated in a 37°C water bath for 30 minutes, cooled, then diluted to give a final saponin concentration of 0.5% to arrest its activity. Wet mounts were prepared of this hemolyzed preparation and examined under the phase contrast microscope.

Both of these methods were found to be less desirable for hemolyzing red cells than freezing. Water hemolysis appeared to cause partial or total disintegration of many of the anaplasma tails and some of the bodies of the anaplasma, while the saponin seemed to "erode" away some of the bodies to give miniature forms.

Actually, it was found that perhaps the best way to observe the bodies with the least amount of disturbance to them was to use defibrinated blood or defibrinated blood stored in Alsever's solution, without any further treatment. By observing preparations of this material bodies could easily be seen, apparently because of some loss of hemoglobin in many of the cells, especially in those containing the marginal parasites.

Saponin diluted in deionized water was also used in higher concentrations in attempts to release the anaplasma

from the red cells. This compound is surface active, and seems to "erode" the stroma of the red cell away from the inclusion body.

To accomplish this much the same procedure was used as for saponin hemolysis of red cells described previously, except frozen hemolyzed cells were generally used for the "erosion" treatment, and varying saponin concentrations were used. The best release of bodies was noted with saponin concentrations between 3.6 and 4.6%. Only fresh solutions of saponin were used. A 10% solution was prepared and diluted to double the final required concentration. 0.25 ml of the frozen blood and 0.25 ml of the double concentration saponin were mixed and incubated for 30 minutes in a 37°C water bath. After cooling the mixture in running tap water, deionized water was added to dilute the saponin concentration to 1% to stop its action. The specimen was then mounted and examined under phase contrast for the presence of released intact bodies.

Some variations of the above procedure were used in an endeavor to obtain better and more uniform release of the bodies. For example, in one experiment cells which had been stored in Alsever's solution, and not previously hemolyzed, were used. These were treated as outlined above, except the saponin used here had been diluted in Alsever's solution rather than water.

Also, shaking of the saponin-blood mixture in a 35°C incubator was tried, after first heating the tubes 5 to 10 minutes in a 37°C water bath. A reciprocating shaker operating at 100 strokes per minute for 25 minutes in one experiment, and 72 spm for 45 minutes in another experiment was used. After shaking, the tubes were cooled, diluted as above, and mounted.

The main difficulty encountered in these experiments was the lack of reproducible results, and the fact that the saponin seemed to act on the bodies as well as the red cell stroma. Fairly good release was obtained at a saponin concentration of 3.85% in one experiment under a particular set of conditions, but another experiment run under the same conditions would give poor release at the same concentration. Shaking of the blood-saponin mixture at 100 strokes per minute definitely had a destructive effect on the bodies, while 72 spm for 45 minutes did not appear to cause this effect. Saponin prepared in Alsever's solution did not appear to be advantageous over that made in water; however, no real conclusions can be derived from the one experiment tried.

## Warburg Experiments

A Warburg respirometer was used in these experiments, employing single sidearm flasks calibrated to contain 3 ml

of fluid in the main reservoir and 0.2 ml in the center well. Manometers contained Brodie fluid and were calibrated to be read at 150. Flasks were shaken at 114 strokes per minute. Temperature of the water bath ranged from 37.4°C in one experiment to 38.0°C in others. For any particular experiment a range of ± 0.1°C was maintained.

For each experiment, oxygen uptake was measured for both anaplasma infected and control blood. In so far as was possible, both the control and infected samples were treated alike from the time of collection. Sterile equipment was used up to the time the samples were distributed to the Warburg flasks.

The procedure followed usually consisted of aseptically transferring each of the blood samples from the collection flasks to Erlenmeyer flasks, then transferring 40 to 45 ml of this to each of two Rockefeller centrifuge tubes. These were centrifuged for 20 to 30 minutes at about 2000 rpm to concentrate the erythrocytes; then the plasma was removed using a 5 ml pipette and an aspirating bulb. If the cells were to be washed, as was done in several experiments, then one centrifuge tube each of normal and infected cells was resuspended in approximately 2 volumes of modified Hank's solution, and centrifuged as previously for about 10 minutes. The supernatant was removed, and the washing procedure repeated. If the "concentrated" cells

were not to be immediately used, they were placed in the refrigerator until the actual run was to start.

It should be noted here that the parasites appear to be very sensitive to excessive agitation. Observations made in the phase contrast microscope on frozen hemolyzed infected blood centrifuged at approximately 2400 rpm for 15 minutes showed the bodies could undergo deterioration. For this reason, the utmost care was taken not to expose the infected cells to excessive jarring or centrifuge speeds.

Throughout the many experiments several variables were introduced in an attempt to provide the optimum experimental conditions for the respiration of erythrocytes and their inclusion bodies. These included:

1. The use of "concentrated" normal and infected blood suspended in modified Hank's solution with Pardee's carbon dioxide buffer in the center well, and with subsequent alveolar aeration.

2. The use of "concentrated" normal and infected blood suspended in modified Hank's solution with 20% KOH in the center well, with and without subsequent alveolar aeration.

3. The use of washed normal and infected cells suspended in modified Hank's solution with Pardee's $CO_2$ buffer in the center well, and with subsequent alveolar aeration.

The first set of conditions was used in every experiment so that oxygen uptake under the other set of conditions could be compared with one common method. In effect, it

served as a standard for the other variables used in each experiment, and for comparative purposes between experiments.

The carbon dioxide buffer was prepared according to the method of Pardee using Kreb's modification of adding 0.1% thiourea to inhibit autooxidation of the solution. The concentration of the reagents used was such that a 3% $CO_2$ atmosphere was maintained in the flasks. The buffer was prepared as follows:

| | |
|---|---|
| Diethanolamine (2,2' iminodiethanol, Eastman) diluted to 60% by volume with deionized water | 10.0 ml |
| 6N HCl ±0.005N | 4.1 ml |
| $KHCO_3$, powdered | 3.0 g |
| Thiourea | 0.015 g |
| Deionized water | 0.9 ml |

This solution was allowed to "age" for at least 24 hours before being used.

Flasks containing Pardee's buffer were aerated with alveolar air for at least 30 seconds in order to introduce the necessary $CO_2$ atmosphere. In all except one of the experiments this was accomplished by exhaling into the flasks, while they were attached to their respective manometers, through the side arm stopper with the manometer stopper open to the atmosphere. Immediately after aeration both stoppers were closed, and the flasks placed in position in the 38°C water bath to allow equilibration of the gas phase.

In the one exception noted above, a commercially compressed 5% carbon dioxide-95% air mixture was used. However, in this case both the infected and the normal cells failed to respire at a measurable rate, so this method of flask aeration was immediately discontinued. It is felt that the reason for this failure of respiratory activity most probably was due to toxic substances in the gas mixture.

Because of the slight chemical activity found to be present in the $CO_2$ buffer with a subsequent slight uptake or emission of gases, three control flasks were run in the later experiments. These flasks contained the usual amounts of Pardee's buffer in the center well, but only a dilute solution of modified Hank's solution in the main reservoir.

The modified Hank's solution was prepared by mixing 10 ml stock solution A with 7 ml stock solution B, then diluting the mixture with deionized water to 100 ml. The stock solutions were prepared according to the following formulae:

Stock Solution A

| | |
|---|---|
| NaCl | 75.0 g |
| KCl | 7.5 g |
| $Na_2HPO_4$, anhydrous | 1.0 g |
| $KH_2PO_4$, " | 1.2 g |
| $K_2HPO_4$, " | 5.0 g |
| Deionized water to make a volume of | 1000 ml |

Stock Solution B

| | |
|---|---|
| $Na_2HPO_4$, anhydrous | 10.0 g |
| Deionized water to make a volume of | 1000 ml |

The mixed solution had a pH of 7.6 and was isotonic for bovine red cells as determined by maintaining these cells for 24 hours at 35.5°C without visible hemolysis.

The suspending media described above was Seitz filter sterilized before being used and stored at 3° to 5°C.

At the time of the run the packed red cells in the Rockefeller tubes were first diluted in 2 parts of the suspending medium. The amount diluted depended, of course, on the number of flasks being run per blood sample, but usually 5 ml of the washed cells or concentrated blood previously packed in the Rockefeller centrifuge tubes was diluted in 10 ml of the suspending medium. This provided sufficient volume of the diluted material to run 3 flasks for each blood sample (i.e., normal, infected, etc.), with enough extra for another flask should accidental spillage occur with any of the other flasks, and also to make a sterility test.

Sterility tests were run by placing 1 ml from each of the diluted samples into separate tubes of thioglycollate broth, then incubating these at 35.5°C for 7 days with an occasional inspection. Because of the high red cell concentration introduced into these tubes it was often

difficult to distinguish contamination in these tests, so if any doubt existed as to the presence of contaminants, a loop transfer was made from the initial culture to a fresh tube of thioglycollate, and this was incubated for 7 days. If no growth appeared by this time, the cultures were discarded and the original material considered sterile.

After the blood had been diluted it was next distributed by adding 3 ml to each of the required number of Warburg flasks. At this point aseptic methods were discontinued since the Warburg flasks themselves were not sterile. The three Pardee's buffer control flasks were set up, if used, as was the thermobarometer, which consisted of water in the main reservoir and $CO_2$ buffer in the center well of the flask.

The center well solutions were added to the flasks and folded filter paper "wicks" inserted into each center well. The glass fittings were well greased with petrolatum; then the side arm stopper was inserted, after which the entire flask was attached to the manometer. The entire unit was aerated with alveolar air and placed on the shaking apparatus in the 38°C constant temperature water bath.

An equilibration period of 45 minutes was used in the early experiments, but it was later found that the Pardee's buffer required more time to stabilize with respect to the

gas phase in the flasks, so the equilibration time was increased to 90 minutes in the later runs.

Following the equilibration period, readings were taken every 15 minutes for two to three hours. Thermobarometer readings were taken with each reading of every other manometer being used. This was done to keep error due to atmospheric changes at a minimum since the total amount of changes in gases involved within the flasks were generally very small.

On termination of the run the flasks were stored in the refrigerator until the packed cell volumes could be determined. Usually this period was not longer than 24 hours after completing the gas exchange measurements.

Packed cell volumes of the diluted blood in the flasks were determined, using Wintrobe hematocrit tubes. Two flasks were selected out of each blood sample group and thoroughly swirled to mix the contents. Duplicate hematocrit measurements were made from each flask. These were centrifuged in the Adams Safeguard centrifuge at 2430 rpm or in the International Clinical centrifuge at 1820 rpm for one hour, then read for the packed cell volume.

To calculate the amount of oxygen taken up per flask per hour, the difference in pressure noted during this time from the manometer readings was multiplied by the flask constant. In the earlier experiments, prior to the use of the $CO_2$ buffer control flasks, any flask having a positive

pressure change was considered to have no oxygen uptake. In later experiments where corrections could be made for Pardee's buffer activity, all pressure changes were included in the calculations.

In determining the respiration rate for a sample of blood (i.e., normal, infected, washed infected, etc.), the number of microliters (μl) of oxygen uptake per flask per hour for each replicate flask was found and an average taken. This figure, along with the average packed cell volume of the diluted blood in each flask containing that sample, was used in the calculation of μl $O_2$ uptake per milliliter of packed cell volume according to the following equation:

$$\mu l\ O_2 \text{ uptake/ml pcv/hr} = \frac{(\text{Average } \mu l\ O_2 \text{ uptake/hr/flask})(0.333)}{\text{Average ml pcv/ml of diluted blood in flasks}}$$

where 0.333 is the factor derived from the fact that the flask had 3.0 ml of diluted blood, and the respiratory rate for 1.0 ml is desired.

Sample calculations for the first hour of a Warburg run are shown below:

| Sample | Flask No. | Flask Constant* | Pressure change per hr, in mm | Avg. ml pcv per ml sample |
|---|---|---|---|---|
| Blood | 1 | 1.455 | -5 | 0.25 |
| | 2 | 1.821 | -3 | |
| | 3 | 1.390 | -6 | |
| $CO_2$ buffer control | 4 | 1.322 | +1 | ---- |
| | 5 | 1.876 | 0 | |
| | 6 | 1.404 | +1 | |

*Flask constants calculated for 38°C.

Step 1: Calculation of the µl $O_2$ uptake per flask.

Flask 1: 1.455 x -5 = -7.275 µl
" 2: 1.821 x -3 = -5.463 µl
" 3: 1.390 x -6 = -8.340 µl

Flask 4: 1.322 x +1 = +1.322 µl
" 5: 1.876 x 0 = 0 µl
" 6: 1.404 x +1 = +1.404 µl

Step 2: Calculation of the average µl $O_2$ uptake per group.

Blood: $\frac{(-7.275)+(-5.463)+(-8.340)}{3} = -7.026$ µl

Buffer: $\frac{(+1.322)+(0)+(+1.404)}{3} = +0.909$ µl

Step 3: Calculation of the average uptake by the sample:

µl $O_2 = -7.026 - (+0.909) = -7.935$ µl

Step 4: Calculation of the µl $O_2$ uptake/ml pcv/hr.

$$\text{µl } O_2 = \frac{(\text{average µl } O_2 \text{ uptake/hr/flask})(0.333)}{\text{average ml pcv/ml of diluted blood in flasks.}}$$

$$= \frac{(7.935)\ (0.333)}{0.25}$$

$$= 10.569 \text{ µl}$$

10.569 µl represents, then, the calculated volume of oxygen that would be taken up by 1 ml of packed cells in one hour under the foregoing conditions.

