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The Louisiana State University and Agricultural and Mechanical College, Ph.D., 1973
Microbiology

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A COMPARISON OF *IN VITRO* NUCLEIC ACID METABOLISM OF NORMAL BOVINE RBC AND RBC FROM CALVES INFECTED WITH *ANAPLASMA MARGINALE*

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Microbiology

by
Michael Edward Billups
B.S., McNeese State College, 1968
M.S., McNeese State University, 1970
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Abstract

Red blood cells from normal calves and calves infected with *Anaplasma marginale* were washed, resuspended in normal plasma, and incubated with one of the following compounds: ^{14}C-hypoxathine, ^{14}C-orotic acid, ^{14}C-sodium bicarbonate, ^{14}C-sodium formate, and ^{3}H-uridine. Tritiated uridine was also incubated with normal RBC resuspended in plasma from infected calves. During incubation, RBC were separated and subjected to fractionation with hot and cold trichloroacetic acid (TCA) and TCA-soluble fractions were assayed for radioactivity. The data indicated that the *Anaplasma* infection decreased the ability of RBC to incorporate these compounds. RBC from *Anaplasma*-infected calves failed to incorporate these nucleic acid precursors into the pool of metabolic intermediates with the same efficiency demonstrated by the normal RBC. Normal RBC showed an increased ability to use uridine for nucleic acid synthesis whereas RBC from infected calves could use orotic acid, but not uridine. Plasma from infected calves caused the normal RBC to resemble RBC from infected calves with respect to uridine uptake and metabolism.

The cold TCA-soluble fractions of the normal and infected erythrocytes which had been incubated with

^{3}H-uridine were hydrolyzed and the hydrolysates were subjected to descending paper chromatography. After detection under UV radiation, the areas containing the nitrogenous bases were cut out and assayed for radioactivity. Data showed that normal RBC converted uridine to cytosine more efficiently, whereas infected cells converted a larger percentage of uridine to thymine.

Orotidine-5'-monophosphate pyrophosphorylase (OMPpase) was assayed in partially-purified marginal bodies by measuring the disappearnace of orotic acid at 295 nm with a Beckman D·B Spectrophotometer. Orotic acid was removed from the incubation mixture slowly which indicated that purified bodies possessed some of the enzymatic capabilities for pyrimidine synthesis.

DNA and RNA extracted from RBC of *A. marginale*-infected calves were compared to that extracted from RBC from a calf made anemic with phenylhydrazine. Sucrose density gradient centrifugation showed that infected RBC contained two species of DNA whereas the RBC from the calf with the chemically-induced anemia possessed only one species of DNA. The second DNA found in the infected RBC appeared to be from *A. marginale*. Most of the RNA found in infected RBC was bovine in origin.

DNA isolated from infected RBC was examined by electron microscopy. Electron micrographs revealed two

species of DNA, one circular and one linear. The linear form was assumed to be bovine.

Introduction

Anaplasmosis is an infectious and transmissible disease of cattle causing progressive anemia which is associated with the presence of intraerythrocytic bodies designated as *Anaplasma marginale* (Ristic, 1960). Mature animals usually have a more severe case of the disease than calves and survivors become carriers for life.

Foote *et al*. (1958) concluded the parasite was a virus whereas Mason and Ristic (1966) considered it to be a rickettsial organism. *Bergey's Manual of Determinative Bacteriology* (Breed, Murray, and Smith, 1957) includes *A. marginale* in the order Rickettsiales and in the family *Anaplasmataceae*.

DNA and RNA were found by Gough (1963) to be present in *A. marginale*-infected RBC. In contrast, Ellender and Dimopoullos (1967) and Senitzer (1969) found no evidence for RNA species associated with *A. marginale*. This study was initiated to study the nucleic acid metabolism of RBC from calves infected with *A. marginale*. Such data would determine the role of RNA in *A. marginale* and would also aid in understanding the site and mode of replication of the organism.

