A STUDY OF THE CRYOGENIC PRESERVATION AND IN VITRO CULTIVATION OF ANAPLASMA MARGINALE

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Scope and Method of Study: This investigation is divided into two parts. The first part of the study was designed to demonstrate the feasibility of preserving the infectivity of Anaplasma marginale over long periods of time by means of deep freezing methods. For this purpose a comparison of freezing and thawing methods, with and without additives, was made. This information was then used to determine the infectivity of blood samples kept in a liquid nitrogen refrigerator, by injection into susceptible calves at 4-week intervals. The refrigerated blood samples were prepared with two different additives, one containing dimethylsulfoxide, the other a solution of sucrose and dextrose, and compared with samples to which no additive was included. Proof of the induced infection was demonstrated by a rising complement fixation titre, and the observation of marginal bodies in their blood. For the second part of this investigation, a series of experiments was designed in an attempt to grow Anaplasma marginale in vitro. Tissue cultures were employed as the medium of growth, and two different stains as well as a specific fluorescent antibody conjugate were used for microscopic observation of the cultures. Subsequent injection of calves, however, was the main criterion for proof of growth and infectivity.

Findings and Conclusions: Maintaining the infectivity of Anaplasma marginale for extended periods of time at the temperature of liquid nitrogen appears entirely practicable. Although observations were confined to a period of 196 days, the uniformly successful results obtained with the use of additives to the blood as contrasted with blood kept without additives, makes it possible to explain the variable results of others in previous years. The integrity of the red cell seems essential to maintaining the infectivity of Anaplasma marginale. With this in mind it seems safe to assume that preservation for many months or even years may be possible. While the results of the second part of this investigation are largely negative, the sustained infectivity of the organism kept at 37° C. for four days on cell culture, (but not without such cell culture) offers some encouragement. The methods of this experiment, when coupled with the pO_2 concept of a latter experiment, might well merit further investigation.

ADVISER'S APPROVAL _____

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To my wife Barbara and daughter Abbie--for their understanding and forbearance of a middle-aged man's desire to play school boy--I tender my love and thanks.

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CHAPTER I

INTRODUCTION

Since Theiler (1911) first offered proof that anaplasmosis was a disease entity separate from babesiosis, and named the causative organism, investigators have been frustrated by the monoxenous nature of Anaplasma marginale. The organism is readily transmitted by certain ticks, tabanids, and other biting flies and insects—none of which, however, is an obligate intermediate host. The definitive bovine host is an obvious handicap to experimental studies, in both size of the required experimental animal, and the increased expense necessitated by use of this animal. Anaplasma marginale has never been adapted to laboratory animals; no practical, quantitative method of in vitro pure culture has been achieved; nor has a feasible method of its long term preservation been offered. The same may be said for the related organisms, Anaplasma centrale and Anaplasma ovis.

While previous studies and the elusive nature of the anaplasmata are discouraging, it nevertheless seems imperative that a sustained effort towards in vitro culture should be inaugurated. Without mention of its controversial classification, one might very well venture an opinion that Anaplasma marginale ought to grow in tissue cultures. Such a premise could be substantiated by recalling the extensive use of tissue cultures for the culture of a wide variety of infective agents—from bacteria, to viruses, rickettsiae, and even many

protozoa (Pipkin, 1960). It would appear that tissue cultures should offer a suitable culture medium for almost any obligate intracellular parasite, since it affords naturally favorable conditions of osmotic pressure, electrolytic balance, and proper pH, only available in or around the living cell. Although Anaplasma marginale may not be a protozoan, a virus, or rickettsia, the following quotation from Pipkin (1960) explains the advantage to be gained from its in vitro culture—as well as the protozoa which he discusses:

> Perhaps the most important advantage of the technique to the protozoologist is the unique opportunity for repeated observation, through plain and phase microscopy, of the growth and development of the intracellular parasite, offering unprecedented advantages in life cycle studies. . . . In parasites of uncertain taxonomic position, many of the details of their developmental cycles in the vertebrate host are vague because of the inaccessibility of these particular stages of development to the protozoologist. Tissue culture may well offer valuable assistance in the elucidation of the intracellular phase of such obscure entities as the Sarcosporidia and Haplosporidia, just as it has with another protozoan, Toxoplasma.

Unfortunate though it is, that the erythrocyte is incapable of reproduction, one can not preclude the possibility of Anaplasma marginale adapting itself to another cell system—for it is not known for certain that an extra-erythrocytic phase does not exist in vivo. With this in mind, two types of cell cultures were used in this study.

Whether or not its culture is successful, it is highly desirable that some practical method of long term in vitro preservation of Anaplasma marginale be devised. Maintaining the organism in a carrier animal presents obvious disadvantages. Furthermore, continued study of this disease might be advanced with the establishment of a repository of topodemic isolates.

CHAPTER II

REVIEW OF SELECTED LITERATURE

The disease which Anaplasma marginale causes in the bovine is characterized by an insidious anemia in the absence of hemoglobinuria. The acute disease as seen in the adult animal, two to three years of age and older, represents a severe economic loss in many tropical and subtropical areas of the world, as well as in the temperate zones of southern and western United States. The loss due to mild, often inapparent, infection in calves has not been determined. (Oglesby, 1962).

Morphology and Staining Characteristics

Anaplasmosis is readily diagnosed by observing the organism as small, round forms attached to the inner margin of erythrocytes in a peripheral blood smear, stained with Romanowsky type stains. These classical anaplasma bodies measure from 0.3 to 1.0 μ in size, and for some time represented the only form of this organism. Early investigators, however, soon began to report bizarre forms (Sieber, 1910; Quevedo, 1914; Descaseaux, 1924; Boynton, 1932) which possessed an appendage to the round body, generally referred to as a tail. The morphology of Anaplasma marginale has been a topic of continued study to the present day. In 1958 Franklin and Redmond reported tailed forms with tails of up to 0.9 μ in length extending outside the erythrocyte.

Espana and Espana (1959) first proved that removal of hemoglobin from the red cell was essential to demonstrate the tailed forms. These tails, or filaments, are demonstrated only by the use of certain stains such as Noland's, Thionin, and silver impregnation (Espana and Espana, 1963); new methylene blue (Schalm, 1962); and Toisson's stain (Kreier et al., 1963b). Lotze and Yiengst (1942) reported seeing large and small round forms and groups of small round forms in erythrocytes of diseased cattle, and also described tailed forms attached to the surface of the red cells.

Both tailed and ring forms of the organism were described by Osebold et al. (1962) by the use of new methylene blue stain. In applying the fluorescent antibody technique to demonstrate anaplasma, Ristic et al. (1957) reported observing only the round marginal forms—possibly as a result of using alcohol fixation of the blood films, thus preventing the de-hemoglobinization of the erythrocytes. (Espana and Espana, 1959). Madden (1962) observed round forms, commas, comets, matchstick and dumbbell forms by use of fluorescein-antibody conjugates. He also noted that while the organisms fluoresced slightly with the application of normal serum, the appearance of the bodies in these control slides differed from the homologous system in that only small round bodies were visible, no tailed forms.

Among the first to apply the electron microscope to a study of Anaplasma marginale were DeRobertis and Epstein (1951), who published pictures showing the classical body to be composed of a central undivided mass and a periphery of elementary bodies of high electron density, 170 to 220 mμ in diameter. In some cases, the entire body was seen to be composed of these smaller units. They also found as

many as 40 elementary units dispersed throughout one erythrocyte. Foote et al. (1958) also studied electron micrographs of ultrathin sections of the infected red cells and found the anaplasma body to be a clear space at the margin of the erythrocyte, containing from 1 to 7 masses of dense particulate matter, measuring 0.2 to 0.7 μ in diameter.

In 1959 Espana and Espana confirmed, by both phase microscopy and electron microscopy, the complex morphology of Anaplasma marginale; ring, match, comet and dumbbell-like forms were observed in both natural and experimental infections. These same workers also purported to have seen extra-erythrocytic organisms, but admitted that proof of this was lacking. Electron microscope studies by Ristic and Watrach (1961) revealed an organism consisting of 1 to 8 round or oval initial bodies, about 300-400 mμ in diameter, and embedded in an apparently homogeneous matrix; both the parent body and the smaller, initial body possessed double limiting membranes. Scott et al. (1961) have published electron micrographs of the Anaplasma marginale organism in which the body is shown to consist of a limiting membrane enclosing smaller units that appeared in three morphologic forms. The first two forms differed in the presence or absence of a dense central mass, and the third form, characterized by numerous dense particles in the smaller units within the body, was later interpreted as artifactual distortion of the body.

Using de-hemoglobinized erythrocytes, Ritchie (1962) was able to demonstrate, by means of electron microscopy, anaplasma forms with an electron dense round structure and an associated sac-like projection which was somewhat electron lucid. The round structures were frequently subdivided into smaller units, and in the case of the double-

ended forms, the author states that they were not generally symmetrical, i.e., one end frequently was seen to be divided into granules.