In the early Warburg experiments red cell counts were made on the diluted blood in the flasks to give the oxygen uptake based on the number of red cells. The method described in Kolmer, Spaulding and Robinson (13, p. 54-59) was used in this determination using the Thoma pipet with Hayem's diluting fluid for dilution of the cells to 1:200.

A Spencer "Bright Line Improved Neubauer" haemacytometer was used for making the counts. Two counts per flask were made, determining the number of cells in 80 small squares per count, then adding four ciphers to give the number of cells per cubic millimeter in that flask.

Oxygen uptake based on the cell counts was discontinued after two experiments in favor of the hematocrit measurements, largely because of the added time involved for making the cell counts without added accuracy. Of interest, however, is the comparison of the average number of cells per cubic millimeter to that of an average hematocrit value for diluted blood in a particular flask group, several of which are given below:

| % rbc | Hematocrit value ml pcv/ml diluted blood | rbc/cu. mm. | rbc/ml |
|---|---|---|---|
| 24.6 | 0.246 | 5,800,000 | $5.8 \times 10^9$ |
| 19.1 | 0.191 | 5,320,000 | $5.32 \times 10^9$ |
| 12.3 | 0.123 | 2,940,000 | $2.94 \times 10^9$ |
| 11.6 | 0.116 | 2,710,000 | $2.71 \times 10^9$ |

## Glassware and Other Equipment

Glassware used for containing blood, suspending media, etc. was processed as follows: New glassware was treated with 25% $H_2SO_4$ for at least 2 hours; then this, as well as dirty glassware that had already been acid treated, was boiled in Labtone for 15 minutes, after which it was rinsed

10 times in tap water and 5 times in deionized, or demineralized water, containing less than 0.3 parts per million electrolyte, expressed as NaCl.

New rubber stoppers, tubing, and other rubber fittings were boiled in approximately 0.5 N NaOH for 15 minutes, rinsed in tap water, then washed according to the procedure followed for glassware.

Glassware and other equipment was usually sterilized by autoclaving at 121°C for at least 30 minutes. Certain other glassware items that did not have rubber fittings, and that could be put up without cotton stoppers, were capped with aluminum foil if necessary and sterilized by dry heat at 180°C for 1½ hours. After sterilization, equipment was held in dust free cabinets until used.

# EXPERIMENTAL RESULTS

## I. Morphological Observations on Anaplasma-Infected Blood:

Based upon observations of Giemsa stained blood films from anaplasma-infected animals, the inclusion bodies found in the red cells, and which are presumed to be the infectious agent of anaplasmosis, have been commonly considered to be small spherical or granular structures located near the periphery of the cells, taking a dark basophilic stain.

It was considered of interest to examine the morphology of these structures present in the infected red cells by means of the phase contrast microscope, thus permitting observation of the parasite in a fresh, unstained, and presumably a living state. As far as was known, this type of study had not previously been reported.

Infected blood from eight calves was examined by the phase contrast method. The blood was first defibrinated or collected in oxalate at various times during each infection, after which it was held at refrigerated temperatures until examination, if such was to be carried out within a week of collection. In some cases, the defibrinated blood was diluted 1:2 with sterile Alsever's solution to aid in the preservation of the blood without freezing it. If a longer holding period than one week was expected, as was often the case, the blood was immediately quick frozen in an ethanol-dry ice mixture, then stored at -65° to -70°C until the time of examination.

At the time of the observations wet mounts were prepared and examined under the oil immersion objective of the phase microscope (900X).

By the use of these techniques it has been revealed that Anaplasma marginale is apparently not merely a small circular body within the red cell, as is usually seen in stained smears of infected blood; instead, one or more of the following morphological forms have been found in the erythrocytes of infected calves:

(1) Those having a single "head" with an approximate average diameter of 1 micron, and a flat tail, which averages approximately 6 μ in length. The head consisted of from one to seven separate segments or units, and was usually located inside the red cell, at or near the periphery, while the tail was flat and rounded at the terminal end, and generally extended laterally across the red cell. The morphology of this type of tailed body gives one the impression of a comet, and for this reason they will be specifically referred to as "comet-like bodies." (See Plates II through VI.)

(2) Those having two heads, each of which was about 1 μ in diameter, and consisted of from one to seven segments, located at the opposite sides of the red cell; these were joined together by an intervening connection, the entire form appearing similar to

"dumbbells." For this reason these will be specifically referred to here as "dumbbell forms." (See Figures 9, 14, 15, 18, and 20.)

(3) Those tailed bodies appearing as combinations of the comet-like forms and of the dumbbell forms. (See Figures 12, 13, and 19.)

(4) Those which consist of the heads only, with or without evidence of the tails within the cell stroma. (See Figure 15.)

To distinguish the parasites observed under the phase contrast microscope from those seen in stained smears, these various morphological forms of Anaplasma described above will be categorically referred to as "tailed bodies."

The tails of the comet-like bodies, and of the combined comet-like bodies-dumbbell forms appeared to be quite thick at the head end, and progressively decreased in thickness toward the terminal end. This variation in thickness gave a fading-out effect, producing in part the comet-like appearance. Usually the width of the tails were fairly uniform throughout their length, being about as wide as the total diameter of the head, which varied as to its number of segments, or, seemingly, its stage of development.

The dumbbell forms appeared to have as the connecting section, material that was similar to the tails of the comet-like forms. The outstanding difference was that the

PLATE I*

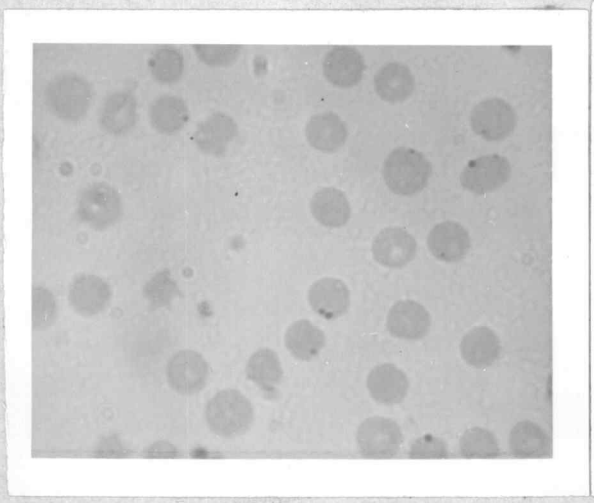


Fig. 1. Giemsa-stained smear of anaplasma-infected blood from Animal 413. Prepared 36 days after carrier transfusion.

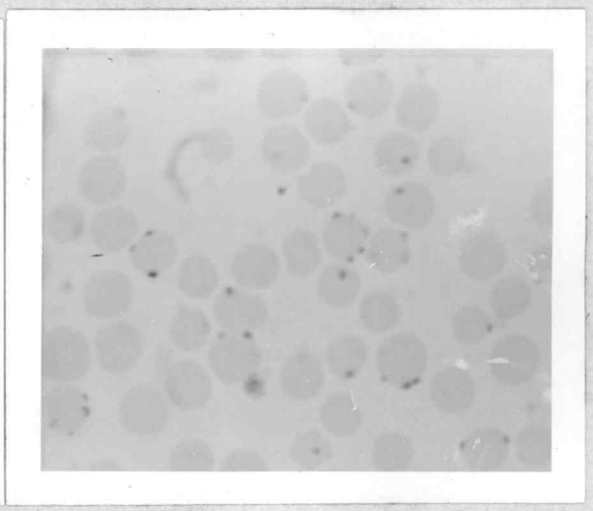


Fig. 2. Giemsa-stained blood smear showing half sphere and slightly curving rod types of atypical bodies from Animal 409. Prepared 10 days after injection with actively infected blood.

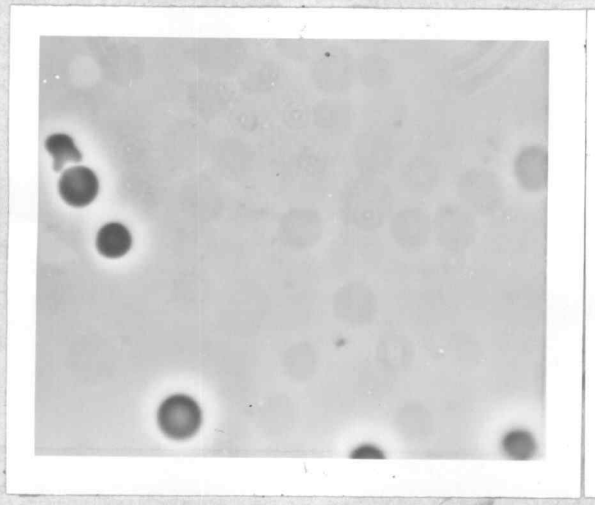


Fig. 3. Defibrinated control blood collected from Animal 415. Held 10 days in the refrigerator after collection.

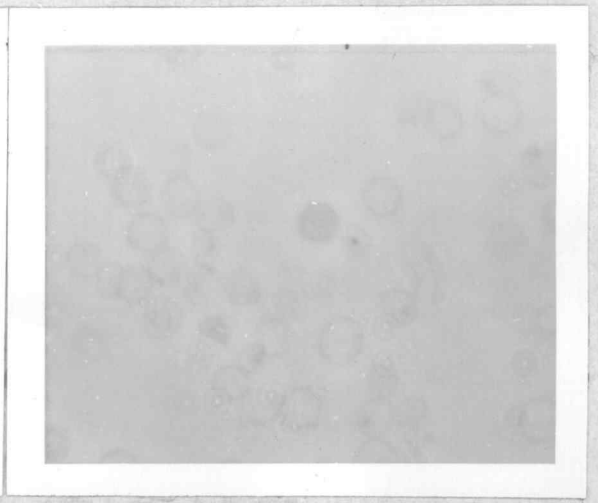


Fig. 4. Frozen-hemolyzed defibrinated control blood collected from Animal 415. Collected the same day as blood shown in Figure 3.

*Photographs show magnification of 900X.

PLATE II

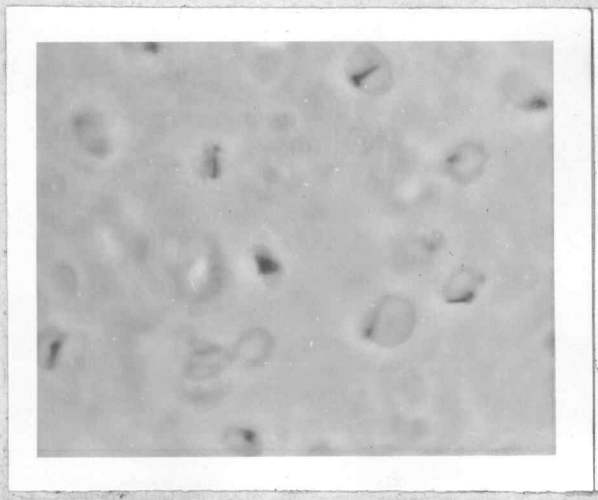


Fig. 5. Defibrinated anaplasma-infected blood hemolyzed by freezing; collected from Animal 402 35 days after injection with carrier blood. Mount prepared in 1% Noble agar.

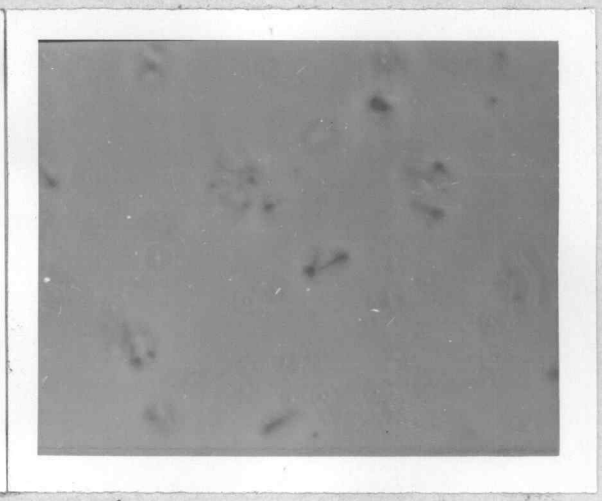


Fig. 6. Defibrinated anaplasma-infected blood hemolyzed by freezing; collected from Animal 402 35 days after injection with carrier blood. Mount prepared with 5% gelatin.

Fig. 7. Frozen-hemolyzed defibrinated infected blood from Animal 402 subjected to saponin treatment (3.95 per cent w/v solution). Blood collected 35 days after injection with carrier blood. Note the free Anaplasma.

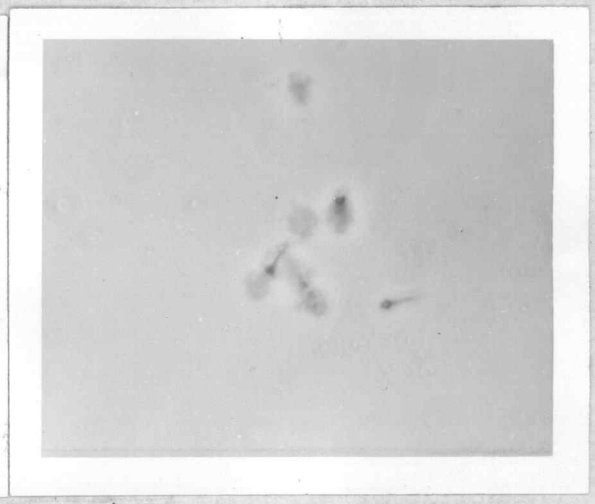


Fig. 8. The same blood sample as was used for treatment in Fig. 7, but subjected to saponin of 3.7% w/v concentration. Again note the free Anaplasma.