Selected Literature

A. *Anaplasmosis*

Smith and Kilborne (1893), as quoted by Ristic (1960), first observed anaplasmosis in conjunction with a study of piroplasmosis of cattle. They concluded that these "coccus-like bodies or marginal points" in the blood of affected cattle were a stage in the life cycle of *Piroplasma bigeminum*. Ristic stated that similar bodies were observed in the RBC of South African cattle by Kolle (1898).

According to Ristic (1960), Theiler (1910a) gathered enough evidence to conclude that the "chromatic bodies" present in the RBC of affected cattle represented a new organism—*Anaplasma marginale*—and it was responsible for the disease, anaplasmosis. Since that announcement, anaplasmosis has been reported in most tropical, subtropical and temperate zones of the world. The first experimental evidence of anaplasmosis in the United States was reported by Meyer in 1913 (Ristic, 1960).

There has been much controversy on the biological classification of *A. marginale*. Theiler, in 1910a (Ristic, 1960) designated it a protozoan parasite due to the similarity of clinical symptoms of anaplasmosis and piroplasmosis. Data collected by Du Toit (1934) and Espana *et al*. (1959), supported this classification.

A. marginale has been considered a virus by some researchers (Foote et al., 1958) and a rickettsial organism by others (Mason and Ristic, 1966). The 7th edition of *Bergey's Manual of Determinative Bacteriology* (Breed, Murray, and Smith, 1957) includes *A. marginale* in the order *Rickettsiales* and the family *Anaplasmataceae*.

Many investigators have shown that ticks and horseflies are responsible for the transmission of anaplasmosis (Howell *et al.*, 1941; Stiles, 1942; Henning, 1949; Dikmans, 1950; Christensen, 1956; Howell, 1957). Mosquitoes have also been shown to play a role in the experimental transmission of the disease (Howell *et al.*, 1941).

Symptoms of anaplasmosis include reduced milk production, labored respiration, increased body temperature, disoriented behavior, icterus, weakness, and anemia (Carricaburu, 1957a,b). Christensen (1956) found that adult animals contracted a more severe form of the disease than calves.

The losses to the cattle industry in the United States due to anaplasmosis have been estimated by Oglesby (1962) to be approximately $35 million annually. Recently, estimated losses have climbed to $100 million per year (Wilkerson, 1972).

Electron microscopy revealed a limiting membrane separating the marginal body from the cytoplasm of the RBC. The initial bodies comprising the marginal body

were round or oval, 300-400 nm in diameter, and enclosed in a double membrane (Ristic and Watrach, 1961; Scott *et al.*, 1961). Each marginal body was found to contain from 1-8 subunits (de Robertis and Epstein, 1951; Foote *et al.*, 1958; Ristic, 1960). Various preparations and staining procedures have also been used to study the morphology of the marginal body (Dickmans, 1933a,b; Lotze and Yiengst, 1942). It has been shown to be a dense, homogenous, round structure measuring 0.3 to 1.0 u in diameter. Using the acridine orange staining method Ristic and Kreier (1963) demonstrated the marginal body to be an inclusion containing several subunits instead of a single compact unit. These subunits have become known as initial bodies.

The development of a successful vaccine against anaplasmosis has been the prime concern of many researchers. Pearson *et al.*, (1953) found that injection of phenol-inactivated tissues of diseased animals into cattle produced longer incubation periods than observed upon challenge with infected blood. Ristic (1960) developed attenuated organism which produced a degree of immunity and was accompanied by a latent infection. A vaccine was developed by Kuttler (1961) that conferred an increased resistance to anaplasmosis but did not possess preventive properties. Recently, Ristic, Sibinovic, and Welter (1968) reported the use of an attenuated vaccine.

This preparation proved effective in reducing the symptons of the disease in over 900 cattle. Furthermore, the organism remained nonvirulent after 10 passages in mature cattle and 5 passages in pregnant cattle.