By means of ultrathin sections, chromium shadow case preparations, and replica technique in electron microscopy, Ristic (1960b) described three distinct morphologic forms of the organism: i.e., the classical marginale inclusion; a smaller initial body, subunit of the large marginal body, 90 to 120 mμ in diameter; and the polyhedral body; a subunit of the initial body, the latter, smaller than 0.1 μ. The marginal body was shown to contain from 1 to 8 subunits (initial bodies) which varied in size from 0.3 to 0.1 μ in size. Of more interest is the author's report of observing initial bodies in nearly every erythrocyte from the blood of acutely infected animals, but in only 1 out of 10 to 20 red cells from blood of carrier animals. From this observation, Ristic claims that the persistence of initial bodies in the blood of carrier animals shows it to be the form necessary for its interepizootic survival.

Filtration experiments would tend to corroborate the size of the organism as measured by electron microscopy. Ristic (1960b) succeeded in passing anaplasmata through a 0.65 μ Millipore filter following prior mechanical disintegration of the erythrocytes in infected blood and proved that the filtrate was infective. Subsequent examination by electron microscope of the fresh, normal blood into which he collected the filtrate showed the anaplasma bodies attached to the erythrocytic membranes. Allbritton (1962) established the size of the infective unit of Anaplasma marginale as between 0.22 to 0.30 μ. The filtrate of sonicated, defibrinated blood passed through a membrane filter with an average pore diameter of 0.30 μ proved to be infective, whereas

that passed through a membrane filter with an average pore diameter of 0.22 μ failed to cause anaplasmosis in susceptible cattle.

Ristic and his associates at Illinois have contributed much in recent years which appears to further confuse the morphology and classification of Anaplasma marginale. Working with only the Florida isolate of the organism, this group has never been able to demonstrate the unusual forms reported by others. For example, Kreier and Ristic (1963b) made a comparative study of the anaplasmata seen in the Florida isolate and those seen in the Oregon isolate. In this study they found that the Florida isolate of the organisms was entirely monomorphic, whereas those seen in the erythrocytes of cattle infected with the Oregon isolate possessed three morphologic forms. These authors claimed that the predominant form of organisms seen in the red cells of cattle infected with the Oregon isolate was identical to that of the Florida isolate. The other two forms of parasites were those having a marginally situated head, a body, and a long tail; and those shaped like a bipolar disc. The bodies and tails of the tailed parasites and the bodies of those shaped like bipolar discs had no affinity for the acridine orange and Giemsa stains, whereas the heads stained as did the Florida isolate of Anaplasma marginale.

Classification

Again, in 1963, Kreier and Ristic (1963c) published a report of an immunoserologic comparison of the Florida isolate and the Oregon isolate of this erythrocytic parasite. By fluorescein-labeled antibody studies and cross-immunity studies with premune cattle, these workers claim to have demonstrated that the round body class of parasites which

occurs in the erythrocytes of cattle infected with the Oregon isolate of Anaplasma is antigenically identical to the parasites which occur in the erythrocytes of cattle infected with the Florida isolate. The class of parasites characterized by a head, a body, and a long tail which occurs in the erythrocytes of cattle infected with the Oregon isolate was demonstrated to be antigenically and immunoserologically distinct. A further inference of the existence of a separate species in the Oregon isolate of Anaplasma is made by the same authors (Kreier and Ristic, 1963d) in a report of a cross infection study. It claimed here that of the three morphologic forms of the organism seen in the Oregon isolate, only the round marginal body type would grow in deer, and the sheep passage of the Oregon isolate, in one case, at least, was said to have eliminated the tailed forms of the Oregon isolate, permitting survival of only the round marginal body type of parasite and the bipolar disc type. On the basis of this work, and that described above, these investigators have gone so far as to suggest that the organisms characterized by a head, body, and tail, and those which resembled bipolar discs were distinct entities for which the names Paranaplasma caudata and Paranaplasma discoides were proposed.

In 1962 Kreier and Ristic reportedly established preliminary evidence from fluorescent antibody studies that the protozoan, Theileria, might react with antiserum to "an organism which has been described as a tailed form of Anaplasma marginale." This is reminiscent of the work of Theiler (1910), who gave the organism its name and unhesitatingly labeled it a protozoan. This early classification has persisted almost to the present day, and likely originated from the association of Anaplasma with Piroplasma (Babesia) bigeminum in

mixed infections. (Smith and Kilborne, 1893; Theiler, 1910; du Toit, 1928). Lotze and Yiengst (1942) observed large and small round forms of anaplasmata, groups of these small forms in red corpuscles, and tailed forms attached to the surface of erythrocytes—which observations led them to believe that Anaplasma were protozoa. More recently, Espana et al. (1959) still believed that they were dealing with a protozoan.

Classification of Anaplasma as a virus was first proposed by Sieber (1910), who suspected that the round bodies attached to the inner margin of the red corpuscles constituted an inclusion body, as a reaction product of some invisible virus. Foote (1954) too, thought that the classical anaplasma body was an inclusion body, resulting from a viral organism—as a result of an experiment in which he recovered an infectious agent from the filtrate of a 3-pound Mandler filter. Still later, Foote et al. (1958), unable to ascertain cellular organelles such as nucleus, mitochondria and endoplasmic reticulum in electron micrographs of Anaplasma marginale, offered this as evidence that the etiologic agent was a virus.

Turner (1944) pointed out certain properties of Anaplasma marginale which might relate to its classification: "Loss of infectivity on standing (in citrated blood) and immunity being contingent on a state of premunition, are two properties peculiar to hemoprotozoan, but both viruses and protozoa are preserved by deep freezing." This too, is devoid of any positive means of classifying Anaplasma marginale.

Ristic and Watrach (1961) suggested that Anaplasma marginale is morphologically similar to rickettsiae and further resembles rickettsiae in its requirement for intracellular multiplication. Based upon

electron microscopy, they proposed binary fission to be the method of replication. This same year Scott et al. (1961), in a report of their electron microscope studies, claimed that "This study indicates the anaplasma body to be neither protozoan nor viral, but probably rickettsial." The filtration experiments of Allbritton and Parker (1962) also led them to suggest that the organism may be similar to the rickettsiae when size is considered. Further identity of Anaplasma with rickettsiae is claimed by Kreier and Ristic (1963a) who reported that the serum from animals infected with Eperythrozoan ovis and E. wenyoni reacted with Anaplasma marginale antigen in complement fixation tests. They stated, "The serological relationships and morphological similarities between the initial Anaplasma bodies and Eperythrozoa suggest that classification of the parasites under the order Rickettsiales as presently done is justified."

Histochemical research of Moulton et al. (1955) revealed that Anaplasma marginale contains both deoxyribose nucleic acid and ribose nucleic acid. Although this may be consistent with another known characteristic of rickettsiae, these authors state that no differentiation can be made on this basis as to whether or not the organism represents a bacterium, virus, or protozoan. Pilcher et al. (1961) compared the oxygen consumption of parasitized erythrocytes with that of uninfected erythrocytes and found that the red cells infected with Anaplasma marginale consumed ten times the amount of oxygen as did those not infected. However, this rate of oxygen consumption is far below that of Plasmodia and other blood parasites, and for this reason the investigators ruled out the protozoan nature of Anaplasma, and stated: "The information reported tends to support classification of

Anaplasma as a separate family, Anaplasmataceae, of the order Rickettsiales."

Espana and Espana (1963) as well as Roby (1960) believe that attempts to classify the parasite on the basis of existing information are premature. But for those who lack the patience and calm deliberation of the above mentioned investigators, there is the suggestion of Neitz et al. (1934), who proposed that Eperythrozoa, Grahamella, Bartonella, and Anaplasma, since they are closely related, should be classed together in the family Anaplasmidae-order Haemosporidia. It should be added, however, that these same people mentioned that Anaplasmidae have many features in common with the rickettsiae.

Life Cycle and Development

Both Brock (1958) and Hansard et al. (1958) have suggested that the bone marrow is the original site of infection and multiplication of Anaplasma marginale. Evidence to the contrary may be inferred from the experiments of Foote et al. (1957), and Ristic (1961a), which showed that infection with Eperythrozoon wenyoni interferes with the inducement of a concommittant infection of Anaplasma marginale. According to Ristic (1961a) this fact, and the doubling growth rate of the organism, with the improbability that the marrow could release erythrocytes at such an accelerated rate, suggest that transmission occurs in the peripheral blood between the red corpuscles there. Intravascular transmission of Anaplasma was further substantiated by readily identifying the organism in deer erythrocytes about 10 hours after transfusion into an infected bovine. The red cells from the deer were easily identified by the extreme sickling which they

characteristically undergo upon removal from the deer (Kreier et al., 1961).