PLATE III

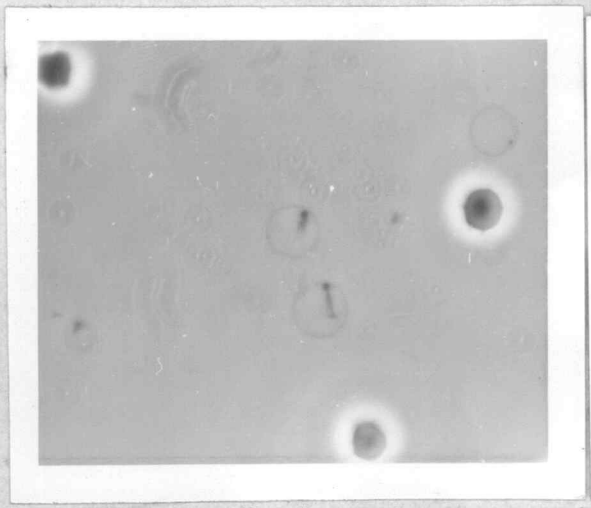


Fig. 9. Defibrinated infected blood collected from Animal 405 during a splenectomy relapse. Blood stored 13 days at 2 to 4°C, then diluted 1:2 with Alsever's solution before examination.

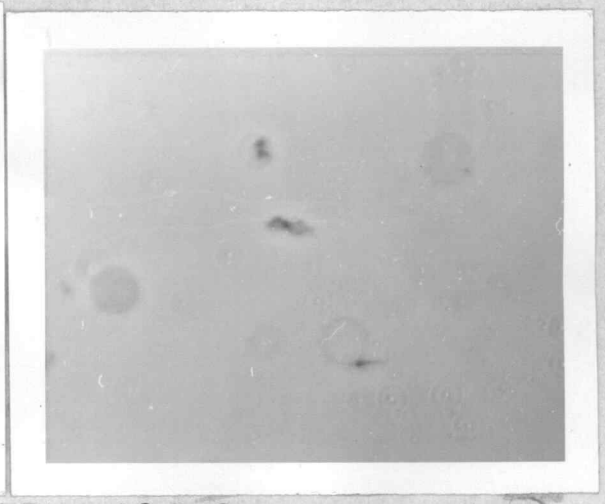


Fig. 10. Same preparation as is shown in Fig. 9, but different field of observation.

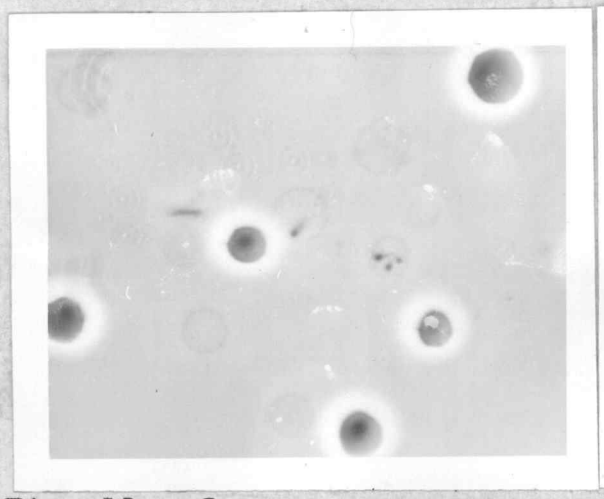


Fig. 11. Same preparation as is shown in Fig. 9, but different field of observation.

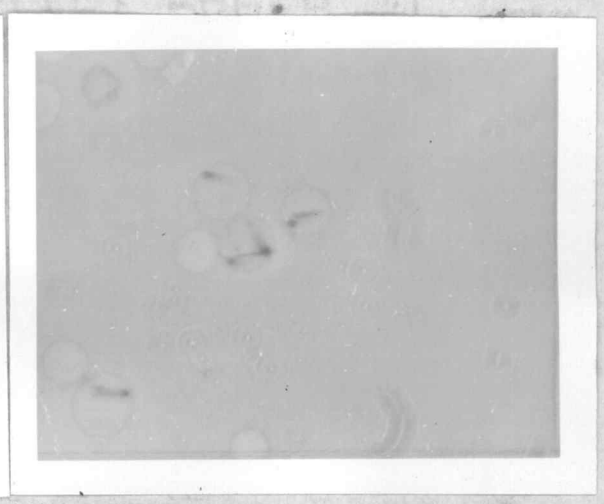


Fig. 12. Defibrinated infected blood collected from Animal 405 during a splenectomy relapse. Blood untreated except for storage at 2 to 4°C for 14 days before examination.

PLATE IV

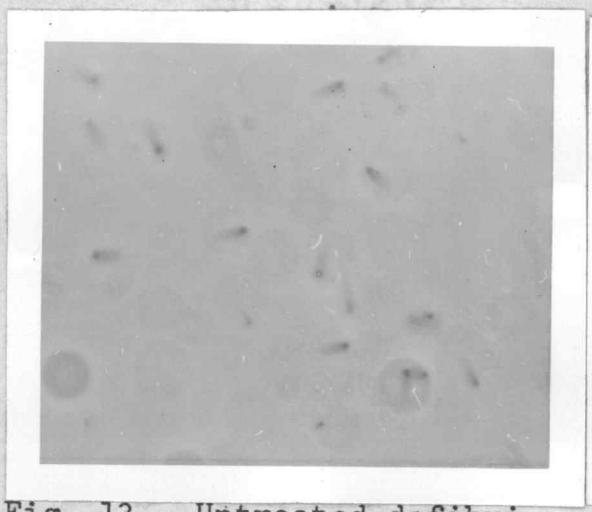


Fig. 13. Untreated defibrinated blood collected from Animal 411 13 days after transfusion with carrier blood. Stored at 2 to 4°C 9 days prior to examination.

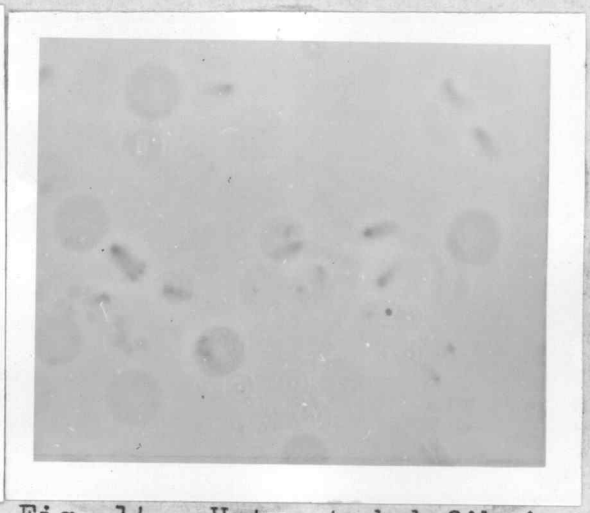


Fig. 14. Untreated defibrinated blood collected from Animal 411 15 days after transfusion with carrier blood. Sample stored at 2 to 4°C seven days prior to examination.

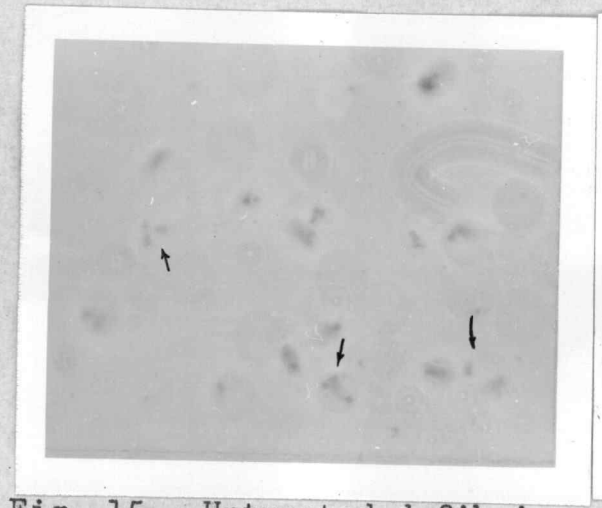


Fig. 15. Untreated defibrinated blood collected from Animal 411 17 days following transfusion with carrier blood. Sample stored at 2 to 4°C for 5 days before examination. Note that some of the heads and tails have become disconnected in some of the parasites.

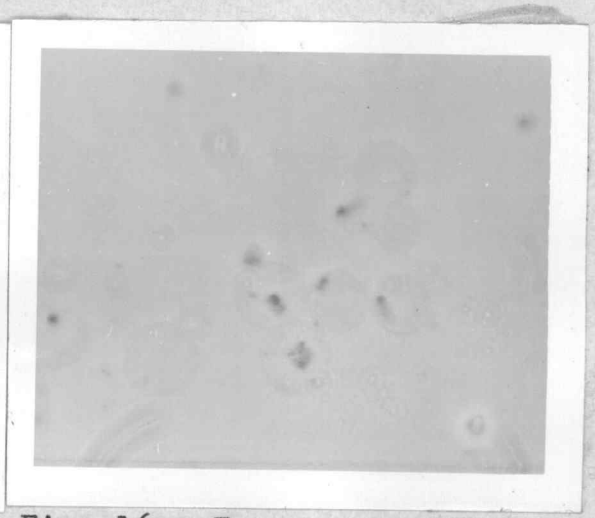


Fig. 16. Frozen hemolyzed oxalated infected blood collected from Animal 411 the same day as that shown in Fig. 15.

PLATE V

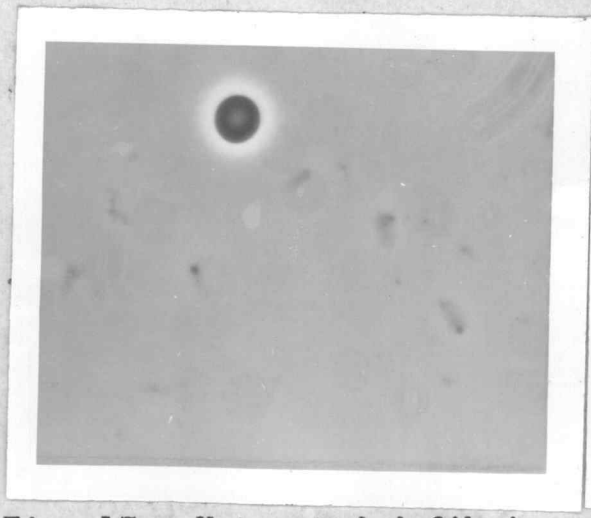


Fig. 17. Untreated defibrinated infected blood from Animal 413 collected 33 days following transfusion with carrier blood. Sample stored 7 days at 2 to 4°C before examination.

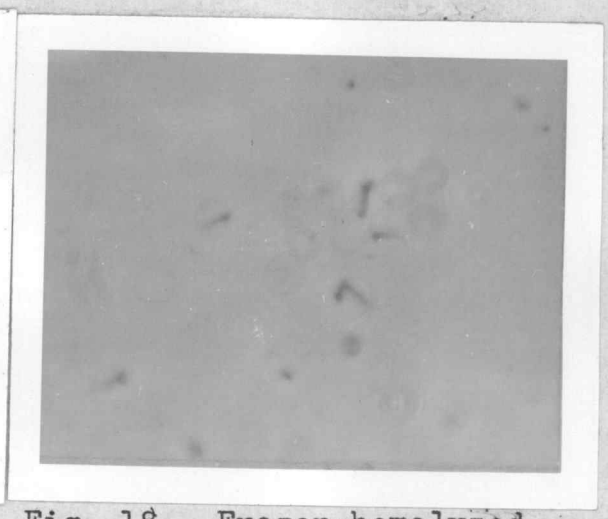


Fig. 18. Frozen-hemolyzed oxalated infected blood from Animal 413 collected the same day as for that shown in Fig. 17.

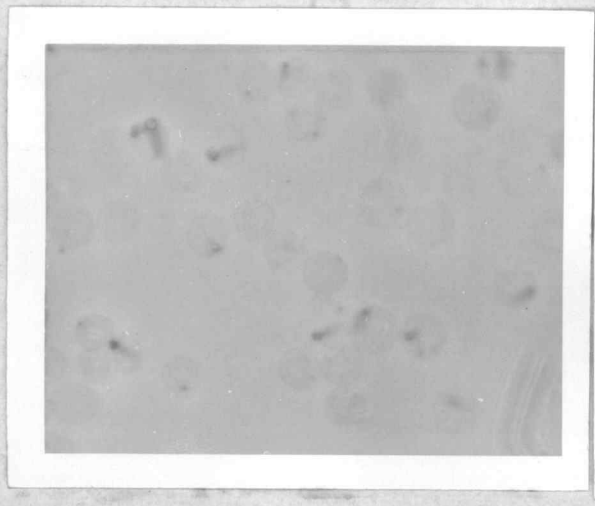


Fig. 19. Frozen-hemolyzed oxalated infected blood from Animal 413 collected one day later than for that shown in Fig. 17.