Very little is known about the chemical nature of *A. marginale*. Various staining procedures have been used to demonstrate the presence of inorganic ferrous iron, amino acids, protein, DNA, and RNA (Moulton and Christensen, 1955; Simpson, Kling, and Love, 1967). The rate of *in vitro* incorporation of radioactive glycine by normal and *Anaplasma*-infected RBC was used by Mason and Ristic (1966) as a criterion for studying protein synthesis of *Anaplasma*. Their data provided evidence for the existence of enhanced intraerythrocytic protein synthesis *in vitro* in RBC from infected cattle. Further studies have shown that *A. marginale*-infected RBC possess a decreased phospholipid concentration (Dimopoullos and Bedell, 1960, 1962), a greater catalase activity (Wallace and Dimopoullos, 1965b), a lactic dehydrogenase activity (Darre', 1967) and an adenosine triphosphatase activity (Garon, 1967). Purified marginal bodies contained large quantities of phospholipids with small amounts of sterols, free fatty acids, triglycerides, and sterol esters (Wallace, *et al.*, 1967).

DNA and RNA were found by Gough (1963) to be present in *A. marginale*-infected RBC at high concentrations. The maximum level of DNA was found at peak infection, whereas

RNA did not reach a maximum level until 3 or 4 days later. Studies by Ellender and Dimopoullos (1967) indicated that DNA isolated from purified marginal bodies was single-stranded in nature. Recently, however, DNA with characteristics of a double-stranded, helical molecule was isolated from purified marginal bodies (Senitzer *et al.*, 1972). Senitzer (1969) found no evidence for RNA in *A. marginale*.

B. Nucleic Acids

1. DNA isolation

The extraction of DNA has become a commonplace procedure in many laboratories but the technique may vary depending upon the nature of the material extracted. In all cases the cell or organism must first be lysed utilizing such techniques as osmotic pressure, freeze-thaw techniques, sonication, bacteriophage, surface-active agents, and enzymes.

Once free of the cell, DNA is susceptible to degradation by various nucleases. For this reason, these enzymes must be inactivated immediately. Chelating agents such as sodium citrate (Peterman and Lamb, 1949) and sodium ethylenediamine tetraacetate (EDTA) (Schildkraut *et al.*, 1962) have proved to be useful in this capacity. Sodium dodecyl sulfate (Kay and Dounce, 1953), Cu^{++} (Zittle, 1945), and nitrogen mustard (Wheeler and Alexander, 1957) have also been used to inhibit the action of these enzymes.

Berns and Thomas (1965) used purified DNA from *Hemophilus* and purified DNA from T2 phage to demonstrate the ability of pronase to destroy nuclease activity.

Upon lysis of the cell, DNA, RNA, protein, polysaccharide, lipid, and inorganic materials are released. These impurities must be removed from the nucleic acid preparation. Protein can be removed by using chloroform and isoamyl alcohol (Du Buy *et al.*, 1965), sodium lauryl sulfate (SLS) (Marmur, 1961), or phenol and pronase (Thomas *et al.*, 1966). Removal of lipid from infected bovine RBC is accomplished by using alcohol-ether extraction (Gough, 1963) and polysaccharide by selectively precipitating the DNA with 2-propanol (Marmur, 1961). Highly purified RNase (DNase free) is used to degrade any contaminating RNA (Marmur, 1961).

Marmur (1961) developed a unique method for the isolation of double-stranded DNA. In his procedure, nucleic acids were precipitated with ethyl alcohol and spooled onto a glass rod. Gafford and Randall (1967) used a modification of this method to obtain high molecular weight DNA from purified fowlpox virus. Thomas *et al.*, (1966) found phenol to be useful in extracting DNA from bacteria and cell nuclei because the need for precipitating the DNA and of winding it on a rod was eliminated.

In all of the above extraction procedures, gentle handling techniques must be employed in order to avoid

cleaving of DNA molecules after lysis of the cell. High DNA concentrations should be used to avoid degradation, and the DNA is redissolved in as small a volume as is practical. DNA preparations of uniform molecular size may be obtained when the extraction procedures are used in conjunction with methyl esterified albumin kieselguhr (MAK) column chromatography.