Although Howell et al. (1941) claimed to have demonstrated the transovarian transmission in D. andersoni ticks, Anthony and Roby (1962), and again Anthony et al. (1964) could only demonstrate stage to stage transmission of Anaplasma in the tick. No one has ever been able to detect the occurrence in the tick of any different stage in the life cycle of the organism. Because of transstadial transmission of the organism, ticks are considered to be biological vectors, but flies and biting insects are merely mechanical vectors of the anaplasmata.

Ristic (1962) described the occurrence of the organism in blood platelets from infected cattle and suggests that the platelets may be a mode of intravascular transmission. Franklin and Redmond (1958) in their report of tailed anaplasmata, propose that this form may represent one stage in their normal development. It was noted by Pilcher et al. (1961) that the dilution of infective blood with water for hemolysis apparently rendered the tails no longer visible by phase-contrast microscopy. It is interesting to speculate on the significance of this in connection with the fact that such hypotonic hemolysis also renders the organism uninfective. (Brock, 1965).

In an attempt to establish some relationship of the filamentous forms of Anaplasma marginale to the development in the life cycle of the organism, Espana and Espana (1963) made a statistical evaluation of the occurrence of such forms in blood from 10 naturally and 12 experimentally infected cattle. They concluded that although the sequence in the infection and the relationship of the several forms

of Anaplasma were not clear, it appeared that the dumbbell-like organisms represented one type of multiplication. Many of the larger round bodies, both those with and without filaments, contained varying numbers of small units, indicating, they thought, the existence of another type of multiplication of the Anaplasma.

Although static representation of dynamic processes can not be considered final proof, several investigators thought they could deduce from electron micrographs certain events which would explain the developmental life cycle of Anaplasma marginale. From their pictures, DeRobertis and Epstein (1951) suggested that the organism replicated by binary fission. Ristic (1960a) thought he could demonstrate the ability of the initial body to transgress the erythrocytic membrane, and said that this completed the extra- and intraerythrocytic cycle of Anaplasma. By daily observance of bovine blood during the course of an infection, Ristic and Watrach (1963) through fluorescent antibody and electron microscope techniques developed a complete hypothesis concerning the cycle of development of the causative agent of anaplasmosis. These authors claimed that the cycle was initiated by penetration of the erythrocyte membrane by the initial body, whereas multiplication by binary fission occurred intracellularly. It is their contention that the complete developmental cycle occurs in the mature erythrocytes and that binary fission of the initial body and its direct transfer between red cells fully explains its mode of development.

Biophysical Characteristics

Early work by Rees (1937) indicated the lethal effect of higher temperatures to Anaplasma marginale. In one instance Rees froze

infective blood for 18 hours on dry ice, and upon thawing at room temperature was able to demonstrate its infectivity when injected into a susceptible heifer. Further experiments indicated that the organisms were killed by exposure for 15 minutes to 60° C.; for 10 minutes at 57° C., 54° C., and 48° C. in mixed infections (with Piroplasma), but survived 10 minutes at 48° C. in a pure infection.

A more detailed study of temperature effects was recently reported by Bedell and Dimopoullos (1962). They reported that Anaplasma marginale was destroyed by exposure at 60° C. for 50 minutes, but not at 15 or 30 minutes; at 45° C. for 8 hours, but not at 4 hours; at 38° C. for 86 hours, but not at 72 hours; at 25° C. for 288 hours, but not at 216 hours; at 4° C. for 21 days, but not at 7 days; at -20° C. for 9 days; and at -66° C. for 112 days, but not at 56 days. It was further shown that the incubation period increased with height of the temperature and the length of exposure at this temperature.

It has been inferred for some time that once the Anaplasma-infected erythrocyte was lysed, the infectivity of the blood sample would be destroyed. (Mott, 1957). More recently, however, Allbritton and Parker (1962) were able to show that the infective agent of anaplasmosis survived sonic oscillation for 5 minutes, and then remained infective when kept in a cell-free filtrate for 8 hours at 25° C.

Continued study along this line by Bedell and Dimopoullos (1963) indicated that the infectivity of Anaplasma marginale was destroyed by exposure to sonic energy treatments for 90 minutes, but not for 75 minutes, when the blood was maintained at 33 to 35° C. Treatments conducted at 19 to 22° C. and 17 to 18° C. did not destroy infectivity after 90 and 210 minutes, respectively. Furthermore, the etiologic

agent remained viable for at least 32 hours in an extra-erythrocytic environment. It was shown, however, that as the time of exposure to sonic energy was increased, there was a lengthening of the incubation time of the disease.

In an attempt to modify the virulence of Anaplasma marginale, Simpson et al. (1964) subjected heavily parasitized blood with the radiation from cobalt-60. Levels of 75,000 roentgens (R) or less did not destroy the disease-producing capacity of the organism, but 100,000 and 300,000 R prevented the development of the disease. Calves inoculated with blood irradiated at the two highest levels did not develop complement-fixing antibodies, and upon challenge with carrier blood, developed an overt case of anaplasmosis.

Further studies of the effect of ionizing radiation upon the infectivity of Anaplasma marginale contained in whole blood was reported by Gough and Dimopoullos (1965). Blood containing approximately 50% anaplasma bodies was subjected to radiation from cobalt-60 at a rate of 3,000 R per minute. Splenectomized calves inoculated with blood samples subjected to 90,000 R or more of radiation did not have signs of infection during an observation period of 90 days. When their resistance was challenged at this time with untreated infected blood, all calves were found to be fully susceptible by the development of typical clinical anaplasmosis. However, samples treated with doses of radiation ranging between 3,000 and 79,000 R were found to be active and produced the disease when inoculated into splenectomized calves, although the incubation periods were correspondingly lengthened.

Review of Previous Cryogenic Preservation

There is a paucity of literature regarding the cryogenic preservation of anaplasmata, especially A. marginale. The earliest evidence of such work is an anonymous and cryptic report in an Australian journal of the successful preservation of A. centrale (Australia, 1944), in which infective blood was quickly frozen, maintained at -80° C., and found to be infective 9 months later when it was quickly thawed. This experiment would appear to be identified with Turner (1944), who gave a more detailed report of such an investigation in the Australian Veterinary Journal that same year. Turner here states that he successfully preserved A. centrale by immersion of 1 ml. aliquots of infective blood in a dry ice-alcohol bath, keeping these in a dry ice chest and thawing them rapidly in a 40° C. water bath. Such blood was found to be infective more than 9 months later.

Another anonymous report (Australia, 1950) states that A. centrale infected blood was preserved in a dry ice box at approximately -80° C., and that 1 ml. of blood was still able to produce typical infection after 739 days. No details of freezing method, nor method of thawing are given.

Mott (1957) presented an account of the variable results he had obtained at that time with the cryogenic preservation of Anaplasma marginale:

> The infective agent from acute cases will survive in undiluted citrated or defibrinated infective blood held at room temperature for only a few days. It may survive for a month or less at ordinary refrigerating temperatures (40° F.), and we have had several successful transmissions with frozen erythrocytes held in a dry-ice storage chest from a few hours to eight years. We had hoped to preserve different experimental field strains in the frozen state rather than to maintain carrier animals for a source of seed material, but this method could

not be relied upon as too many frozen samples failed to survive in storage.

History of Attempted Culture

Carpano (1913) reported the occurrence of Anaplasma-like forms in his blood cultures of Nuttalia equi. Ignorance of the host specificity of anaplasma, and its existence as a distinct etiologic entity, made possible his speculation on their appearance as a phase in the reproductive cycle of the piroplasms under his study.

Perhaps inspired by the methods of Carpano, Veglia (1915), two years later, published a detailed and optimistic account of his in vitro culture of Anaplasma marginale in liquid blood cultures, modified by the addition of various substances. He used pure defibrinated blood, blood added to salt solution, ordinary bouillon, and sodium citrate solution, which he termed the "Carpano medium". Growth was demonstrated by his observation of the increase in the number of anaplasma bodies over a period of up to 40 days. In one case, blood containing 7% anaplasma bodies, in 20 days had reached 20% bodies, and by the 40th day, 25% of the erythrocytes were infected; another culture was begun with 17% of infected red cells, and within 21 days the number of bodies observed gradually rose to 30%; and the infected red cells rose from 6% to 20% within 8 days in still another culture—all the aforementioned being attributed to cultures in citrated blood. Numerous other cultures were tried by Veglia using the other three media, but only the defibrinated blood gave results comparable to the citrated blood—the bouillon and sodium chloride solutions being somewhat less successful. When kept at temperatures of 7° C., 15° C., and 26° C.,

little difference in the development of the anaplasmata could be noted. Veglia also claimed that the rate of increase of anaplasma bodies observed in the culture corresponded to that seen in the living animal, i.e., a doubling effect every 24 hours. He also recorded the culture of blood infected with both Anaplasma and Babesia, whereat it was stated that the latter did not develop, and the culture proved to contain Anaplasma alone.