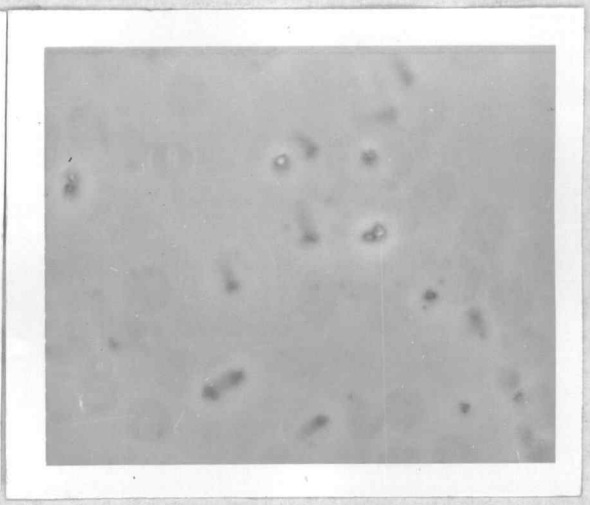


Fig. 20. Frozen-hemolyzed oxalated infected blood collected from Animal 413 two days later than for that shown in Fig. 17.

PLATE VI

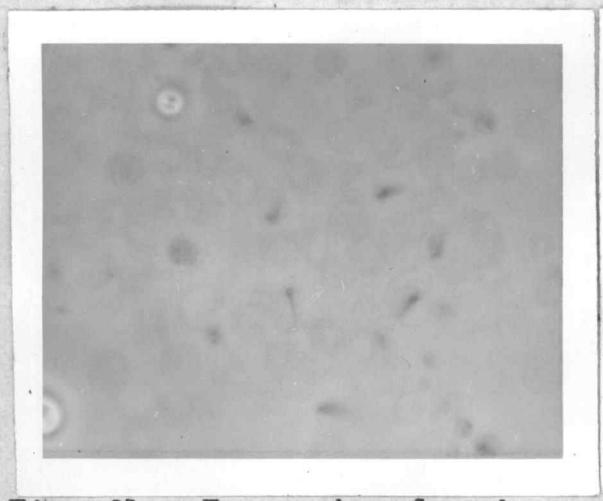


Fig. 21. Frozen-hemolyzed oxalated blood collected from Animal 412 22 days following transfusion with actively infected blood.

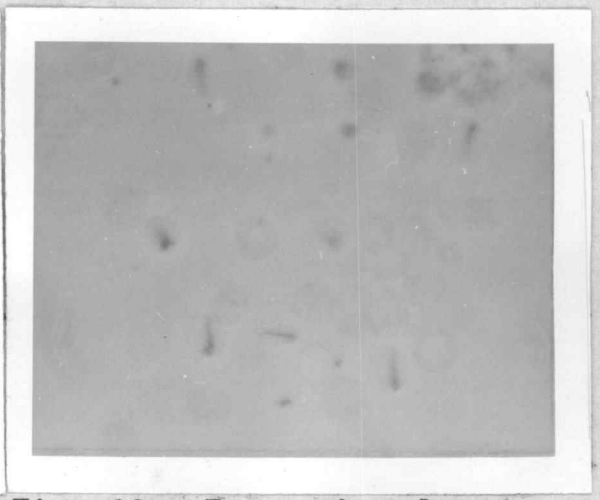


Fig. 22. Frozen-hemolyzed oxalated blood collected from Animal 412 three days later than for that shown in Fig. 21.

thickness of the connecting band seemed to be uniform throughout its length. These forms were generally found to appear several days following the appearance of the active infection.

It is interesting to note that in one observation of defibrinated infected blood diluted 1:2 with Alsever's solution at the time of the wet mount preparation, an apparently "split" dumbbell form was observed (Figure 9). This hemolyzed erythrocyte contained what appeared to be two comet-like forms with pointed tails. The heads of these tailed bodies were at opposite sides of the red cell, such that their tails pointed in opposite directions. Due to the slight currents within the mount the two forms appeared to move about inside the stroma. At various times they were observed to come very close to each other, and under this circumstance, appeared very similar to a single dumbbell form.

Combinations of the comet-like form and the dumbbell form were composed of a typical dumbbell form with its two heads. However, from one of these heads also projected a tail, thus forming the combined bodies. Anaplasma of this morphology were not common.

One of the most interesting phenomenon observed among the tailed bodies was the ability of the head and the tail to become separated or disconnected, several of which are shown in Figure 15, as designated by the pointers. This was

found to occur to a higher degree in the later stages of the active infection, with few or none of these forms being found in the earlier stages. The tails of these separated forms were often absent, such that the only portion of the tailed body remaining within the stroma was the head, as can be seen in Figure 16.

As mentioned previously, the heads of the tailed bodies could be found to consist of from one to seven segments. This apparently represented varying stages of division as reported by Lotze and Yiengst (14, p. 312-320), and others (5, p. 254-258; 9, p. 148). Each of the component parts were usually circular or oval; however, the arrangement of the units varied with respect to one another in forming the entire head. In some cases, for example, when less than three parts per head were found, they could be arranged laterally across the end of the tail; or with heads containing from four to seven parts they could be found in apparently spherical packets. Triangular arrangements, as seen in the dumbbell form in Figure 20, were also seen. Still others were found with no seemingly orderly arrangements.

By and large the form of head most commonly found was undivided, as contrasted to those having more than five sections, which were comparatively few. However, even among those having undivided heads differences in morphology could be found other than size.

In the forms of smaller diameter, the heads usually appeared of uniform density, as illustrated in the tailed bodies of Figure 13. On the other hand in many of the larger heads, the density appeared irregular, especially about their center, as can be seen in Figure 20. It was very common to see these latter forms with what appeared to be large to small openings. And these openings, or vacuoles, were not restricted to heads of only one segment, but were also common in those having many parts, with possibly each one having these seemingly "void" areas.

Several observations were made from samples from three different animals where the red cell envelope appeared to contain many small granules or clusters of granules, as illustrated in Figure 16. This was noted to occur near the peak of the infection and later, and was never found to occur in normal or control blood, nor in infected blood collected early in the active infection. It is felt that these granules may represent a stage of the parasite in which the once vacuolated head shattered, giving rise to these many small forms. Usually these granules were found in stroma without evidence of the tail, but in one substantiating observation evidence was found where an intact comet-like form was seen with only one section of its head. In the cell stroma, close to the position where the remaining portions of the head should have been, were a group of very small granules. The intact sections of the head

corresponding to the empty spaces were absent, and it is felt that the granules represented what was once these sections. However, one must be guarded in making definite assumptions since these morphological effects could be due to some physical treatment of the red cells during collection or mounting.

The optimum morphological observations were obtained, it is felt, from untreated defibrinated blood. That is, it was in these preparations that the parasite was thought to be most nearly in its in vivo form, largely because of the fact that these underwent the least treatment and handling after collection. Freezing of the tailed bodies, or diluting in Alsever's solution, or hemolyzing the infected red cells with distilled water was found to have a deleterious effect on the tails. This was particularly true of the latter treatment, which caused, in many preparations, complete deterioration of that part of the anaplasma. Freezing, and diluting the infected red cells in Alsever's solution, had a less drastic effect than did the water hemolysis; never the less, some of the tails did become pointed at the ends, and of seemingly equal thickness throughout their length (See Plates II and VI).

Although an occasional form of this type was found in untreated defibrinated blood, it was only in the later stages of the active infection; they were never observed in

such numbers in these preparations as in the frozen hemolyzed red cells, or those suspended in Alsever's solution.

Several attempts were made to stain the tail of the parasite using various methods of staining, so that the entire form might be observed under the light microscope. These methods include Giemsa stain, and several variations of this method; Fontana stain (Spirochete stain); nigrosin negative stain; combination of Wright's stain and Giemsa; Leifson modified flagella stain; Celestine blue in combination with eosin, with acid fuchsin, and with Giemsa; Feulgen stain; and an auramine O stain. The bodies were detected by practically all the above methods; however, none of the stains showed the presence of the tailed forms with the exception of the Leifson modified flagella method, and even this was observed with doubt as to their presence.

To ascertain that the tailed bodies seen in the hemoglobin-deficient erythrocyte using the phase contrast microscope were actually A. marginale and not some artifact, the per cent of infected cells was determined for 500 red cells from several different samples, collected at varying intervals after inoculation of the animals. These figures were then compared to those obtained from Giemsa stained smears prepared on the same day as the respective wet mount counts were made, and to oxalated or defibrinated frozen hemolyzed blood from normal calves, also collected the same days. Results of these determinations are given in Tables 2, 3, 4, 5, 6, 7, and 8.

TABLE 2

A COMPARISON OF THE PER CENT OF INFECTED RED CELLS IN ANAPLASMOSIS BLOOD AS DETERMINED IN WET MOUNTS BY PHASE MICROSCOPY AND ON STAINED SMEARS

ANIMAL 407

| Date | Days following inoculation | % infected red cells* | |
|---|---|---|---|
| | | wet mount (phase contrast) | smears (Giemsa stain) |
| 2-23-59 | 12 | 0 | 0 |
| 2-26 | 15 | 0 | 0 |
| 3-2 | 19 | 0 | 0 |
| 3-4 | 21 | 0 | 0 |
| 3-5 | 22 | 0 | 0 |
| 3-6 | 23 | 0 | 0 |
| 3-9** | 26 | 0 | 0 |
| 3-11 | 28 | 0 | 0.2 |
| 3-13 | 30 | 0.4 | 0.3 |
| 3-14 | 31 | 1.4 | 0.6 |
| 3-16 | 33 | 2.6 | 1.2 |
| 3-17 | 34 | 3.6 | 3.1 |
| 3-19 | 36 | 6.4 | 5.5 |
| 3-20 | 37 | 10.8 | 8.1 |
| 3-21 | 38 | 9.8 | 11.9 |
| 3-23 | 40 | 15.0 | 17.7 |
| 3-24 | 41 | 12.0 | 14.0 |

* Based on counts of 500 rbc in wet mounts and 500 to 1000 rbc in stained smears.

** Second inoculation with 5 cc carrier blood.

TABLE 3

A COMPARISON OF THE PER CENT OF INFECTED RED CELLS IN ANAPLASMOSIS BLOOD AS DETERMINED IN WET MOUNTS BY PHASE MICROSCOPY AND STAINED SMEARS

ANIMAL 409

| Date | Days following inoculation | % infected red cells* | |
|---|---|---|---|
| | | wet mount (phase contrast | smears (Giemsa stain) |
| 3-31-59 | 7 | 0 | 0 |
| 4-3 | 10 | 0 | 0** |
| 4-4 | 11 | 0 | 0** |
| 4-6 | 13 | 0.2 | 0** |
| 4-7 | 14 | 0 | 0** |
| 4-8 | 15 | 0 | 0** |
| 4-10 | 17 | 0 | 0 |
| 4-13 | 20 | 0 | 0 |
| 4-15 | 22 | 0.2 | 0 |
| 4-17 | 24 | 0 | 0.1 |
| 4-20 | 27 | 0.2 | 0.3 |
| 4-21 | 28 | 0.6 | 0.9 |
| 4-22 | 29 | 1.6 | 0.6 |
| 4-23 | 30 | 3.2 | 2.1 |
| 4-24 | 31 | 3.6 | 4.2 |
| 4-25 | 32 | 7.4 | 4.8 |
| 4-27 | 34 | 10.2 | 9.0 |
| 4-28 | 35 | 18.2 | 13.4 |
| 4-29 | 36 | 19.4 | 13.3 |
| 4-30 | 37 | 20.0 | 25.5 |
| 5-1 | 38 | 27.2 | 22.4 |
| 5-5 | 42 | 12.0 | ---- |
| 5-6 | 43 | 8.4 | 6.3 |
| 5-8 | 45 | 4.6 | 3.4 |
| 5-12 | 49 | 5.4 | 1.2 |
| 5-19 | 56 | 10.0 | 4.8 |
| 5-22 | 59 | 14.0 | 7.8 |
| 5-29 | 66 | 0.6 | 0.7 |
| 6-11 | 79 | 3.6 | 2.0 |
| 6-15 | 83 | 2.6 | 1.6 |

* Based on counts of 500 rbc in wet mounts and 500 to 1000 rbc in stained smears.

** Presence of atypical forms.

TABLE 4

A COMPARISON OF THE PER CENT OF INFECTED RED CELLS IN ANAPLASMOSIS BLOOD AS DETERMINED IN WET MOUNTS BY PHASE MICROSCOPY AND STAINED SMEARS

ANIMAL 410

| Date | Days following inoculation | % infected red cells* | |
|---|---|---|---|
| | | wet mount (phase contrast) | smears (Giemsa stain) |
| 4-21-59 | 3** | 0 | 0 |
| 4-28 | 10 | 0 | 0 |
| 5-1 | 13 | 0 | 0 |
| 5-12 | 24 | 0 | 0 |
| 5-15 | 27 | 0 | 0 |
| 5-19 | 31 | 0 | 0 |
| 5-22 | 34 | 0 | 0 |
| 5-29 | 41 | 0 | 0 |
| 6-12 | 55 | 0 | 0 |
| 6-15 | 58 | 0 | 0 |
| 6-22 | 0*** | 0 | 0 |
| 6-26 | 4 | 0 | 0.1 |
| 7-1 | 9 | 0 | 0 |
| 7-7 | 15 | 1.6 | 2.5 |
| 7-10 | 18 | 7.4 | 4.3 |
| 7-13 | 21 | 8.4 | 9.7 |
| 7-15 | 23 | 5.4 | 9.7 |
| 7-17 | 25 | 9.8 | 8.6 |

* Based on counts of 500 rbc in wet mounts and 500 to 1000 rbc in stained smears.
** 5 cc carrier blood.
*** Second inoculation carrier blood (5 cc).