2. RNA isolation

The initial step for isolating RNA is identical to that in DNA isolation. The organism or cell is lysed utilizing one of the methods previously mentioned, thus releasing the RNA along with DNA, nucleases, and other proteins. As in DNA extraction, these contaminants must be eliminated.

Sherrer (1969) eliminated traces of RNase by treating the RNA preparations with bentonite or macaloid. Pronase, which digests most proteins, can be used as an alternative to this treatment (Huppert and Semmel, 1964). The pronase can be inactivated with phenol at room temperature.

DNA contaminating an RNA preparation is unimportant if gradient, MAK colum, or electrophoretic analysis is employed. However, DNA disrupts hybridization experiments and must be removed by treatment with pancreatic DNase I. The DNase should be eliminated with sodium docecyl sulfate (SDS) or precipitation with phenol, depending on the type of experiment being performed (Scherrer, 1969).

Hot phenol extraction has become a standard method for preparation of RNA from bacterial and animal cells. This procedure has been used to isolate the precursors to rRNA and DNA-like RNA with molecular weights up to eight million daltons (Scherrer and Darnell, 1962; Scherrer et al., 1963; Scherrer and Marcaud, 1965). Hot phenol has been used to isolate the biologically active messenger RNA which is capable of directing the synthesis of phage T4 lysozyme in a cell-free ribosome system. Scherrer (1969) demonstrated that this method permits the extraction of RNA in its physiological form. Hot phenol was more effective than cold phenol in the isolation of infectious WEE virus RNA (Wecker, 1958). Approximately 95% of the RNA can be extracted from whole cells with hot phenol as compared to 70% with cold phenol (Larsen and Frederiksen, 1972).

Heat treatment which is used in the phenolic extraction procedure can be rationalized in several ways. The heat plus the effects of a strong detergent immediately dissolve the nucleoprotein complexes of chromatin and ribosomes and disassociate glycoprotein and lipid all of which can be resistant to cold phenol (Wecker, 1958). At higher temperatures artificial associations of RNA, DNA, and protein which form during the initial denaturation by phenol are reduced. At pH 5 heat treatment degrades the DNA and allows it to pass into the phenol phase. Therefore, heating plays a dual role by increasing the

efficiency of the phenol extraction and by eliminating DNA.

3. Base composition analysis

a. Hydrolysis

The first step in determining the base composition of nucleic acids is digestion by chemical or enzymatic means (Mangold, 1965). The standard chemical procedure involves the hydrolysis of acid-labile glycosidic bonds with hot perchloric or formic acid. The purine-pentose bond is extremely labile to perchloric and formic acid, whereas, the pyrimidine-pentose bond is not. Hot hydrochloric acid has also been used with success in this type of chemical hydrolysis (Walsh and Sherman, 1968).

Mangold (1965) utilized dilute solutions of sodium and potassium hydroxide in degradation of DNA to a mixture of high molecular weight polynucleotides. When subjected to this treatment, RNA is reduced to mononucleotides.

Enzymatic procedures have proved useful for the analysis of small amounts of DNA. Liberation of 3'-mononucleotides is achieved by digestion with micrococcal nuclease followed by calf spleen phosphodiesterase (Josse *et al.*, 1961). A combination of pancreatic DNase and snake venom phosphodiesterase is used to release 5'-mononucleotides (Lehman, 1966). Pancreatic DNase I hydrolyzes native DNA thus producing fragments terminated with 5'-monophosphates (Laskowski, 1966).

b. Separation and analysis

Paper chromatography is a common technique used in the separation of complex mixtures including nitrogenous bases. Individual compounds are identified by comparing their locations to those of known samples. The differences in rate of movement of the components are caused by their different solubility in mobile and stationary phases (Merck and Darmstadt, 1963).