Lestoquard (1924) reported the successful culture of Anaplasma ovis in citrated blood, according to the methods of Veglia. Weakly infected blood, with citrate, was placed in sterile tubes and incubated at 37° C., upon which a 25-fold increase of organisms was noted a week later. The same results were obtained with cultures kept at 22° C., and no difference could be detected between the blood of sheep or that of goats. The addition of about 1 drop of 30% glucose solution to 1 cc. of parasitized blood was said to have greatly enhanced the growth of Anaplasma ovis in such cultures. Neither the aerobic, nor anaerobic state of the culture was said to influence its multiplication. Studies of cultures made in serial passage to uninfected blood, however, were uniformly fruitless. Lestoquard claims that the same results were obtained with the in vitro culture of Anaplasma marginale, with the exception that a less intense multiplication of this organism occurred.

In similar experimental cultures, Helm (1924) corroborated the results recorded by Lestoquard and Veglia, and further claimed that in the subcultures of the first series, the anaplasmata could be made to increase.

Using Noguchi's agar for leptospira, to which had been added rabbit kidney and a vitamin agar broth with the inclusion of cerebral tissue, de Faria (1928) was unable to ascertain any definite growth of Anaplasma marginale. Conservation of the organisms in liquid media that preserved the erythrocyte was observed, but actual multiplication in these cultures and subsequent serial passage could not be demonstrated. Upon duplicating the method of Veglia, de Faria noted only a slight increase in the number of parasitized red cells, 2 to 5%, which he attributed to statistical error. de Faria could not confirm the variant forms, diplococcal and quadruplex as recorded by Veglia, as signs of intraglobular multiplication. Animal inoculations, according to de Faria, were also negative, or at best, inconclusive:

> Also, the attempts to obtain infections with cultures of more than 30 days of age were always negative. The inoculations of younger cultures have no great value . . . the blood by itself can preserve its infectivity for a period of 23 days at least.

Dikmans (1933) attempted culture of Anaplasma marginale by use of defibrinated or citrated blood to which 1% of a 50% dextrose solution was added. Noguchi's leptospira agar and blood agar were also tried, but without success. Dikmans did believe, however, that he obtained multiplication in his tube cultures, and concluded that the forms he observed in culture were dividing and multiplying forms of Anaplasma. These various forms were compared with those observed in the blood smears from an infected, splenectomized bovine and were described as "diads, triads, multiple points, and rod forms".

Applying the same technique Lestoquard described for the cultivation of Anaplasma ovis, Rossi and Triozon (1953) attempted to cultivate Anaplasma marginale in citrated and heparinized blood.

They were never able to observe the massive multiplication described by Lestoquard, nor could they observe the various forms related by Dikmans in his work. A slight increase of organisms was ascertained during the first two days only—thereafter a decrease occurred. The heparanized blood was less successful than the citrated blood. These investigators did not regard its culture possible.

CHAPTER III

CRYOGENIC PRESERVATION OF ANAPLASMA MARGINALE

Materials and Methods

For this experiment, about 40 mls. of infective blood was withdrawn aseptically into a sterile tube containing 0.5 ml. of 1:1000 heparin (as the NH_4 salt). This blood possessed a marginal body count of 27% and a hematocrit of 12.5 volumes percent.

Two additives had been previously prepared as follows: (1) a dimethylsulfoxide (DMS) additive, consisting of 10% DMS and 15% calf serum in a single strength solution of #199 tissue culture medium; and (2) a sugar solution containing 5% glucose and 9.35% sucrose in once distilled, demineralized water. The formulae for these preparations are essentially those given by Greaves et al. (1963), with the exception that tissue culture medium #199 was substituted for Gey's balanced salt solution. In preparing the DMS additive, it was found necessary to dilute first the required amount of DMS in the #199 medium and, by constant stirring, to add slowly the calf serum. Failure to do this produced a fine precipitate of the serum. Subsequent work with DMS later revealed that anaplasma infective blood, collected with EDTA as an anticoagulant, caused the blood to clot. Normal blood collected with EDTA did not clot when DMS was added.

The blood was prepared for freezing by sedimenting the erythrocytes

in two 10 ml. aliquots by centrifuging for 20 minutes at 1000 rpm in a Model UV International Centrifuge. The plasma from each tube was carefully removed. In one tube, the plasma was substituted for an equal volume of the DMS additive, and in the other tube, the plasma was substituted by adding a volume of sugar solution equal to that formerly occupied by the plasma. The red cells were gently mixed with their respective additives.

To three 1/2 dram screw cap vials was added 1 ml. each of blood reconstituted with the DMS additive; to another three 1/2 dram screw cap vials was added 1 ml. each of blood containing the sugar additive; and 1 ml. each of whole, heparinized blood was added to three other 1/2 dram screw cap vials. These vials were slowly frozen in the Linde Company BF-5 Biological Freezer, with the apparatus set at the 0 ring marked "E". The cooling rates for this position of the freezer are given as 1.3° C./hour in the fluid state and 1.1° C./hour in the solid state, plus or minus 40%. The formula furnished by the Linde Company indicated that these blood samples would be frozen to -70° C. in about 2 hours. These vials were removed at the end of 2 hours and immersed in liquid in a Linde Model LR-35-9 liquid nitrogen refrigerator.

Similarly, three groups of 1 1/2 dram screw cap vials were filled with 1 ml. each of blood with DMS additive, blood with sugar additive, and heparinized blood with no additive. These vials were placed in the cannisters of the liquid nitrogen refrigerator and immediately plunged into the liquid nitrogen.

It was intended that calves be inoculated every 4 weeks with the fast frozen blood samples and every 8 weeks with the slow frozen blood. This procedure would make possible a comparison of the methods of

freezing, the relative merits of the additives, and the blood without additive. All blood samples were to be thawed in a 45° C. water bath. Subsequent difficulties caused by leakage of liquid nitrogen into the vials when freezing necessitated altering this plan. It was possible to thaw rapidly two blood samples in the 45° C. water bath—after which three vials of blood were lost by explosion due to rapid expansion of trapped liquid nitrogen. Only the rapidly frozen samples with DMS and sugar solution were thawed rapidly for the first 4-week inoculations. All other blood samples were wrapped in cheese cloth when removed from the refrigerator and allowed to thaw at room temperature for about 20 minutes. This method of thawing was used for the remainder of the experiment.

After thawing, the blood samples were placed on ice in a styrofoam ice chest and taken to the Pawhuska Veterinary Research Station for calf inoculation. Prior to inoculation, a blood sample was withdrawn from each calf to check for possible anaplasma infection by means of the complement fixation (CF) test. The CF test was conducted according to the method of Price et al. (1954). The slightly modified government test (U.S.D.A., 1958) has been found by Merriman et al. (1962) to be very accurate. Merriman (1962) and co-workers found 2.3% false positives in a herd of 129, and these animals were found to be negative 18 months later.

Each calf was inoculated subcutaneously in the flank with 0.5 ml. of the blood sample being tested. The course of infection of each calf was followed by CF tests performed three times weekly and a check of the blood for marginal body counts, as well as hematocrit, hemoglobin and red cell count was made. Negative animals were studied for a

minimum of 90 days. After three animals remained negative when inoculated with blood samples not containing additive—regardless of freezing technique—it was decided to discontinue testing these blood samples. When thawed, those blood samples without additives appeared to be completely lysed, whereas those with additives were quite opaque. The blood samples were injected without removal of the additive.

Results

Viability of the Anaplasma marginale organisms was readily demonstrated in every case, except those in which additive was not included and in which complete lysis was apparent when thawed. Calves so infected developed a 4+ reaction to the CF 1:10 titre in an average of 19.2 days, and 31.7 days was the mean time required to demonstrate the maximum number of bodies. These results are summarized in Table I.

When setting up the experiment, it was unfortunate that an evaluation of the additives and methods used was not made to determine the relative number of erythrocytes which survived freezing. This was impossible at the time because no special glass ampules were available, nor had the BF-3 Biological Freezer (for controlled freezing) arrived. Some time later, however, by simulating the conditions under which the original experiment was performed, it was possible to make comparisons between the hematocrits before and after freezing, to obtain in this manner a percentage of hemolysis resulting from the freezing process.