TABLE 5

A COMPARISON OF THE PER CENT OF INFECTED RED CELLS IN ANAPLASMOSIS BLOOD AS DETERMINED IN WET MOUNTS BY PHASE MICROSCOPY AND STAINED SMEARS

ANIMAL 411

| Date | Days following inoculation | % infected red cells* | |
|---|---|---|---|
| | | wet mount (phase contrast) | smears (Giemsa stain) |
| 5-15-59 | 7** | 0 | 0 |
| 5-19 | 11 | 0 | 0.4*** |
| 5-22 | 14 | 0 | 0 |
| 5-29 | 21 | 0 | 0 |
| 6-12 | 2**** | 0 | 0 |
| 6-18 | 8 | 1.6 | 1.9 |
| 6-22 | 12 | -- | 21.5 |
| 6-23 | 13 | 36.0 | 27.8 |
| 6-25 | 15 | 35.8 | 37.8 |
| 6-27 | 17 | 30.8 | 32.8 |
| 6-30 | 20 | 11.0 | 12.1 |
| 7-2 | 22 | 1.0 | 5.6 |

* Based on counts of 500 rbc in wet mounts and 500 to 1000 rbc in stained smears.
** After inoculation with deer blood.
*** Atypical infection.
****Transfusion of 350 ml carrier blood.

TABLE 6

A COMPARISON OF THE PER CENT OF INFECTED RED CELLS IN ANAPLASMOSIS BLOOD AS DETERMINED IN WET MOUNTS BY PHASE MICROSCOPY AND ON STAINED SMEARS

ANIMAL 412

| Date | Days following inoculation | % infected red cells* | |
|---|---|---|---|
| | | wet mounts (phase contrast) | smears (Giemsa stain) |
| 6-12-59 | 21** | 0 | 0 |
| 6-22 | 31 | 0 | 0 |
| 6-26 | 35 | 0 | 0 |
| 6-29 | 0*** | 0 | 0 |
| 7-3 | 4 | 0 | 0 |
| 7-6 | 7 | 0 | 0 |
| 7-9 | 10 | 0 | 0 |
| 7-13 | 14 | 0 | 0 |
| 7-16 | 17 | 0 | 0 |
| 7-23 | 7**** | 0 | 0 |
| 7-27 | 11 | 0.2 | 0 |
| 7-29 | 13 | 0 | 0 |
| 8-3 | 18 | 2.0 | 1.4 |
| 8-6 | 21 | 8.4 | 4.4 |
| 8-7 | 22 | 9.8 | 5.3 |
| 8-10 | 25 | 9.4 | 6.3 |
| 8-12 | 27 | 8.2 | 6.8 |
| 8-13 | 28 | 9.4 | 6.8 |

* Based on counts of 500 rbc in wet mounts and 500 to 1000 rbc in stained smears.
** After 5 cc injection with carrier blood.
*** After 400 ml transfusion with carrier blood.
****After 200 ml transfusion with actively infected blood.

TABLE 7

A COMPARISON OF THE PER CENT OF INFECTED RED CELLS IN ANAPLASMOSIS BLOOD AS DETERMINED IN WET MOUNTS BY PHASE MICROSCOPY AND ON STAINED SMEARS

ANIMAL 413

| Date | Days following inoculation | % infected red cells* | |
|---|---|---|---|
| | | wet mounts (phase contrast) | smears (Giemsa stain) |
| 6-5-59 | 0** | 0 | 0 |
| 6-12 | 7 | 0 | 0 |
| 6-26 | 21 | 0 | 0 |
| 7-2 | 27 | 0 | 0 |
| 7-9 | 1*** | 0 | 0 |
| 7-13 | 5 | ?**** | ?**** |
| 7-16 | 8 | ? | ? |
| 7-23 | 15 | 0 | 0 |
| 7-27 | 19 | 0 | 0 |
| 8-3 | 26 | 2.2 | 1.2 |
| 8-6 | 29 | 6.0 | 3.8 |
| 8-10 | 33 | 21.0 | 10.9 |
| 8-11 | 34 | 19.0 | 16.6 |
| 8-12 | 35 | 18.9 | 17.8 |

* Based on counts of 500 rbc in wet mounts and 500 to 1000 rbc in stained smears.
** After 5 cc injection with carrier blood.
*** After 400 ml transfusion with carrier blood.
****Severe atypical infection.

TABLE 8

NORMAL CALF BLOOD EXAMINED FOR THE PRESENCE OF TAILED ANAPLASMA BODIES EMPLOYING THE PHASE CONTRAST MICROSCOPE

| Animal No. | Total number of samples examined | Examination period, days | % infected red cells |
|---|---|---|---|
| 403* | 1 | 1 | 0 |
| 409* | 15 | 30 | 0 in all samples |
| 410* | 12 | 47 | 0 " " " |
| 411* | 11 | 17 | 0 " " " |
| 412* | 6 | 39 | 0 " " " |
| 413* | 1 | 1 | 0 |
| 415 | 15 | 52 | 0 in all samples |
| 27-0 | 1 | 1 | 0 |
| H-70 | 1 | 1 | 0 |

*Examined before inoculation with infected blood.

Control animals examined for the presence of tailed anaplasma bodies under the phase contrast microscope. Oxalated or defibrinated samples were collected at irregular intervals, frozen hemolyzed, then examined as wet mounts, at least 500 red cells per sample being examined.

Analysis of the Tables 2 through 7 will show that between the phase microscope observations on wet mounts and the ordinary light microscope counts on the stained smears, there is a very close correlation for: (1) the time of appearance of the bodies in the red cells; (2) the time that the highest counts occur; and (3) the general trend of the per cent infected red cells for the period of infection studied for each animal.

For example, Animal 409 (Table 3) gave positive evidence for the presence of tailed bodies in the wet mounts 22 days after inoculation, and 2 days later the bodies were found in the stained smears.

It should be explained here that this animal showed the presence of an atypical infection from the 10th to the 15th days following inoculation. During this period one anaplasma was found in the wet mount preparation of the 13th day, but none could be found in the stained smear; a large number of atypical bodies were present, however.

Following the appearance of the anaplasma infection in Animal 409, it can be seen that the per cent infected red cells rose to a peak at 37 days in the stained preparations, and at 38 days in the mounts examined by phase microscopy. From this point the infection regressed, after which a secondary rise appeared with both methods of examination, reaching a peak on the 59th day. Again the per cent of

infected red cells decreased according to both counts, and another slight rise was noted on the 79th day, giving way to another fall.

Thus, for this animal, where 30 separate hemolyzed samples were examined and compared to stained smears over a period of 80 days, the tailed bodies appeared at approximately the same time as the stained bodies; both reached maximum numbers at approximately the same time, and decreased at about the same rate. Trends such as these were noted in each of the other animals examined.

Normal animals examined in conjunction with the infected samples were uniformly negative for the presence of the tailed bodies (Table 8).

Close analysis of the tables correlating the per cent infected red cells in wet mounts with that of the stained smears will reveal, in some cases, large differences in the actual percentage of infected cells for a particular day. This can be accounted for in part by the large sampling errors inherent in counting methods of this sort, and in part by the fact that non-infected hemolyzed cells were often difficult to distinguish under phase contrast. Hemolyzed cells containing the tailed bodies were much more readily distinguished than those not containing the inclusion bodies, largely because of the high microscopic density of the latter. Error could result, then, by not actually counting

all the non-infected erythrocytes in the wet mounts, just because they couldn't be seen.

But, even with these large differences as noted for certain days in Tables 2 through 7, there appears to be sufficient correlation to indicate the identity of the tailed bodies and Anaplasma marginale with a high degree of probability.

## II. Respiration of Anaplasma-Infected Blood

In an attempt to obtain some additional information on the nature of the causative agent of anaplasmosis, respiration experiments were carried out on anaplasma-infected blood from six different splenectomized animals. Normal bovine blood specimens were collected at the same time as the specimens from the infected animals and used as experimental controls.

Both washed cell and unwashed cell experiments were carried out, using modified Hank's solution as the suspending medium in the ratio of 1 part of blood to 2 parts of the diluent. After the distribution of the blood to the Warburg flasks, Pardee's buffer, which gave a 3% $CO_2$-air atmosphere in the flasks, or 20% KOH, which gave a $CO_2$-free atmosphere, was added to the center wells. Then all flasks were aerated with alveolar air for at least 30 seconds. Aeration was

omitted in some cases where 20% KOH was employed in the center well to determine the effect of this procedure upon respiration.

The flasks were next incubated at approximately 38°C in the Warburg constant temperature water bath for 45 to 90 minutes, after which the changes in pressure per flask were observed for at least 2 hours. Upon completion of each run hematocrits were determined on the flask contents to determine the packed cell volume; then the amount of oxygen taken up per milliliter of packed cell volume was computed.

A summary of the results of 15 separate experiments is given in Table 9. As can be seen, under these conditions there were only two experiments where the control, or normal blood, gave a higher rate of respiration than the infected blood for the same day, both being in the second hour of Experiments 6 and 11.

It should be pointed out that in Experiments 6 through, and including Experiment 13, technical difficulties were encountered with the Warburg apparatus causing local variation in temperature. These are reflected in the marked variation in measurements on the control blood specimens through these experiments.

Examination of Table 9 will reveal, for instance, that in the above experiments the range of variation of the $O_2$ uptake of the normal samples extended from 1.5 $\mu$l $O_2$/ml

packed cell volume to 9.1 µl $O_2$/ml pcv for the first hour of measurements. In the second hour, the range of variation was even more pronounced, extending from 0 µl $O_2$/ml pcv to 17.4 µl $O_2$/ml pcv.

These values are contrasted to those in Experiments 15 through 23 for the measurements on the control blood samples. The range here was only from 1.0 µl $O_2$/ml pcv to 4.9 µl $O_2$/ml pcv in the first hour of readings, and from 0 µl $O_2$/ml pcv to 7.1 µl $O_2$/ml pcv in the second hour of measurements.

For these latter runs the difficulties causing the local temperature variations between flasks had been eliminated. Thus the data obtained represent a more accurate and uniform picture of the respiration rates of infected and normal erythrocytes.

In reporting the data, however, it is believed that Experiments 6 to 13, even though somewhat erratic, are sufficiently accurate to be included with the later experiments in Table 9. They lend the additional weight of 7 experiments and 2 infected animals to the results. However, cognizance of the erratic results in the early data has been made in the following analysis of the data for the range of significance.

The data obtained for Experiments 6 through 23, and that obtained for Experiments 15 through 23 were separately subjected to statistical analysis, employing Student's t test

TABLE 9

SUMMARY OF RESPIRATION EXPERIMENTS: THE OXYGEN UPTAKE OF ANAPLASMA-INFECTED AND NORMAL BOVINE BLOOD

| Experiment No. | Infected Animal No. | Infected Blood µl $O_2$ uptake/ml pcv | | Control Animal No. | Control Blood µl $O_2$ uptake/ml pcv | |
|---|---|---|---|---|---|---|
| | | 1st hr. | 2nd hr. | | 1st hr. | 2nd hr. |
| 6 | 407 | 26.5 | 6.7 | 409 | 6.3 | 9.6 |
| 7 | " | 26.1 | 23.6 | " | 1.5 | 4.8 |
| 9 | 409 | 7.9 | 10.2 | 411 | 6.1 | 2.2 |
| 10 | " | 10.6 | 3.5 | " | 9.1 | 0.0 |
| 11 | " | 12.5 | 12.5 | " | 3.1 | 17.4 |
| 12 | " | 14.3 | 11.2 | " | 3.6 | 1.6 |
| 13 | " | 22.9 | 5.4 | 412 | 7.5 | 1.7 |
| 15 | 411 | 5.1 | 7.8 | 415 | 3.9 | 5.1 |
| 16 | " | 9.0 | 11.5 | " | 4.6 | 9.4 |
| 18 | 410 | 7.3 | 9.9 | H-11 | 1.0 | 0.0 |
| 19 | " | 8.7 | 9.5 | 358 | 4.9 | 2.2 |
| 20 | " | 7.7 | 9.9 | C-50 | 3.5 | 3.4 |

TABLE 9 (CONTINUED)

SUMMARY OF RESPIRATION EXPERIMENTS: THE OXYGEN UPTAKE OF ANAPLASMA-INFECTED AND NORMAL BOVINE BLOOD

| Experiment No. | Infected Animal No. | Infected Blood $\mu l$ $O_2$ uptake/ml pcv | | Control Animal No. | Control Blood $\mu l$ $O_2$ uptake/ml pcv | |
|---|---|---|---|---|---|---|
| | | 1st hr. | 2nd hr. | | 1st hr. | 2nd hr. |
| 21 | 412 | 2.5 | 6.6 | H-70 | 2.5 | 4.6 |
| 22 | " | 7.4 | 8.5 | 415 | 2.5 | 7.1 |
| 23 | " | 12.3 | 13.1 | 27-0 | 4.7 | 3.6 |
| 21 | 413 | 8.5 | 10.8 | H-70 | 2.5* | 4.6* |
| 22 | " | 12.9 | 9.8 | 415 | 2.5* | 7.1* |
| 23 | " | 17.9 | 17.1 | 27-0 | 4.7* | 3.6* |
| Mean | | 12.2 | 10.4 | | 4.3 | 4.8 |

*Single control samples used in Experiments 21, 22, and 23; therefore, control values for Animals 412 and 413 in these experiments are alike.