Whatman No. 1 filter paper is ideal for the separation of nitrogenous bases because it requires no preptreatment and exhibits a high resolving capacity (Kream and Chargaff, 1952). One could also use Schleicher and Schuller filter paper 2043 a, which is equivalent in weight and flow rate to the Whatman No. 1.

The choice of a solvent system is extremely important since it must not react with the substance applied. In the case of nitrogenous bases, the solvent should not interfere with viewing under the UV lamp. Wyatt (1951) used a 2-propanol/HCl/H_2O (65/16.7/18.3; v/v/v) which proved to be an excellent system for the separation of nucleic acid components. Other solvent systems used are glacial formic acid/N-butanol saturated with water (90/10; v/v) (Markham and Smith, 1949) and 5% NA_2HPO_4-isoamyl alcohol (Carter, 1950).

The individual purines and pyrimidines are best detected with long wave ultraviolet light (Bendich, 1957).

Elution of the bases can be achieved by immersing the paper in 0.1 N HCl for six hours (Heppel, 1967). The quantity of the base present in the eluates is calculated according to the formula: u moles base = optical density at maximum absorption x volume of eluate x 1/E, where E is the molecular extinction coefficient.

Nucleobases, nucleosides, mononucleotides, and oligonucleotides can also be separated by thin-layer chromatography (TLC) (Mangold, 1969). Silica gel, cellulose, dextran gel, and ion exchangers are used as adsorbants. Nitrogenous bases and nucleosides are separated using diethylaminoethyl (DEAE)-cellulose or ECTEOLA-cellulose (reaction product from epichlorohydrin, triethanol-amine and alkali-cellulose) (Coffey and Newburgh, 1963) and polyphosphate-impregnated (PP)-cellulose (Randerath, 1966). A good separation of oligonucleotides can be accomplished through thin-layer electrophoresis and thin-layer electrophoresis-chromatography.

Water has been used as solvent in DEAE and ECTEOLA-cellulose layers (Coffey and Newburgh, 1963). Saturated ammonium sulfate solution/N sodium acetate/isopropanol (80/17/12; v/v/v) is particularly good for separating purine and pyrimidine bases on layers of DEAE-cellulose.

4. Enzymatic assays

An extremely important step in pyrimidine biosynthesis is the formation of orotidine-5'-monophosphate (OMP) from the condensation of orotic acid and

phosphoribosyl-pyrophosphate (PRPP). The enzyme, orotidine-5'-monophosphate-pyrophosphorylase (OMPpase) catalyzes this reaction.

OMPpase can be assayed for by following the disappearance of orotic acid at 295 nm with a spectrophotometer (Beckwith *et al.*, 1962). Walsh and Sherman (1968) utilized this technique in the study of pyrimidine synthesis by *Plasmodium lophurae*.

5. Electron microscopy

Kleinschmidt and Zahn (1959) first described a method whereby DNA could be viewed with the electron microscope. This technique involved the spreading of DNA molecules into a monomolecular layer on an aqueous subphase. Protein films were found to produce insoluble surface films onto which DNA could be absorbed (Kleinschmidt *et al.*, 1962). The nucleic acids are bound by the basic side groups of the protein and are brought into a two-dimensional configuration. Cytochrome C is the protein chosen by many researchers (Kleinschmidt, 1968; Westmorland and Szybalski, 1969; Mosharrafa *et al.*, 1970). The resulting film is transferred to electron microscope grids and dehydrated with ethyl alcohol. Deposition of metal along each side of the nucleic acid strands enables one to take electron micrographs of the preparation (Kleinschmidt, 1968). Gordon and Kleinschmidt (1968) described a method for high contrast staining of nucleic acids using uranyl acetate in acetone.

C. Radiotracer methodology

Researchers have developed the use of radioisotopes as tracers into a powerful research tool. Using radiotracers, a given compound in a biological system can be readily traced and detected in extremely minute amounts. The magnifying power of a typical radiotracer experiment may be as high at 10^8 fold with respect to the amount that can be detected by chemical means (Wang and Willis, 1965).