The conduct of this experiment varied only in that 1 ml. blood samples were sealed in 1.2 ml. ampules; the BF-3 Biological Freezer

TABLE I

SUMMARY OF RESULTS OF CRYOGENIC PRESERVATION

Calf No.	Freezing Method	Type Additive	Days Frozen	CF 1:10	Maximum Infected RBCs
*728	fast	sugar	28	4+, 11th day	50%, 25th day
*744	fast	DMS	28	4+, 9th day	35%, 28th day
730	fast	none	28	neg.	none
737	slow	sugar	28	4+, 16th day	27%, 30th day
733	slow	DMS	28	4+, 11th day	31%, 25th day
734	slow	none	28	neg.	none
727	fast	sugar	56	4+, 23rd day	15%, 35th day
735	fast	DMS	56	4+, 14th day	25%, 28th day
726	fast	none	56	neg.	none
766	fast	sugar	84	4+, 56th day	10%, 46th day
768	fast	DMS	84	4+, 21st day	56%, 32nd day
767	slow	sugar	84	4+, 16th day	28%, 32nd day
756	slow	DMS	84	4+, 21st day	27%, 32nd day
765	fast	sugar	112	4+, 16th day	8%, 37th day
752	fast	DMS	112	4+, 16th day	35%, 32nd day
769	slow	sugar	112	4+, 16th day	25%, 32nd day
**763	slow	DMS	112	4+, 25th day	21%, 39th day
762	fast	sugar	169	4+, 27th day	30%, 34th day
757	fast	DMS	169	4+, 13th day	11%, 24th day
746	fast	sugar	196	4+, 16th day	12%, 28th day

*Thawed rapidly in 45° C. water bath - all others thawed slowly at room temperature.

**Only "1 drop" given, since the vial leaked upon thawing.

was used to control cooling at a rate of approximately 1^{o} C. per minute, and the formula for the DMS additive was altered by the substitution of Hank's balanced salt solution for the #199 tissue culture medium. The blood used had 3.1% infected red cells. Three different groups of blood were tested; i.e., heparinized blood without additives, heparinized blood in which the plasma was replaced by the DMS preparation, and heparinized blood in which the sugar solution was substituted for the plasma. Four methods of freezing and thawing were also checked: rapid freezing and rapid thawing (RR), rapid freezing and slow thawing (RS), slow freezing and slow thawing (SS), and slow freezing and rapid thawing (SR). Normal, i.e., uninfected blood with EDTA, rapidly frozen and rapidly thawed, is also included. The results are summarized in Table II.

Discussion and Conclusion

Cryobiology has been given great impetus in recent years by the discovery of Polge et al. (1949) that glycerin would protect bovine spermatozoa from injury by freezing. Luyet (1965) showed that the protective pattern of 10% DMS is the same as 10% glycerol. Glycerol, according to Lehmann (1965), protects cells from freezing injury by binding water that does not freeze. One mole of glycerin binds up to 3 moles of water (Meryman, 1960). Rey (1960) also attributes other qualities to the protective action of glycerol, such as, decrease of the crystallization velocity and dilution of the hypertonic solutions resulting from the separation of pure ice, thus facilitating super-cooling and permitting the formation of amorphous structures at low temperatures. Dimethylsulfoxide was used as one of the additives in

TABLE II

COMPARISON OF CRYOGENIC METHODS

	*Hct. Before Freezing	Hct. After Freezing	Percent Hemolysis
Blood with no additive, RR**	20.5	3.5	82.92
Blood with no additive, RS	20.5	0.0	100.00
Blood with no additive, SS	20.5	0.0	100.00
Blood with no additive, SR	20.5	0.0	100.00
Blood with DMS, RR	20.5	9.5	53.65
Blood with DMS, RS	20.5	11.0	46.34
Blood with DMS, SS	20.5	3.0	85.36
Blood with DMS, SR	20.5	2.5	87.80
Blood with sugars, RR	16.0	10.5	34.37
Blood with sugars, RS	16.0	5.5	65.62
Blood with sugars, SS	16.0	2.0	87.50
Blood with sugars, SR	16.0	2.0	87.50
Normal blood, no additive, RR	29.5	20.0	32.00

*Hematocrit
**Rapid freeze, rapid thaw, etc.

this investigation, since Ashwood-Smith (1961) demonstrated that the bovine erythrocyte is impermeable to glycerol and, therefore, without protective value. This same author also claimed that DMS was generally superior to glycerol as a preservative.

Luyet (1949) stated:

> The purpose of the ultra-rapid change of temperature, it may be recalled, is to avoid the formation of ice crystals which apparently takes place if protoplasmic material is given enough time, in the "dangerous range of temperatures," to undergo the molecular rearrangement involved in crystallization. . . . Crystal formation may occur either during the cooling or during the rewarming through that range, that is, respectively, at the freezing and at the "devitrification" temperatures.

According to Rinfret (1960), this critical temperature zone is 0^{o} to -50^{o} C. Luyet, quoted above, was referring to work he did with the mammalian erythrocyte; such explanations, however, being somewhat in contrast with the more recent hypothesis of Meryman (1960). Meryman notes that the red cell is the one outstanding exception to the cooling rate requirements of most cells—being the only cell that can be frozen rapidly in liquid nitrogen with the recovery of about 80% of the cells intact. The explanation for the phenomenon is given by his observation that very rapid freezing rates produce ice crystals inside the cells rather than exclusively outside them—such mechanical injury being the likely cause of cell death in this case. As Meryman says: "Perhaps the erythrocyte survives rapid freezing since its lack of internal structure enables it to tolerate this insult." Lehman (1965) has also observed that slow freezing of cells causes crystals to form outside the cell, whereas very rapid freezing produces random crystallization inside and outside the cell.

Luyet, as early as 1949, discovered that the cooling of oxalated

bovine blood, at a velocity of some 200° C./second, and rewarming at about the same velocity by immersion in physiological saline at room temperature, " . . . gave opaque suspensions in which 72% of the red cells were intact (as measured by the hematocrit)." Similar blood smears frozen and thawed at a much slower rate of but a few degrees per second resulted in transparent solutions in which only 4% of the red cells were not hemolyzed. Confirmation of these results is made by Rinfret (1960) and by Greaves (1963), who claim that blood without additives may be frozen and thawed rapidly without suffering complete lysis. Rinfret, in fact, claimed an 80 to 85% recovery from such treatment—as did Meryman (1960). Rinfret (1960), however, did suggest that the erythrocyte could be frozen too rapidly, although admitting that rapid thawing could compensate, to some extent, for such technique. Luyet (1965), in a more extensive investigation, showed that even with additives, blood could be frozen too fast and result in 95% hemolysis.

The above facts seem to agree well with the results published herein. As seen in Table II, only that blood without additives, which was both frozen and thawed rapidly, survived partially. Other blood samples without additives, treated at various cooling and thawing rates, were hemolyzed completely. The results of the calf inoculations summarized in Table I indicate that such hemolyzed samples were the only ones not proven infective. This coincides with the report of Mott (1957) who said:

> One freezing and thawing of infected blood cells will lyse most of the red cells, and correspondingly, will destroy 95% or more of the infectious agent. Infectivity is totally destroyed by more than one freezing and thawing.

Allbritton (1962) demonstrated that the anaplasma organism remained viable in a cell-free filtrate for 8 hours at 25° C. It is difficult to correlate Allbritton's results with those published in this paper, conducted under quite different temperatures and conditions. But it would seem, from the results obtained, that the viability of Anaplasma marginale is dependent upon, or least enhanced by, maintaining the integrity of its host cell, the erythrocyte. While no record of cooling rates were made for those samples rapidly frozen, the rapid freezing conditions of this experiment effected a favorable red cell recovery.

In a like manner, it appears impossible to correlate the work of Mazur (1960) in his studies of the deep freezing preservation of a fungus, yeast, and bacterium—the classification and nature of Anaplasma marginale still remaining an enigma. Mazur's work indicated that the survival of the organisms he investigated was dependent upon a slow cooling to -70° C. If red cell preservation is the sole criterion for maintaining the viability of Anaplasma marginale, it would seem that the addition of 40% DMS, the optimum amount for red cell preservation (Ashwood-Smith, 1965), might be the answer. Nevertheless, one might consider the possibility that such a high concentration of DMS could be inimical to anaplasmata.

It will be noted in Table II that there exists a marked difference in the effects produced in infective blood without additive, rapidly frozen and thawed, and normal blood without additive, rapidly frozen and thawed. These results further indicate that a greater concentration of DMS may not be advisable. The probable explanation may be the increased fragility of the erythrocyte infected with Anaplasma marginale,

(Ristic, 1961b; Dimopoullos et al., 1962). Dimopoullos et al. (1962) report a change in the ultra-structure of the red cell membrane occurring as a result of a decrease in concentration of total phospholipid in the infected cell. It was discovered that the concentration of the phospholipid of that stromata was inversely proportional to the osmotic fragility of the erythrocyte. (Dimopoullos, 1962). Schrader et al. (1963) were able to demonstrate that the chemical alteration of the erythrocyte membrane is mainly due to the decrease in the concentration of lecithins and cephalins, with a slight decrease in sphingomyelins.

The coagulation of infective blood containing EDTA upon the addition of the DMS preparation cannot be explained at this time. The fact that normal blood, i.e., uninfective, did not coagulate under these conditions would seem to indicate that infective blood differs chemically in some way.