The oxygen uptake of anaplasma-infected and normal, or control blood suspended in modified Hank's solution, with 1 part of blood to 2 parts of the diluent. Each Warburg flask contained 3 ml of the diluted blood with 0.2 ml Pardee's buffer in the center well, and was aerated with alveolar air to provide a 3 to 4% initial $CO_2$ atmosphere, then allowed to equilibrate for 45 to 90 minutes before readings were taken. The buffer has been shown to maintain an atmosphere of about 3% $CO_2$.

to determine whether the difference between the means obtained for the infected and normal blood for both the first and second hours are of significance. (Refer to the appendix for actual statistical analysis.)

By this method t values of 4.3 ($P=<0.01$) and 3.6 ($P=<0.01$) were obtained for the first and second hours, respectively, for data from all experiments, while values of 3.6 ($P=<0.01$) and 4.6 ($P=<0.01$) were found for data obtained from Experiments 15 to 23. All of these figures are highly significant, according to Fisher's Table of t. That is, the difference between the mean respiratory rate of infected blood and normal blood is, in each case, highly significant, and cannot be accounted for on the basis of the normal variation expected in the respiration of control blood.

A question might be posed as to whether the increase in respiration could be due to small numbers of bacteria, either present as secondary invaders in the animal itself, or present as contaminants after collection of the blood. Sterility tests in thioglycollate broth, however, made immediately prior to each run essentially rules out this possibility, since all such tests were negative for the presence of bacteria.

Thus, these experiments seem to have demonstrated that the blood of calves in the active stage of anaplasmosis has

a respiratory rate two to three times as great on the average as the blood of normal calves.

Experiment 17, not reported in Table 9, gave unaccountable results. This particular run was made with infected blood from Animal 411, which had, at the time, an average of 12.1% infected red cells. Using the standard procedure of suspending the cells in modified Hank's solution, using Pardee's buffer in the center well, then aerating for 30 seconds with alveolar air and allowing 90 minutes for equilibration of the gas phase, readings were obtained from this infected blood, as well as control blood from Animal 415.

The results of this experiment in the first hour gave, for the infected blood, readings of 57.3 $\mu$l $O_2$/ml pcv, and in the second hour a value of 47.0 $\mu$l $O_2$/ml pcv. The control blood gave respiration rates of 6.3 $\mu$l and 0.3 $\mu$l $O_2$/ml pcv for the first and second hours, respectively.

Obviously the figures obtained for the infected blood are somewhat out of the range of values obtained from all other animals reported, being over twice that found for the closest value. They are over 4 times the highest readings obtained for infected blood in Experiments 15 to 23, where experimental conditions were under better control.

It appears quite evident that this particular infected blood sample was respiring at a rate not in line with all other past experiments, and the most likely reason for this

appeared to be bacterial contamination. However, phase contrast and ordinary light microscope observations, sterility tests in thioglycollate broth, and blood agar streaks with the original blood sample failed to reveal any evidence of contamination. So it is felt that the results of this experiment are due to unknown factors, and because of the extremely high readings of the infected blood, the data was excluded from Table 9.

Since oxygen appears to be utilized by the anaplasma-infected blood at an increased rate, an attempt was made to determine whether there might be some correlation between the per cent of infected erythrocytes and the amount of oxygen consumed by the cells; however, there apparently is no logical relation between these factors (See Table 10). That is, more highly infected blood may have higher or lower rates of respiration than blood which had only a low grade infection.

On the other hand, there does appear to be a general rise in the amount of oxygen taken up with respect to the stage of infection (See Graph 1). That is, the infected blood of all animals upon which respiration measurements were made, with the exception of Animal 410, showed a general increase in the rate of oxygen consumption with increasing time in days during the period in which such measurements were made. For example, averages of the first and second hour

TABLE 10

OXYGEN UPTAKE OF INFECTED BLOOD WITH RESPECT TO THE PER CENT INFECTED ERYTHROCYTES

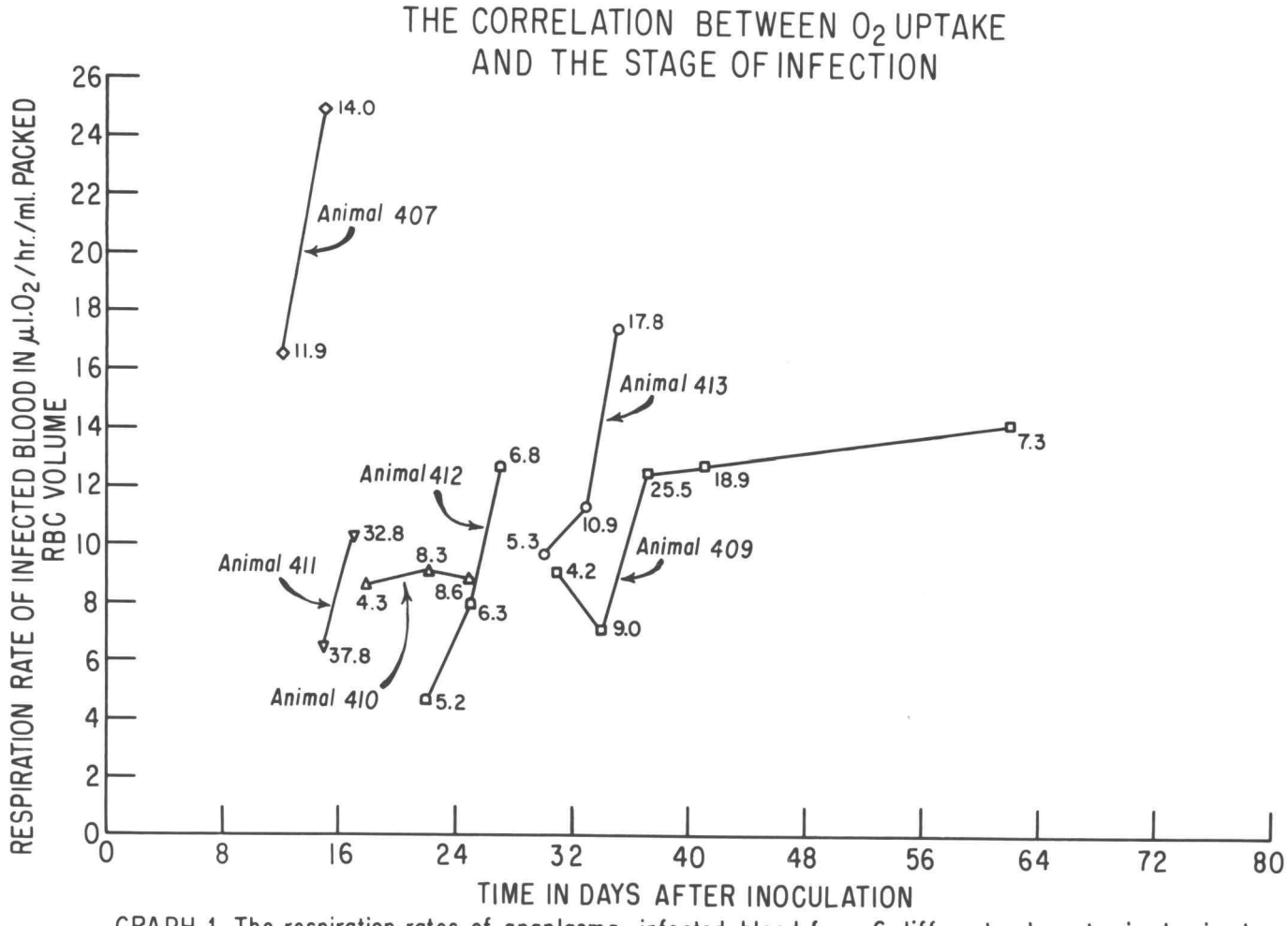
| Experiment No. | Animal No. | % infected red cells | $\mu l$ $O_2$ uptake/ml packed cell volume | |
|---|---|---|---|---|
| | | | 1st hr. | 2nd hr. |
| 9 | 409 | 4.2 | 7.9 | 10.2 |
| 18 | 410 | 4.3 | 7.3 | 9.9 |
| 21 | 412 | 5.2 | 2.5 | 6.6 |
| 21 | 413 | 5.3 | 8.5 | 10.8 |
| 22 | 412 | 6.3 | 7.4 | 8.5 |
| 23 | 412 | 6.8 | 12.3 | 13.1 |
| 13 | 409 | 7.3 | 22.9 | 5.4 |
| 19 | 410 | 8.3 | 8.7 | 9.5 |
| 20 | 410 | 8.6 | 7.7 | 9.9 |
| 10 | 409 | 9.0 | 10.6 | 3.5 |
| 22 | 413 | 10.9 | 12.9 | 9.8 |
| 6 | 407 | 11.9 | 26.5 | 6.7 |
| 7 | 407 | 14.0 | 26.1 | 23.6 |
| 23 | 413 | 17.8 | 17.9 | 17.1 |
| 12 | 409 | 18.9 | 14.3 | 11.2 |
| 11 | 409 | 25.5 | 12.5 | 12.5 |
| 16 | 411 | 32.8 | 9.0 | 11.5 |
| 15 | 411 | 37.8 | 5.1 | 7.8 |
| Mean | | 13.0 | 12.2 | 10.4 |

The oxygen uptake of infected erythrocytes as compared to the per cent of infected cells. Note the lack of correlation between rates of $O_2$ consumption and the % of infected cells.

measurements for blood from Animals 407, 412, and 413 showed net increases of 8.3, 8.1, and 7.8 µl $O_2$/ml pcv/hr, respectively, between the first measurements and those made 3 to 5 days later.

Only the infected blood from Animal 410 did not follow this general increase over the period of measurements, having an average net rise of only 0.2 µl $O_2$/ml pcv/hr during the measured period. This animal, however, had previously been inoculated with deer blood, which, it had been hoped, might be carrying the marginal parasite. After approximately two months, when it was evident that an infection was not induced by the deer blood injection, the calf was then transfused with carrier blood. Thus, the anaplasma-infected blood may have been suppressed in its ability to respire by some factor present due to the inoculation of deer blood.

The observation that there is a general trend toward increase in the respiratory capacity of the infected blood with all the other animals suggests that there may be some factor other than infected red cells which is increasing in the blood. The increases, as mentioned previously, are not correlated with the per cent of infected red cells found in stained smears; perhaps they could be due to some form of the agent not found, or not seen in the stained smears, or due to an increase in the total number of bodies in the blood, which was not determined in the stained smears.




GRAPH 1. The respiration rates of anaplasma-infected blood from 6 different splenectomized animals. Plotted points correspond to actual measurements, and figures indicate the percent of infected red cells at the time of measurement.

The possibility also exists that reticulocytosis may have caused the general increase in the rate of respiration. It has been found by several workers that reticulocytes respire at rates higher than that of mature red cells. For example, Wright (27, p. 201-213) reported that if the reticulocyte count in anemic rabbit blood was as great as 10% of the total red cell count respiration was increased about threefold. With anaplasmosis being characterized by anemia, one cannot discount the possible role of reticulocytes as a factor in the increased respiratory rates found in infected blood.

Unfortunately this factor was not investigated as it was not appreciated that reticulocytes respire at a higher rate than mature erythrocytes until after this work was completed.

Several experiments were run where the infected and normal cells were washed twice with modified Hank's solution prior to Warburg measurements (Table 11). Results indicate that the washed infected cells did respire at a higher rate than did the normal cells in 2 experiments, but did not do so in 2 others.

It is interesting to find that the washing of the cells appeared to lower the respiration rates, in most cases, when compared to the unwashed cells, the $O_2$ uptake rates of which were measured at the same time. (Compare values of Table 11 with that of comparable experiments in Table 9.)

Only one reading in Experiment 16, which was for infected blood, was higher than that for comparable unwashed cells. In fact, washing of normal cells, in many cases, reduced the respiratory capacity to below measurable rates. Perhaps this was due, in part, to the absence of the normal physiological cell environment produced by the washing of the cells. In any case, no conclusions are possible from these few experiments.

Aeration of the flasks with alveolar air appeared to stimulate the respiration of both infected and control cells during the first hour of measurements. This is suggested by the data of Tables 12 and 13. The data of Table 12 for 2 experiments suggests that both Pardee's buffer and 20% KOH give similar respiratory rates if all flasks are aerated with alveolar air. Table 13 suggests that aeration is important, the uptake for the aerated flasks with Pardee's buffer being generally higher during the first hour than that for the non-aerated flasks containing 20% KOH in the center well. These observations are too few to be statistically significant.

The explanation for this apparent difference is presumably the stimulatory effect of $CO_2$. Perhaps only minute amounts of $CO_2$ are necessary for increased respiration rates. Following aeration, perhaps enough of this gas may have been absorbed in the medium to satisfy the requirements

TABLE 11

OXYGEN UPTAKE OF WASHED INFECTED AND NORMAL ERYTHROCYTES

| Experiment No. | Infected Animal No. | Infected Blood µl $O_2$ uptake/ml pcv | | Control Animal No. | Control Blood µl $O_2$ uptake/ml pcv | |
|---|---|---|---|---|---|---|
| | | 1st hr. | 2nd hr. | | 1st hr. | 2nd hr. |
| 16 | 411 | 10.1 | 8.4 | 415 | 2.1 | 0 |
| 18 | 410 | 1.2 | 0.0 | H-11 | 0 | 0 |
| 19 | 410 | 0.9 | 2.0 | 358 | 1.8 | 1.9 |
| 20 | 410 | 5.7 | 3.9 | C-50 | 1.0 | 2.9 |

The oxygen uptake of infected and normal, or control erythrocytes that had been washed twice in modified Hank's solution, suspended in this same medium, and added to the Warburg flasks. Pardee's buffer in center well of flasks; then aerated 1 minute with alveolar air.