Radiotracers have been used extensively in the study of bovine lipid metabolism. ^{14}C labeling was used to show that saturated fatty acids were incorporated into bovine RBC to a greater extent than unsaturated fatty acids.

Radioactive precursors have been extremely useful in the study of DNA and RNA of *Plasmodium knowlesi*. It was found that when incubated *in vitro*, intraerythrocytic stages of *P. knowlesi* utilized all the preformed radioactive purines. Of the pyrimidines surveyed, only orotic acid was incorporated (Gutteridge and Trigg, 1970). All precursors analyzed were incorporated into both DNA and RNA (1:3 ratio). Purine and pyrimidine biosynthesis in the avian malaria parasite, *P. lophurae*, and its host cell, the duck RBC, has been investigated by Walsh and Sherman (1968). Pyrimidine synthesis was measured using the incorporation of ^{14}C sodium bicarbonate whereas ^{14}C sodium formate incorporation was used to measure purine

synthesis. The individual nucleobases were separated by paper chromatography and assayed for radioactivity utilizing liquid scintillation techniques. It was suggested that *P. lophurae* synthesized purines to a limited extent but derived most of its purines from the host cell. It appeared that the parasite synthesized pyrimidines *de novo*.

Autoradiography has been a useful application of radioisotopes in recent years. The basis of the technique involves the placing of a sample containing radioactive material in contact with a suitable film or plate. Following exposure and processing, the location of the tracer in the sample is revealed in the photographic layer. The image is thus called an autoradiograph. In a classic example of autoradiography, Cairns (1963) demonstrated the method by which the circular *E. coli* chromosome replicates. Caro *et al.* (1962) described a procedure for obtaining autoradiographs of high resolution using various emulsions.

Materials and Methods

A. Experimental animals

Calves of mixed breeds and sex for use in this study were purchased from local farms and auction sales. When necessary, the younger calves were fed starter rations and rehydrated milk or placed with a nurse cow until they were of age to be fed grain.

Approximately two weeks before infection, all animals were splenectomized. Splenectomy reduces natural resistance to anaplasmosis and augments susceptibility to the disease (Roby, Gates and Mott, 1961; Jones and Brock, 1966).

Anaplasmosis was produced by inoculating calves via the jugular vein with 100 ml of infected whole blood. The inoculum was drawn from an animal in the carrier state or from one with an infection in the acute phase of the disease.

B. Hematology

Blood for hematologic examination was taken each day from infected animals. Samples were collected by jugular venipuncture into evacuated glass tubes (16 x 100 mm) which contained 12 mg disodium ethylenediamine tetraacetic acid (EDTA) as anticoagulant. Blood was examined for percent infected RBC and packed cell volume (PCV). Blood smears

were made on glass slides, fixed with methanol for two minutes, and stained with Giemsa stain (10%) for 25-30 minutes. Observation with the oil immersion objective of the light microscope revealed the dark blue *Anaplasma* bodies located in the periphery of the pink-colored RBC. Percent infected RBC was determined by counting the number of infected and non-infected cells in several fields. Packed cell volumes were determined using the microhematocrit technique.

When parasitemia in the calves reached 50-80%, blood was taken aseptically by one of the following methods: 1) with a syringe by jugular venipuncture for small volumes (50-100 ml); 2) exsanguination by cardiac puncture into 4-liter glass containers; 3) jugular venipuncture into 4-liter glass containers using a sterile bell bleeding apparatus. In all cases, heparin sodium solution (1000 U.S.P. units per ml) was used as anticoagulant at a volume of 0.5 ml/50 ml blood. Cardiac puncture proved most efficient when large volumes of blood were required.

C. Preparation of materials

1. RBC and plasma

After collection, blood was centrifuged at 4 C at 2,000 x g for 20 minutes. RBC were washed three times in physiological saline (0.9%) and the buffy coat removed each time by aspiration.

Plasma was centrifuged (10,000 x g for 20 minutes) to remove remaining cells and used in later experiments as a suspending medium for washed RBC.