Should a longer term preservation of Anaplasma marginale, other than that eventually to be shown by the use of liquid nitrogen become desirable, the use of phase II liquid helium would seem to be the answer. Viability in the frozen state, it appears, is dependent upon a complete cessation of all metabolic activities. Liquid helium approximates the temperature of absolute zero at which, theoretically, all life's functions cease. It is commonly assumed that all biochemical and biophysical processes concerned with the metabolism of organisms are arrested at the temperature of liquid nitrogen (-195° C.). According to Fernendez-Moran (1960),

> . . . recent studies indicate that many chemical reactions involving additions of hydrogen atoms, can take place at these temperatures. Moreover, many unstable chemical species such as free radicals still show considerable reactivity (at 70° K.), and it has only recently

been possible to trap these chemical fragments by freezing them into an inert solid at the extremely low temperatures obtained with liquid helium. Free radicals and other transient intermediates with unpaired electron spin play a key role in enzymatic reactions and metabolic electron transfer.

Fernandez-Moran at that time (1960) knew of no reports concerning detection of metabolic activity at liquid nitrogen temperatures, but did record that the reduction of metabolic rate at liquid helium temperatures had been calculated as approximately 10^{13} times greater than at the temperature of liquid nitrogen. However, Lehmann (1965) was able to report that after three years the original aldolase activity of an organism lost more than 50% of its initial value when stored at liquid nitrogen temperature; -196° C. This author stated that the evidence for deterioration of enzymes, even at the low temperature of liquid nitrogen, cannot be ignored.

Within the limited time conditions of this investigation, it appears feasible to maintain, for extended periods, stock isolates of Anaplasma marginale in liquid nitrogen refrigerators. The maintenance of such viability is apparently dependent upon maintaining the integrity of the infected erythrocyte.

CHAPTER IV

IN VITRO CULTURE OF ANAPLASMA MARGINALE

Materials and Methods

Because of the variations introduced into each culture experiment, it is only possible at this point to relate in a general way the materials and methods utilized in this phase of the study. Where indicated, the exceptions or deviations from the following procedures will be given at the beginning of each attempted culture.

The cell cultures used consisted of cells from the 2nd to 7th subculture of primary cultures made in our own laboratory; i.e., they were not of commercial origin. Other than the bone marrow cultures, these were all of embryonic origin—such embryos being procured from abatoirs of neighboring cities. The bone marrow cultures were derived from an uninfected, adult cow kept at the veterinary college clinic.

While the bone marrow culture methods of Plum (1947) and Berman et al. (1955) were studied, the necessity of obtaining large amounts of such a tissue from the intact animal induced this writer to develop his own methods of marrow culture. Previous workers had utilized the marrow from the severed bones of freshly killed, small laboratory animals. For the purposes of this investigation, two methods of marrow culture were devised: (1) about 4 to 5 mls. of a bone marrow aspirate were put into 50 mls. of Alsever's solution and gently mixed. The

marrow flecks were then reclaimed from this solution by filtering through a Millipore funnel without a filter membrane, and the flecks were collected from the sintered glass. These flecks were scraped off the funnel top, by the use of a rubber policeman, into one or more Cooper dishes containing 5 mls. each of culture medium. (2) several trephine cores, of about 1/4 inch diameter, were collected into a 50 ml. centrifuge tube containing Hank's balanced salt solution (BSS). That portion of each core possessing marrow tissue was placed in a Cooper dish and minced into small fragments by means of a scissors. All procedures were asceptically conducted, and antibiotics used in all solutions—100 units of penicillin/ml. and 100 ug. of dihydrostreptomycin/ml.

To obtain the desired number of cells, it was necessary to make serial subcultures—up to seven times. Although it was hoped that such cultures might contain basic stem cell types of mesenchymal origin, for which the anaplasma organism might have an affinity, the laborious and time consuming work involved prevented its use beyond the first experiment. For the other experiments, bovine embryonic kidney cultures were used.

Tissue culture medium #199 with 10% calf serum was used throughout, in addition to which 0.5% each of lactalbumin hydrolysate and bacto-peptone was included for the first two experiments. The serum was of commercial origin, from pooled sources, and each batch purchased was tested for CF antibodies. Only negative sera were used. No serum with positive CF test was found among those purchased.

In each experiment cells were cultured in Cooper dishes containing three halves of 22x30 mm. cover slips for use in microscopic examination.

Cell cultures to inoculate with anaplasmata were made in 2 oz. prescription bottles. Those cultures utilized in the experiment were harvested mechanically with a rubber policeman to avoid possible injury to cells induced by enzymatic harvesting procedures. This precaution was taken to insure the ready availability of subcultures, since source material was not always easily obtained. The transfer of such cultures was usually computed on an area basis—i.e., the culture vessels to be used in the experiment represented approximately one-half of the total area of those vessels containing fully confluent stock cultures from which the cells were derived. Transfers to experimental vessels were usually made 24 to 48 hours before beginning the experiment, and hence, the cultures were only about 30 to 40% confluent. The purpose of using incompletely confluent cultures was to obtain discrete cells for microscopic observation. Because of the Cooper dishes, these cultures were kept in a CO_2 incubator at 5 to 7% CO_2 and 37° C.

The cover slips from the Cooper dishes, on which the cells were cultured, were stained with three different stains and examined at intervals during the experiment. The stains employed for this purpose were Wright's, modified Noland's (Anthony et al., 1964), and specific fluorescent antibody stain (FA). The method used in preparation of the FA stain was essentially that of Madden (1962), wherein the dye Rivanol (or Ethodin, Winthrop Labs.) was used to fractionate antiserum possessing a high CF titre. (Saifer and Lipkin, 1959). The principle deviation from Madden's method was the lyophilization of the solution of gamma globulin and its reconstitution in the reaction mixture at a concentration of 10 mg. of gamma globulin per 1 ml. of reaction

mixture. Since a pure isomer of crystalline fluorescein isothiocyanate was employed, it was necessary to add only 0.0125 mg. of the dye to 1 mg. of protein in the conjugation procedure[1].

The FA controls were prepared by the application of the specific FA conjugate to known positive blood films which previously had been overlain with specific antiserum—resulting in no fluorescence—and compared with known positive blood films, stained by the direct method. All FA stained slides were examined by means of a Zeiss microscope having a built-in BHO 200 mercury lamp. Exciting filter BG-12 and barrier filter 44 were used to provide transmission of the correct spectrum of ultra violet light.

The specific FA stain and periodic injection of calves were the two methods used to assess possible growth of the anaplasma organism. In the case of the first experiment, conducted during the winter, the calves were kept at the anaplasmosis barn at Stillwater. For the other experiments, the calves were kept at the Pawhuska Veterinary Research Station in screened, vector-proof pens when so indicated by the season of the year. Blood samples were withdrawn from each experimental animal immediately prior to injection of test materials to check for CF antibodies and thus preclude the use of carrier calves. Materials to be injected were carried to Pawhuska on ice in a styrofoam ice box and were injected within two hours of leaving Stillwater.

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[1]Baltimore Biological Laboratories, Baltimore, Maryland.

Experiment 1

Bovine bone marrow cell cultures (BM), about 30% confluent, were inoculated with two different preparations. Infective blood with 12% of the erythrocytes possessing marginal bodies, was washed twice in physiological saline. One inoculum consisted of a suspension of these washed whole red cells in the serum-free tissue culture medium, at a concentration of 1×10^6 infected erythrocytes per ml. of suspension. This same dilution of a lysate of the infective cells was used to effect the same level of infectivity of the second inoculum. Lysis of the blood was accomplished by means of glass beads and a Teflon covered magnetic stirring bar, and was somewhat incomplete. The cell cultures were inoculated by replacing the medium on the cultures with the two different inocula, which consisted of the tissue culture medium and infective agent. This inoculum was replaced with serum-free medium 24 hours later.

One ml. of each inoculum was injected intravenously (IV) into calves the first day to prove its infectivity. Cover slips were examined on the first and eighth day after inoculation of the cultures. On the eighth day, also, the cells were scraped from the 2 oz. bottles (which had been prepared in duplicate), and 0.1 ml. of this tissue cell suspension was used to subinoculate new cell cultures. The remainder of this infected cell suspension, 9.6 mls. each with the respective inocula, was used to inject two calves IV. No further subinoculations were made, nor were any other calves injected with test materials.

Results

It was not possible at any time to identify anaplasma bodies in any of the stained cell cultures on cover slips, even with the use of the FA stain. Nor was it possible to detect any cytopathogenic effect (CPE) which might be attributed to the organism under study. Of the two calves injected with the original inocula at the beginning of the experiment, that animal injected with the whole, washed red cells became ill with the disease. The other calf displayed a transient 1+ reaction in 1:10 titre on the 30th day, but never became ill. The two calves inoculated on the 5th day of the experiment with a suspension of tissue cells in medium, showed a transient 3+ reaction to a 1:10 titre, and never developed anaplasma bodies. These latter two animals were splenectomized 54 days after inoculation, but no CF reaction or overt disease developed in these calves. A summary of these results may be seen in Table III.