TABLE 12

A COMPARISON OF $O_2$ UPTAKE OF INFECTED AND NORMAL BLOOD IN AERATED FLASKS CONTAINING PARDEE'S BUFFER WITH FLASKS CONTAINING 20% KOH IN THE CENTER WELL

| Experiment No. | µl $O_2$ uptake/ml packed cell volume | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1st hour | | | | 2nd hour | | | |
| | Pardee's Buffer | | 20% KOH | | Pardee's buffer | | 20% KOH | |
| | Infected Blood | Normal Blood | Infected Blood | Normal Blood | Infected Blood | Normal Blood | Infected Blood | Normal Blood |
| 7 | 26.1 | 1.5 | 27.9 | 4.5 | 23.6 | 4.8 | 22.5 | 7.2 |
| 13 | 22.9 | 7.5 | 19.2 | 8.2 | 5.4 | 1.7 | 10.2 | 4.4 |

The oxygen uptake of normal and infected blood suspended in modified Hank's solution with Pardee's buffer or 20% KOH in the center well. All flasks were aerated one minute.

TABLE 13

A COMPARISON OF THE $O_2$ UPTAKE OF INFECTED AND NORMAL BLOOD IN AERATED FLASKS CONTAINING PARDEE'S BUFFER AND NONAERATED FLASKS CONTAINING 20% KOH IN THEIR CENTER WELLS

| Experiment No. | µl $O_2$ uptake/ml packed cell volume | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1st hour | | | | 2nd hour | | | |
| | Pardee's Buffer | | 20% KOH | | Pardee's Buffer | | 20% KOH | |
| | Infected Blood | Normal Blood | Infected Blood | Normal Blood | Infected Blood | Normal Blood | Infected Blood | Normal Blood |
| 9 | 7.9 | 6.1 | E* | E* | 10.2 | 2.2 | 2.5 | E* |
| 10 | 10.6 | 9.1 | 7.0 | 2.0 | 3.5 | 0 | 0.4 | 1.9 |
| 11 | 12.5 | 3.1 | 3.5 | 1.4 | 12.5 | 17.4 | 10.0 | 7.1 |
| 12 | 14.3 | 3.6 | 12.3 | 4.7 | 11.2 | 1.6 | 14.1 | 9.5 |
| 13 | 22.9 | 7.5 | 10.0 | 4.9 | 5.4 | 1.7 | 7.6 | 8.3 |

*E indicates erratic results.

Oxygen uptake of normal and infected blood suspended in modified Hank's medium, with Pardee's buffer or 20% KOH in the center wells. Flasks containing Pardee's buffer were aerated for one minute, while those containing KOH were not aerated. The purpose of the alveolar air was to establish an atmosphere of 3 to 4% $CO_2$ in the flasks containing Pardee's buffer without delay.

of the cells, even in the case where KOH was used in the center well. Red cells are well known to bind $CO_2$ chemically. Flasks with the same KOH in the center well, but not aerated did not, apparently, have sufficient $CO_2$ to support the maximum respiratory capacity of the cells. So, even though 20% KOH in the center well of the aerated flasks may have left enough $CO_2$ bound to the cells, a margin of safety was presumably provided by using Pardee's buffer which maintained carbon dioxide in the flasks at a 3% level at all times.

# DISCUSSION

## I. Morphological Observation on Anaplasma-Infected Blood:

Throughout the detailed examination of the infected blood with the phase contrast microscope it was found that the anaplasms consisted not only of a head, which is apparently the stainable portion of the parasite, but also an unstainable tail, both of which were intracellular. When one head was located at the end of a tail, a "comet-like" appearance was assumed, while if a head was found on each end of the tail, the parasite resembled a "dumbbell." Combinations of the comet-like forms and the dumbbell forms were also observed. During the latter stages of the active infection heads could be found without evidence of the tails, and some of these often appeared to be "shattered" giving rise to a large number of small granules.

The observation of these various forms is largely attributed to the fact that the phase contrast microscope enables one to observe blood preparations in an unfixed and unstained state, whereas the general technique up to this time has been to prepare a stained smear of the blood, then observe this under the ordinary light microscope. This latter method implies that the blood must first be dried, perhaps fixed by some method, and then stained with an appropriate dye. Any one of these processes may have an

adverse effect on the overall morphology of the intracellular parasite.

The only change in the red cells required to make the tailed bodies visible was hemolysis or release of hemoglobin. This was accomplished by freezing and thawing, or simple aging of blood in the refrigerator. However, it is felt that the anaplasms observed in hemolyzed cells were in the state of morphology found in vivo, especially when unfrozen freshly defibrinated blood, or oxalated blood was examined.

The morphological forms of Anaplasma marginale as observed here with the phase contrast microscope are in part comparable to those reported in the literature for bodies seen in stained preparations and in electron microscope studies. This is especially true with regard to observations of the stainable head portion.

For example, the ring forms observed by Boynton (3, p. 10-12) could certainly have been the result of staining the "heads," which under phase contrast appeared to have small or large central openings. The "buds" or protuberances, which Boynton also observed, could be explained by the segmenting of the heads into several units, and one or more of these protruding out to give a budding appearance when stained.

Some observations made by Lotze and Yiengst employing staining techniques (14, p. 312-320) also correspond quite

closely with what was found under the phase microscope. These investigators reported, for instance, that the anaplasma body could be classed as (1) smooth types, which were spherical, of smooth contour, and 0.2 to 0.5 microns in diameter, and (2) rough anaplasms which were 0.6 to 0.9 microns in diameter, roughly spherical, although occasionally cuboidal, and apparently consisting of a number of small spheroid bodies.

The smooth type, which these investigators described as being homogenous in nature, appears to correspond closely to what is described here as the smaller diametered tailed bodies, which were also of uniform density. The rough anaplasms, on the other hand, seem to relate to what has been referred to here as heads which were segmented, the segments corresponding to the spheroids. However, while the spheroids were reported as being in a tetrad arrangement, one tetrad being on top of another to give a box-like structure with a clear central space, or in a roughly circular form with a clear central space, phase contrast observations revealed that there could be many arrangements of the two to seven segments forming the head. These included the spherical packets, triangular arrangements, lateral configurations, or no orderly organization. In the case of the spherical packets and the triangular arrangements a clear central space could be found in many instances, but

it is interesting to note that each of the segments making up the entire head might also have a clear central space. The difference in observations in this case might be due to better definition in the unstained preparations.

These same investigators also reported that some red cells, in the acute cases, contained large numbers of what appeared to be the spheroid forms of the rough anaplasms, which were similar to the smooth types, except the latter were found only near the margin of the red blood cell. Here again, this might be explained by the dispersion of the segments of the head into the stroma without actually shattering to give rise to the small granule-like forms.

Electron microscope studies reported by others may be correlated with what was found here concerning the granular structure of the segments. It was observed here that during the later stages of the infection, some of the cell stroma could be found with a large number of small granules. These granules, as pointed out previously, appeared to be the result of a shattering or dispersion of one or more segments of the head. In this regard, Foote et al. (7, p. 19-21) found in their studies of A. marginale with the electron microscope that the parasite was made up of 1 to seven masses of dense particulate matter arranged about a clear central space. Here again, these masses may be what have been regarded as segments in this study. In contrast,

De Robertis and Epstein (5, p. 254-258), using the same instrument, found that the parasites, in one form, were undivided in the central area, but that the periphery consisted of 170 to 200 millimicron rounded bodies protruding from the surface, while in another stage these small rounded forms were scattered throughout the erythrocyte, with as many as 40 being found in one red cell. This latter observation conforms with what has been found here, within limits of the resolution of the phase contrast microscope. The undivided form, however, could only be explained by the fact that these investigators, like Lotze and Yiengst, were observing the dense, undivided, or unsegmented forms.

The tail portion of the parasites as found under the phase microscope is not as well documented as the head portion. But, Lotze and Yiengst (14, p. 312-320) and Franklin and Redmond (10, p. 252-253) do report the observation of tails on Anaplasma marginale using stained blood preparations. The former group found these to occur, with knob-like heads, outside the cell stroma, and largely on non-infected erythrocytes. In one case report these extracellular tailed bodies were found throughout the infection. Franklin and Redmond, on the other hand, found only the tails extending out of the cell; also, the tailed forms were observed only occasionally throughout the active infection.

This is somewhat in contrast, of course, to what was observed here, with the entire parasite, both tail and head, being found intracellularly. The fact that the marginal body is found throughout the active infection, though, does agree with what was found by Lotze and Yiengst.

It is interesting to note that the tails give an answer as to why the bodies, as seen in stained preparations, appear only near the margin of the erythrocyte. It is apparent that if the tail extends across the red cell, as was found in this study, there actually is no other position available for the head except at the cell margin.

From the overall observations made here, it appears that the parasite does consist of a head and a tail. The tail is an integral portion of the anaplasma until the later stages of the infection when it may become degenerate.

The head, on the other hand, seems to go through a series of divisions from a single compact unit until it may have from one to seven segments. The segments seem to have a granular structure, such that after a certain period of maturation they may shatter, giving rise to a large number of free granules within the cell stroma.

Just what becomes of these granules is a matter of pure speculation, but from reports of electron microscope studies, perhaps these too consist of even smaller granules which are undistinguishable with the ordinary light microscope. But, again, this leads the investigator to the unanswered

question of what role, if any, do these very small particles play in furthering the infection in the host, or even between hosts.

II. Respiration of Anaplasma-Infected Blood:

Warburg respirometer measurements carried out on anaplasma-infected blood, employing the procedure previously outlined, indicate that there is an increased rate of respiration when compared to measurements made on non-infected, or control blood. Data of this type, in as far as it is known, has not previously been reported in the published literature.

The average amount of increased respiration was found to be about three times that of the control blood during the first hour of measurements, and about twofold for the second hour of measurements, as found by averaging all experiments. There was no apparent correlation between the per cent of infected red cells and the amount of oxygen consumed; however, a general increase in consumption was found as the disease progressed, during the period from the 12th to 36th day after infection, except in the case of one animal.

This latter trend, along with the lack of correlation of the oxygen consumed with the percentage of infected cells, raises some question as to whether the increases were actually due to the parasite, or to some other factor.

It is possible, as mentioned previously, that the general increase during each particular infection could be due, in part, to some undetected form of the anaplasma. However, another explanation could be reticulocytosis, a condition generally accompanying a severe anemia, such as is often found in anaplasmosis. Reticulocytes have been reported to respire at a significantly higher rate than mature red cells. Ponder (21, p. 357), for example, reported that rabbit reticulocytes respire at rates from 30 to 50 times that of mature red cells. Rubinstein, Ottolenghi, and Denstedt (22, p. 222-235) actually demonstrated the presence of an aerobic metabolism in reticulocytes by showing the presence of a tricarboxylic acid cycle and a cytochrome system.

The possible presence of reticulocytosis in the anaplasma infections investigated here was not determined; but its possible role in causing the increased respiratory rates cannot be ruled out.

Since the general consensus is that A. marginale is protozoon, it is of interest to compare some respiration measurements obtained in these experiments with those made by others on protozoan parasites to determine whether some correlation might be made.

Maier and Coggelshall (15, p. 87-96), in one study of malarial parasites, found that the oxygen uptake by monkey

plasmodium (Plasmodium knowlesi and P. cynomolgi) was dependent on the stage of growth. Ring forms utilized the least oxygen, using only 7.7 and 3.4 $mm^3/10^8$ parasites, respectively, for the first hour, while segmentors and/or 3/4 grown parasites utilized 33.8 and 47.0 $mm^3$ during the first hour, respectively. von Brand, Tobie, and Mehlman (26, p. 273-300) found that blood forms of Trypanosoma lewisi utilized 62 $mm^3$ $O_2$ in one hour for $10^8$ parasites and 194 $mm^3$ $O_2$ in one hour for $10^8$ blood forms of T. rhodesiense.

In order that a direct comparison to these reported data might be made, several conversions must be carried out with the data obtained in these respiration experiments. Employing the overall average oxygen uptake of 12.3 µl/ml pcv, and an overall average of 13% infected red cells, the following are obtained.

First, 12.3 µl is equivalent to about 12.3 $mm^3$. The total number of red cells per milliliter of packed cell volume is computed to be approximately $23.6 \times 10^9$ red cells, as determined by ratios of pcv to direct cell counts, given on page 33. The number of parasites in $23.6 \times 10^9$ red cells is found by multiplying the number of red cells per ml pcv by the average per cent infected cells, or $23.6 \times 10^9$ times 13%. From this, then, it is found that there were approximately $3.07 \times 10^9$ parasites present in this number of red cells, assuming 1 parasite per cell.

Thus, it is indicated that about 12.3 $mm^3$ $O_2$ was being taken up by approximately $3.07 \times 10^9$ parasites during the first hour of measurements, disregarding the normal respiration of the red cells themselves. Since the average uptake of non-parasitized red cells during the first hour of measurements was 4.3 µl, or 4.3 $mm^3$, then the actual uptake by $3.06 \times 10^9$ parasites is reduced to only 8.0 $mm^3$ during the first hour of measurements. Now, when this is based on $10^8$ parasites, as was reported in other protozoan respiration experiments, a value of only 0.26 $mm^3$ is obtained, or, in other words, 0.26 $mm^3$ for $10^8$ parasites during the first hour of measurements.