2. Marginal bodies

Anaplasma bodies were collected and purified utilizing the procedure of Slack (1970). This method employs sucrose density gradients and ultracentrifugation. Discontinuous gradients were formed by carefully layering 4 ml of 60% sucrose, and 7 ml each of 50%, 40% and 30% sucrose. Relatively pure *Anaplasma marginale* was found at the 30 and 40 percent interface. An outline of this technique is shown in Figure 1. A curved-tipped Pasteur pipette was used to remove the purified bodies. *Anaplasma* bodies were then dialyzed against phosphate buffered physiological saline (pH 7.6), sedimented by centrifugation at 27,000 x g for 30 minutes at 4 C and resuspended in 5 ml of phosphate buffered saline (pH 7).

3. Chemically-induced anemia

An anemia occurs in anaplasmosis as a result of the infection and is therefore a secondary response. Intravenous injections of phenylhydrazine HCl were used to produce an anemic condition in an experimental calf. This was done to compare DNA and RNA levels in RBC of infected calves with the chemically-induced anemia. Dosage, injection times, hematologic values, and RBC count are shown in Table 1.

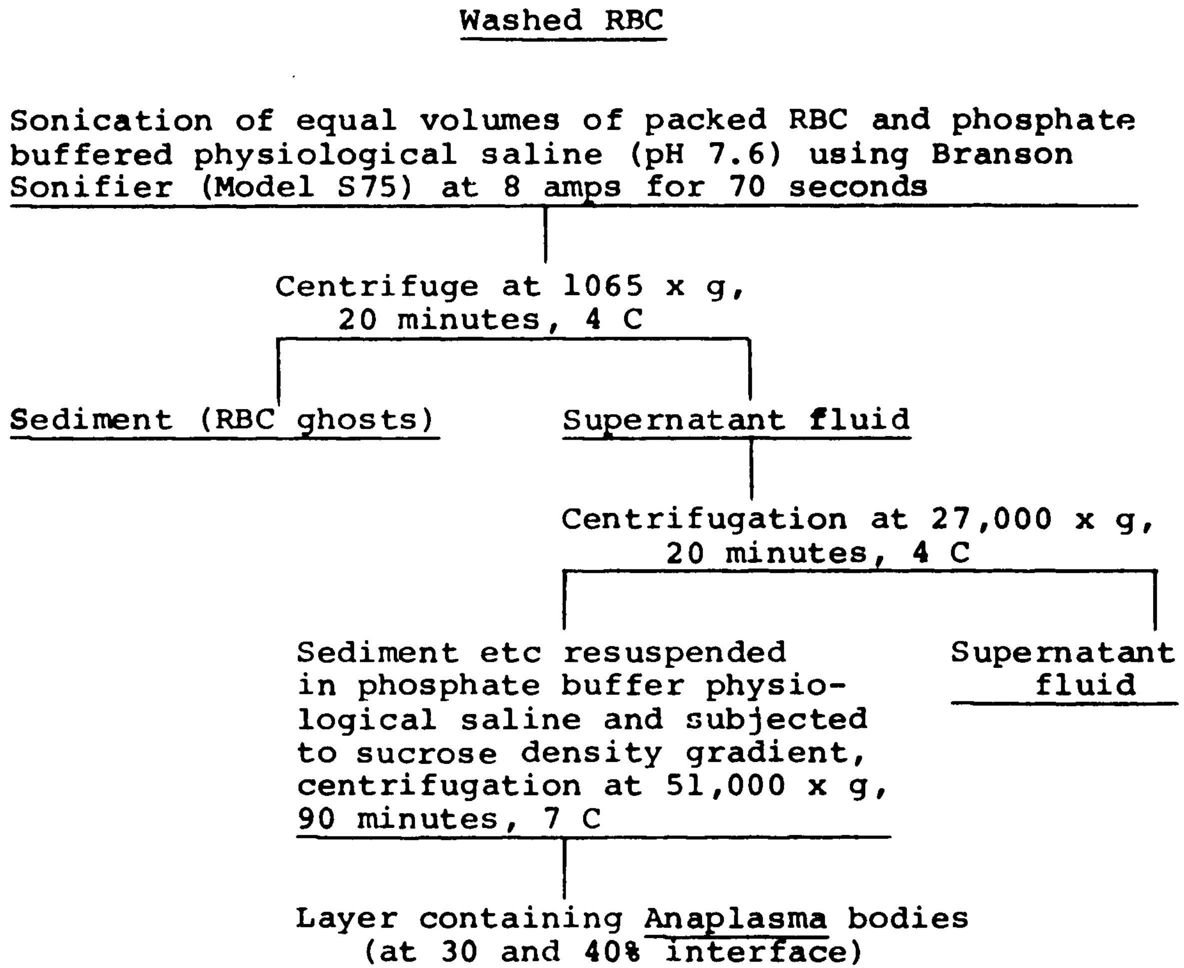


Figure 1. Diagrammatic scheme showing procedure utilizing sucrose density gradients and ultracentrifugation to purify *Anaplasma* bodies.

Table 1. Dosage, injection time and hematologic values, for a calf with anemia induced with phenylhydrazine HCl.

Time of injection (Days)	Dose (Mg)	PCV	RBC count x 10^6
1	250	29	5.76
2	250	32	5.61
3	-		
4	-		
5	250	29	5.27
6	250	28	5.44
7	500	27	5.99
8	500	25	4.91
9	250	20	3.81
10	250		
11	-		
12	-		
13	500	20	2.69
14	250	21	2.59
15	500	20	2.53
16	-	19	2.22

D. Incubation of materials

Approximately 10 ml of packed normal RBC and RBC from infected calves were resuspended in 20 ml cell-free normal plasma. In one experiment normal cells were suspended in plasma from infected calves. This resulted in a PCV of approximately 25%. EDTA (0.01 ml of 0.1% solution per ml) and the appropriate radioactive