TABLE III

SUMMARY OF EXPERIMENT 1

Calf No.	Cell Culture	Type Inoculum	CF 1:10	Max. Bodies
596 "0" day	none	whole RBCs	4+ 16th day	9%, 30th day
598 "0" day	none	lysed RBCs**	4+* 30th day	none
597 8 days	BM	whole RBCs	3+* 8th day	none
755 8 days	BM	lysed RBCs**	3+* 15th day	none

*Transient titres
**Incomplete lysis

Experiment 2

Bovine embryonic kidney cell cultures (BEK), about 40% confluent, were inoculated with a suspension of infected red cells in medium containing serum. The concentration of this inoculum was such that it contained 1×10^6 infected red cells per ml. of suspension. Two sets of controls were used in this experiment. One set of controls was inoculated with a suspension of red cells from negative blood, at a concentration equal to the total number of red cells in the infective inoculum. Another set of Cooper dishes was maintained without any inoculum. These controls were kept for microscopic observation only, which with the inoculated cultures, were examined every other day. Calves were injected at weekly intervals with a tissue cell suspension of cultures exposed to the infective inoculum. Subsequent to the first week, these cultures, as well as those designed for microscopic observation, were subinoculated by transfer of 0.1 ml. of the tissue cell suspension from the previous week's cultures.

The calves injected were checked for the prior existence of CF antibodies, and each was given 10 mls. IV of the tissue cell suspension being tested. The original inoculum was not injected into calves, but its infectivity was assumed upon the observation that 9% of the erythrocytes, in the blood from which it was prepared, were infected.

Results

Neither the results of microscopic observation of the cell cultures, nor the injection of calves with cell suspensions of such cultures revealed definite results. No identification of anaplasmata

was possible by the use of either Wright's or modified Noland's stains. No specific fluorescence could be detected by means of the FA stain. No CPE was observed in any of the cultures. Nor was it possible to discern any difference in the appearance of the control cultures when compared to those that were inoculated with a suspension of infected red cells. The calves injected with the inoculated cultures displayed only transient CF reactions to a 1:10 titre. The results are summarized in Table IV.

TABLE IV

SUMMARY OF EXPERIMENT 2

Calf No.	Cell Culture	Type Inoculum	CF 1:10	Max. Bodies
742			1+*	
8 days	BEK	whole RBCs	49th day	none
731			2+*	
15 days	BEK	whole RBCs	60th day	none
752			trace*	
22 days	BEK	whole RBCs	51st day	none
746			trace*	
29 days	BEK	whole RBCs	28th day	none

*Transient titres

Experiment 3

This is a short term, paired experiment, designed to demonstrate any possible enhanced growth or viability of the organism in primary BEK cultures, when compared with the inoculum alone. The inocula used were prepared as suspensions of sonicated[1] infective blood and

[1]Branson Instruments Sonifier Model #LS-75, Stamford, Connecticut.

sonicated negative blood in the standard culture medium, i.e., #199 with 10% calf serum. The sonification was designed to release the infective agents from the erythrocytes. The energy input could not be measured on this apparatus, but by experimentation, the minimum time and energy required to effect such lysis was determined. Therefore, the respective blood samples used in the preparation of the inocula were lysed by placing 2.5 ml. aliquots of each sample in autoclavable, plastic tubes with screw caps, and by immersing these tubes at about a 30° angle in a tray of ice water and adjacent to the sonifier head for 30 seconds. The infective blood was completely lysed, and the negative blood revealed a barely discernable microhematocrit of less than 1%.

No complete body count of the infective blood could be made inasmuch as only an occasional marginal body was detected. Because of this an arbitrary amount of 0.013 ml. of sonicated blood per ml. of suspension in culture medium was used. The concentration of the negative blood used for the control cultures was computed in the same manner.

Cultures of BEK in Cooper dishes with slip covers were set up for daily observation, including a group of controls inoculated with negative, lysed blood suspension. One group of 2 oz. prescription bottles contained BEK cultures which were inoculated, as before, with the suspension of infective blood. An equal amount, 5 mls., of the infective blood suspension was placed in another group of 2 oz. bottles which did not contain cell cultures. Both groups of bottles were incubated under identical conditions. A calf was injected initially to prove the infectivity of the inoculum used. Thereafter,

a total of three pairs of calves were injected at about 48-hour intervals with both the inoculum alone, and a suspension of the harvested BEK cells in the inoculum. All animals in this and succeeding experiments were injected subcutaneously in the flank with 5 mls. of the test material.

Results

Anaplasmata could not be demonstrated in the slip cover cultures with any of the stains employed. Neither was it possible to detect cytopathic effects which might indicate growth of such an organism. The calf injected on day "0" of the experiment, to prove infectivity of the inoculum used, died 36 days later. Two days before this animal died, 65% of its erythrocytes were infected with one or more Anaplasma marginale. All other calves injected with test materials did not develop the disease and remained negative to the CF test for three months. A summary of these results appears in Table V.

Experiment 4

The purpose of this experiment was to determine any possible effects of antibiotics on Anaplasma marginale, in vitro. In previous experiments, the tissue culture medium used always contained the usual amount of antibiotics, i.e., 100 units of penicillin, and 100 ug. of dihydrostreptomycin per ml. of medium. Blood possessing a marginal body count of 3.6% infected red cells was sonicated as before, and diluted with medium exactly as in the last experiment. The tissue culture medium used in this instance, however, contained no antibiotics. Five ml. aliquots of such infective blood suspension were placed in

2 oz. prescription bottles (without cell cultures) and incubated in a CO_2 incubator at 37° C.

TABLE V

SUMMARY OF EXPERIMENT 3

Calf No.	Cell Culture	Type Inoculum	CF 1:10	Max. Bodies
743 "0" day*	none	sonicated RBCs	4+ 22d day	65% 34th day
736 2 days	none	sonicated RBCs	neg.	none
738 2 days	BEK	sonicated RBCs	neg.	none
745 4 days	none	sonicated RBCs	neg.	none
739 4 days	BEK	sonicated RBCs	neg.	none
741 6 days	none	sonicated RBCs	neg.	none
740 6 days	BEK	sonicated RBCs	neg.	none

*i.e., day "0" of the experiment.

Results

As in the previous experiment, only that calf injected on day "0" developed anaplasmosis. A summary appears in Table VI.

Experiment 5

This is a double paired experiment in which it was attempted to demonstrate any possible enhanced growth or viability of the organism in two different types of inocula, with and without BEK cell cultures. One inoculum was prepared as a suspension of red cells (washed 3 times), in such a concentration that each ml. of suspension contained 1×10^6 infected erythrocytes. The other inoculum consisted of a suspension

of defibrinated blood in tissue culture medium, calculated to contain the same number of infected red cells as the first. The blood was defibrinated by stirring with glass beads. The same donor calf was used as a source of infective blood for both inocula, and this blood possessed a marginal body count of 4.5% parasitized RBCs.

TABLE VI

SUMMARY OF EXPERIMENT 4

Calf No.	Type Inoculum	CF 1:10	Maximum Bodies
991		4+	
"0" day	sonicated RBCs	27th day	27%, 36th day
729			
2 days	sonicated RBCs	neg.	none
746			
6 days*	sonicated RBCs	neg.	none
747			
8 days	sonicated RBCs	neg.	none

*Calf not injected the 4th day because of severe sleet storm which even prevented air travel.

Two groups of 2 oz. bottles with 40% confluent BEK cell cultures were inoculated with the two respective media. Another two groups of such bottles without cell cultures were filled with the same amount of inoculum, 5 mls., and incubated in an identical manner. The medium used in this experiment contained antibiotics, since the last experiment indicated no deleterious effects of these on the anaplasma organism.

Cooper dishes, in which BEK cells had been cultured on slip covers, were also inoculated with the two different media so that daily observations might be made.

Microscopic examination of the cultures revealed nothing

significant at any time, including a lack of specific staining by use of the fluorescent antibody conjugate. Results obtained from the injected calves were, on the other hand, somewhat different. All four calves injected the 2d day became ill with a clinical case of the disease, and two animals out of the four injected on the 4th day of the experiment developed anaplasmosis.

One of these latter two calves developed a 4+ reaction to the 1:10 CF titre on the 21st day after injection, and died on the 38th day. The other animal developed a positive CF reaction on the 79th day, and anaplasma bodies were demonstrable in its blood 8 days later. It is of further interest to note that both of these calves injected on the 4th day of the experiment, were injected with a suspension of cultured cells and inoculum, while those injected this same day with only the inoculum, never showed evidence of disease. The calf which had a positive CF test on the 21st day was injected with a suspension of BEK cells and defibrinated blood inoculum; the other calf was injected with a suspension of BEK cells and washed red cell inoculum. The test material contained, as in similar experiments, the cultured cells in a suspension of the original inoculum, which meant that the anaplasma organism had at least survived longer than in other experiments.