This amount of uptake is obviously not nearly as high as that obtained by Maier and Coggelshall or von Brand, Tobie, and Mehlman investigating malarial parasites and trypanosomes, respectively. The above results suggest that Anaplasma marginale, if it is indeed a protozoon, must have a very different type of metabolism from the malarial plasmodia and trypanosomes, which show very active oxidative activity.

It is of interest to consider whether the observations reported here lend support to any one of the 3 theories concerning the true nature of the agent anaplasmosis, which have been proposed.

One theory, held by Foote (7, p. 19; 8, p. 498) considers the agent to be a virus. It has been found that host tissue cells, infected with a number of different

viruses, do not show a stimulation or inhibition of respiration. This was true for influenza virus (1, p. 421-428); vaccinia, (19, p. 173-184); Newcastle disease (16, p. 475-485); and mouse encephalomyelitis (20, p. 577-582). The low respiratory rate found in this work for anaplasma infected red cells does not necessarily rule out the virus theory. However, if the tailed bodies and dumbbell forms seen in the red cells represent the infectious agent, these are larger than any agents now classified as viruses. Furthermore, anaplasmosis responds to therapy with the tetracyclenes. Only one group of viruses, the psittacosis lymphogranuloma group, are susceptible to these drugs, and there is some question as to whether they should be regarded as true viruses. Thus the evidence for the virus nature of the anaplasma is not very impressive.

The oldest theory considers Anaplasma marginale to be a protozoan parasite of the erythrocytes. The morphological observations described earlier in this report are in keeping with this theory. However, the respiration measurements do not lend it much support. Protozoan parasites, where studied, have all been found to consume considerable amounts of oxygen under aerobic conditions (von Brand, 25, p. 146-161). The oxygen consumption of anaplasma infected blood is about 3 fold greater than normal bovine blood, but far less than that of malarial blood when compared on the basis of the per cent of infected cells. If the increased respiration is due

to the anaplasma bodies they consume very little oxygen compared to either Plasmodium malariae or Trypanosoma lewisi. Thus they do not appear to be closely related to these typical pathogenic protozoa metabolically.

The third concept concerning the nature of the anaplasma is that proposed in Bergey's Manual of Determinative Bacteriology (4, p. 931-934). Here these organisms are classified under the Order, Rickettsiales; Family, Anaplasmataceae; Genus, Anaplasma marginale. They are described as very small intracellular parasites found in erythrocytes of vertebrates and transmitted by arthropods. The morphological concept of these organisms from phase contrast observations is not incompatible with this classification. Little is known concerning metabolism of organisms in the Order, Rickettsiales. It has been shown that some of the Rickettsiaceae consume oxygen in the presence of suitable substrates (2, p. 561-565; 12, p. 175-197). The findings reported here concerning respiration of anaplasma infected blood suggest that these organisms may utilize oxygen at a very low rate.

All in all, the results of the present studies of Anaplasma marginale considered together with other known properties seem to support the classification of Bergey, wherein this organism is regarded as a genus of the Family, Anaplasmataceae, under the Order, Rickettsiales.

# CONCLUSIONS

## I. Morphology of *Anaplasma marginale*:

Phase contrast microscope observations of hemolyzed infected blood have shown that *Anaplasma marginale* may consist of any one of several intracellular forms classified here as: (1) comet-like forms; (2) dumbbell forms; (3) combined comet-like forms and dumbbell forms; and (4) tailless forms.

The comet-like forms consisted of one head, which was about 1 u in diameter, and a flat tail about 6 u in length, which generally extended across the red cell. The dumbbell forms consisted of two heads, each of which were about 1 u in diameter, and each of which was located at one end of an intervening connection. The intervening connection appeared very similar in substance to that of the tails of the comet-like forms. The combination forms, which were less prevalent than the former two forms, consisted of a dumbbell form in which one of the heads also had a tail, giving a combined comet-like and dumbbell form. The tailless forms were most common during the latter stage of the active infection.

The heads of these variously described parasites were found to consist of one to seven segments; the density of some of these units was uniform, while other appeared vacuolated, or hollow. Late in the active infection, these

segments were frequently found to be "shattered" giving rise to a large number of small granules.

The tails of the parasites appeared to be very fragile as noted by their deformation by exposure to freezing and Alsever's solution, as compared to their morphology in untreated defibrinated blood. The tails were also noted to deteriorate on exposure to distilled water, and, apparently, to various techniques used in the preparation of stained smears.

The heads of these forms seen in red cell stroma by phase contrast were found to be apparently identical to the marginal bodies which have been known for many years in stained films of infected blood.

II. Respiration of Anaplasma-Infected Blood:

Anaplasma-infected blood appears to respire at a significantly higher rate than does normal, or non-infected blood when measured on a standard Warburg apparatus under comparable conditions.

Eighteen separate respiration measurements were made on six different anaplasma-infected animals during the active infection. During the first hour of measurements an overall average increase of about threefold was found for infected blood over that for control blood, while in the second hour of measurements the increase was found to be about twofold.

There was no apparent correlation between the per cent of infected red cells and the rate of oxygen consumption; however, a general increase in this rate was noted as the disease progressed in all animals except one.

When the oxygen uptake was based upon the per cent of infected red cells, it was found that there was only about 0.26 $mm^3$ (0.26 ul) per $10^8$ marginal bodies being consumed during the first hour of measurements. A comparison of this value with data from other published reports on protozoan parasites, leads to the conclusion that anaplasma is relatively inactive from a respiratory point of view.

This, of course, brings out the question as to whether this parasite is the actual cause of the increased $O_2$ uptake. There is a possibility that other factors may be present causing this effect or contributing to it. One of these may be reticulocytosis, a condition presumably present due to the anemic nature of anaplasmosis. Other investigators have found that reticulocytes respire at a significantly higher rate than mature red cells. Thus, the observed increase could be due to the increased presence of these immature red cells.

With this factor complicating the picture, it appears that further investigation should be carried out on this aspect of anaplasmosis before any definite conclusions are made.

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# APPENDIX

A. Test of Significance for the Difference in Mean Respiratory Rate of Infected Blood and Normal Blood, Including All Reported Experiments, Employing Student's t Test.

Table of Data

| Expt. No. | $O_2$ Uptake µl | | | |
|---|---|---|---|---|
| | 1st hr. | | 2nd hr. | |
| | Infected | Controls | Infected | Controls |
| 6 | 26.5 | 6.3 | 6.7 | 9.6 |
| 7 | 26.1 | 1.5 | 23.6 | 4.8 |
| 9 | 7.9 | 6.1 | 10.2 | 2.2 |
| 10 | 10.6 | 9.1 | 3.5 | 0.0 |
| 11 | 12.5 | 3.1 | 12.5 | 17.4 |
| 12 | 14.3 | 3.6 | 11.2 | 1.6 |
| 13 | 22.9 | 7.5 | 5.4 | 1.7 |
| 15 | 5.1 | 3.9 | 7.8 | 5.1 |
| 16 | 9.0 | 4.6 | 11.5 | 9.4 |
| 18 | 7.3 | 1.0 | 9.9 | 0.0 |
| 19 | 8.7 | 4.9 | 9.5 | 2.2 |
| 20 | 7.7 | 3.5 | 9.9 | 3.4 |
| 21 | 2.5 | 2.5 | 6.6 | 4.6 |
| | 8.5 | | 10.8 | |
| 22 | 7.4 | 2.5 | 8.5 | 7.1 |
| | 12.9 | | 9.8 | |
| 23 | 12.3 | 4.7 | 13.1 | 3.6 |
| | 17.9 | | 17.1 | |
| Total | 220.1 | 64.8 | 187.6 | 72.7 |
| Mean | 12.2 | 4.3 | 10.4 | 4.8 |
| Difference | 7.9 | | 5.6 | |

1st hr.
Infected $\Sigma d = (14.3)^2 + (13.9)^2 + (4.3)^2 + (1.6)^2 + (0.3)^2 + (2.1)^2 + (10.7)^2 + (7.1)^2 + (3.2)^2 + (4.9)^2 + (3.5)^2 + (4.5)^2 + (9.7)^2 + (3.7)^2 + (4.8)^2 + (0.7)^2 + (0.1)^2 + (5.7)^2$

$= 818.7$

Normal $\Sigma d = (2.0)^2 + (2.8)^2 + (1.8)^2 + (4.8)^2 + (1.2)^2 + (0.7)^2 + (3.2)^2 + (0.4)^2 + (0.3)^2 + (3.3)^2 + (0.6)^2 + (0.8)^2 + (1.8)^2 + (1.8)^2 + (0.4)^2$

$= 69.1$

2nd hr.
Infected $\Sigma d = (3.7)^2 + (13.2)^2 + (0.2)^2 + (6.9)^2 + (2.1)^2 + (0.8)^2 + (5.0)^2 + (2.6)^2 + (1.1)^2 + (0.5)^2 + (0.9)^2 + (0.5)^2 + (3.8)^2 + (0.4)^2 + (1.9)^2 + (0.6)^2 + (2.7)^2 + (6.7)^2$
$= 345.7$

Normal $\Sigma d = (4.8)^2 + (0.0)^2 + (2.6)^2 + (4.8)^2 + (12.6)^2 + (3.2)^2 + (3.1)^2 + (0.3)^2 + (4.6)^2 + (4.8)^2 + (2.6)^2 + (1.4)^2 + (0.2)^2 + (2.3)^2 + (0.8)^2$
$= 290.4$

Combined $\Sigma$ squares, 1st hr. = 887.8
Combined $\Sigma$ squares, 2nd hr. = 636.1

Divisor = (18-1) + (15-1) = 31

Mean square
1st hr. = $\frac{887.8}{31} = 28.7$

2nd hr. = $\frac{636.1}{31} = 20.5$

Est. Std. deviation
1st hr. = $\pm\sqrt{28.7} = \pm 5.4$

2nd hr. = $\pm\sqrt{20.5} = \pm 4.5$

Standard error of difference between means
1st hr. = $\pm 5.4\sqrt{\frac{1}{18} + \frac{1}{15}}$
$= \pm (5.4)(0.35)$
$= 1.89$

$t = \frac{7.9}{1.85} = 4.3$
Entering Fisher's Table of t, with n = 31,
P = < 0.01

2nd hr. = $\pm$ 4.5 (0.35) = 1.57

$t = \frac{5.6}{1.57} = 3.6$
For this value of t, P = < 0.01

B. A Test of Significance for the Difference in Mean Respiratory Rate of Infected Blood and Non-infected Blood, Including Experiments 15 through 23, Employing Student's t Test.

Table of Data

| Expt. No. | Respiration rates, µl | | | |
|---|---|---|---|---|
| | 1st hr. | | 2nd hr. | |
| | Infected | Normal | Infected | Normal |
| 15 | 5.1 | 3.9 | 7.8 | 5.1 |
| 16 | 9.0 | 4.6 | 11.5 | 9.4 |
| 18 | 7.3 | 1.0 | 9.9 | 0.0 |
| 19 | 8.7 | 4.9 | 9.5 | 2.2 |
| 20 | 7.7 | 3.5 | 9.9 | 3.4 |
| 21 | 2.5 | 2.5 | 6.6 | 4.6 |
| | 8.5 | | 10.8 | |
| 22 | 7.4 | 2.5 | 8.5 | 7.1 |
| | 12.9 | | 9.8 | |
| 23 | 12.3 | 4.7 | 13.1 | 3.6 |
| | 17.9 | | 17.1 | |
| Total | 99.3 | 27.6 | 114.5 | 35.4 |
| Mean | 9.0 | 3.4 | 10.4 | 4.4 |
| Difference | 5.6 | | 6.0 | |

1st hr.
Infected $\sum d = (3.9)^2 + (0)^2 + (1.7)^2 + (0.3)^2 + (1.3)^2 + (6.5)^2 + (0.5)^2 + (1.6)^2 + (3.9)^2 + (3.3)^2 + (8.9)^2$
$= 170.2$

Normal $\sum d = (0.5)^2 + (1.2)^2 + (2.4)^2 + (1.5)^2 + (0.1)^2 + (0.9)^2 + (0.9)^2 + (1.3)^2$
$= 13.0$

2nd hr.
Infected $\sum d = (2.6)^2 + (1.1)^2 + (0.5)^2 + (0.9)^2 + (0.5)^2 + (3.8)^2 + (0.4)^2 + (1.9)^2 + (0.6)^2 + (2.7)^2 + (6.7)^2$
$= 80.0$

Normal $\sum d = (0.7)^2 + (5.0)^2 + (4.4)^2 + (2.2)^2 + (1.0)^2 + (0.2)^2 + (2.7)^2 + (0.8)^2$
$= 58.7$

Combined sum of squares

1st hr. = 183.2
2nd hr. = 138.3

Divisor = (11-1) + (8-1) = 17

Mean square

$$\text{1st hr.} = \frac{183.2}{17} = 10.8$$

$$\text{2nd hr.} = \frac{138.3}{17} = 8.1$$

Estimated Std. deviation

$$\text{1st hr.} = \sqrt{10.8} = 3.3$$

$$\text{2nd hr.} = \sqrt{8.1} = 2.8$$

Standard error of difference between means

$$\text{1st hr.} = \pm 3.3\sqrt{\frac{1}{11} + \frac{1}{8}} = \pm (3.3)(0.47) = 1.55$$

$$t = \frac{5.6}{1.55} = 3.61$$

for n = 17
P =< 0.01

$$\text{2nd hr.} = \pm (2.8)(0.47) = 1.32$$

$$t = \frac{6.0}{1.32} = 4.55$$

for n = 17
P =< 0.01