The disparity in time between which Calf No. 734 and Calf No. 726 became ill might cause one to suspect that the latter was a naturally occurring infection. However, the early season, March 22 to June 9, makes this unlikely since no vectors have been noted there during this time. The fact that no other animals in this area became ill also tends to discredit the chance of its being a natural infection. Table VII

summarizes the results.

TABLE VII

SUMMARY OF EXPERIMENT 5

Calf No.	Cell Culture	Type Inoculum	CF 1:10	Max. Bodies
759			4+	15%
"0" day	none	washed RBCs	25th day	32d day
764			4+	15%
"0" day	none	defibr. blood	25th day	27th day
739			4+	23%
2 days	BEK	washed RBCs	23d day	30th day
736			4+	38%
2 days	BEK	defibr. blood	30th day	39th day
745			4+	11%
2 days	none	washed RBCs	25th day	34th day
738			4+	25%
2 days	none	defibr. blood	23d day	30th day
726			4+	<1%
4 days	BEK	washed RBCs	79th day	87th day
734			4+	35%
4 days	BEK	defibr. blood	21st day	37th day
741				
4 days	none	washed RBCs	neg.	none
809				
4 days	none	defibr. blood	neg.	none
849				
*7 days	BEK	washed RBCs	neg.	none
772				
7 days	BEK	defibr. blood	neg.	none
729				
7 days	none	washed RBCs	neg.	none
750				
7 days	none	defibr. blood	neg.	none

*Inclement weather made it impossible to inject calves at the 6th day interval as originally intended.

Experiment 6

In this experiment it was decided to study the possible effects of increased oxygen tension on growth, or increased survival of the organism. For this purpose a hyperbaric chamber was improvised by

attaching a pressure gauge to a stainless steel Millipore Filter Corporation filtration tank. In this tank were placed three open milk dilution bottles with 30% confluent BEK cell cultures, four Cooper dishes with slip cover cultures, and one open milk dilution bottle containing only the inoculum. The inoculum, like previous ones, consisted of a suspension, in tissue culture medium, of 1×10^6 washed infected red cells per ml. of suspension. The hyperbaric chamber was filled with a gas mixture of 5% CO_2 and 95% oxygen to a pressure of 10 pounds per square inch (psi). Each time Cooper dishes were removed, the chamber was slowly decompressed, and slowly refilled with this gas mixture.

Slides were examined on the 1st, 2nd, and 4th day—and two calves were injected on the 6th day, with inoculum only in one case, and another with inoculum plus a suspension of BEK cells. Five mls. of each test material were injected subcutaneously into the flank of each calf, such material being carried to Pawhuska on ice.

Results

It was not possible at any time to detect anaplasmata in the cell cultures, even with the FA stain, and the degenerating cells which began to appear on the 4th day of the experiment were attributed to the toxic effect of the increased oxygen tension. (Graff, 1957; Pace, 1962). Neither calf developed an overt case of the disease, or even a transient titre to the CF test.

Discussion and Conclusion

The transient CF titres occurring in the first two experiments may represent a carry over of dead anaplasma organisms, or extremely minute amounts of live organisms, into serial cultures. (Brock, 1965). From the first, third and fourth experiments it can be seen that disruption of the erythrocyte is detrimental to the survival of Anaplasma marginale. In the first experiment, where lysis was achieved by means of rotating glass beads at room temperature, the organism did not survive the few hours necessary to prepare it for injection on day "zero". The report of Bedell et al. (1963) was verified in Experiments 3 and 4, in that the organism survived the brief exposure to sonic energy at the temperature of ice water. Bedell et al. (1963) stated that infectivity was not destroyed when the organism was exposed to sonic energy for 210 minutes at 17 to 18° C. These same authors also stated that the etiologic agent of anaplasmosis remained viable for at least 32 hours in an extra-erythrocytic environment, which fact would coincide with the results of Experiments 3 and 4.

The results of the calf injections in Experiment 5 were, on the basis of previous experience, quite unexpected. Bedell et al. (1962) reported that the infectivity of Anaplasma marginale was destroyed by exposure to 38° C. for 86 hours, but not for 72 hours. The incubator temperature in the case of Experiment 5 was 37°, plus or minus about 0.5° C. The possibility exists that this deviation in temperature could cause results that differed from Bedell's observations. On the other hand, it is also possible that the calves that became ill as a result of the injection of inoculum and BEK cells on the fourth day,

represent growth of the organism—since the other calves injected this same day with the respective inoculum minus BEK cells did not even display a transient CF titre. Negative microscopic observations in this regard could be due to faulty technique, or impossibility of demonstrating submicroscopic units of the organism. It is also possible that the calves which became ill following injections on the 4th day, represent increased survival of the organism due to the nature of the inoculum—i.e., something in the serum which enhances viability of anaplasmata. It might also be explained by the normal occurrence of extra-erythrocytic forms of the organism. Neither of these latter hypotheses would apply to Calf No. 726 which was injected with BEK cells suspended in an inoculum of washed red cells. Whatever the explanation for this 4-day survival of anaplasmata seen in Experiment 5, the number of animals represented in this experiment make any valid conclusion on a statistical basis impossible.

Dimopoullos et al. (1962) have said:

> Data on the source of metabolites for A. marginale are not available. The present studies provide suggestive evidence that the erythrocyte, per se, may supply nutrients for the organism. The propensity of A. marginale for metabolic products derived from the cell may be the prime cause for the decrease that is observed in the phospholipid concentration (of the erythrocytic stromata).

However, according to Bedell et al. (1962), and the data of the experiments herein presented, it is to be noted that even in whole blood preparations the organism has a limited survival time, especially at higher temperatures. This survival time is inversely related to temperature, since Bedell et al. (1962) observed that Anaplasma marginale survived for 56 days at -66° C. and by deep freezing techniques it has survived for over 9 months providing that hemolysis

on thawing was not complete. (Turner, 1944). At this point, one must inject the concept of increased oxygen tension as a factor in the growth and survival of anaplasmata. This is suggested by the very nature of the organism's environment, within the erythrocyte. Segre (1964) was able to demonstrate CPE within 48 hours in cell cultures inoculated with a strain of hog cholera virus which is normally noncytopathogenic, when such cultures were subjected to an increased oxygen tension of 10 lbs. psi. The negative results obtained in the hyperbaric chamber study presented in this thesis do not entirely preclude the significance of increased oxygen tension, since the experiment is much too limited in scope. The writer was unable to find any figures regarding the intracellular oxygen tension of the red cell. Cooper et al. (1958), in working with suspended (Maitland type) cell cultures demonstrated that the liquid-phase oxygen level or equivalent redox potential is the important factor for optimum growth of such cell cultures. Perhaps this might apply to an organism such as Anaplasma marginale and, with this in mind, it would be well to investigate further the use of increased oxygen in such cultures and to correlate its use with pO_2 levels of the medium.

The dams from which the embryos used in these cultures were obtained, were not checked in any way for possible CF titres or actual presence of the organism. However, it is known that neither antibodies nor anaplasmata cross the bovine placenta (Kutler, 1962) so that this may be ruled out as a possible factor in the negative results obtained in this study.

It can only be concluded from this study that the growth of Anaplasma marginale has not been demonstrated. But from the data of

this study and that of the cryogenic study, it can be inferred that low temperatures and intact host erythrocytes enhance the survival time of this organism.

CHAPTER V

SUMMARY AND CONCLUSIONS

This project is divided into two phases: Phase I concerns the cryogenic preservation of Anaplasma marginale by means of deep freeze methods in liquid nitrogen. Phase II involves the attempted in vitro cultivation of this organism.

The first part of this study demonstrates the feasibility of preserving the infectivity of Anaplasma marginale for 196 days. Such preservation, it is shown, is apparently dependent upon maintaining the integrity of the erythrocyte, for which purpose two additives were tested, one containing dimethylsulfoxide, and the other a sugar solution of sucrose and dextrose. Both lysis and exposure to warm temperatures are detrimental to preserving the infectivity of the organism.

In the second part of this study several experiments were conducted in an attempt to culture Anaplasma marginale in vitro. The methods used herein were radically different from those employed by early investigators in that tissue cultures were used, but the results were equally futile. Only the results of calf injections seen in Experiment 5 offer a faint glimmer of hope for its possible culture, although any inferred conclusions are statistically invalid because of the limited scope of this experiment. Nevertheless, it would seem that further investigation along the lines of Experiment 5 are

indicated—especially if correlated with the variable pO_2 concept as introduced in Experiment 6.

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