precursor, ^{14}C-hypoxanthine, ^{14}C orotic acid, ^{14}C sodium bicarbonate, ^{14}C sodium formate, or ^{3}H uridine (20-25 uCi), were added. Initially, incubations were from one hour to eighteen hours at 38 C on a rotary shaker water-bath. However, it was subsequently found that uptake of precursors peaked at 5 hours and therefore incubations were terminated at 7-8 hours. At various time intervals, 2 ml samples of the incubation mixture were centrifuged at 2,000 x g for 5 minutes in order to separate RBC from plasma. The RBC were then washed 3 times in physiological saline to remove the isotope not incorporated. Plasma was assayed for radioactivity as described in section G. Washed RBC were fractionated with TCA and the fractions assayed for radioactivity as described in section G.

E. TCA fractionation of RBC

After a given precursor was added to an incubation mixture, it became important to determine its fate in the cell (pool of metabolic intermediates or nucleic acids). Trichloroacetic acid (TCA) was used to fractionate the RBC

into their various components. Cold TCA precipitates the proteins and nucleic acids and solubilizes the pool of metabolic intermediates and polynucleotides. Hot TCA dissolves nucleic acids.

After incubation with the various precursors, washed normal and infected RBC (100 ul) were precipitated by resuspension in 1 ml of cold 5% w/v TCA. The TCA-precipitated material was extracted twice at 0 C for 30 minutes with 1 ml of cold 5% w/v TCA. After each extraction, the residue was sedimented by centrifugation (2,000 x g for 5 minutes) and the supernatants pooled. This supernatant was labeled as the cold TCA-soluble fraction. In some experiments the residual material was suspended in 1 ml of 10% w/v TCA and extracted at 90 C for 60 minutes. The residue was sedimented by centrifugation (2,000 x g for 5 minutes) and the supernatant was labeled hot TCA-soluble fraction.

F. Hydrolysis and separation of bases

Following incubation of normal and infected RBC with ^{3}H-uridine and TCA fractionation, the cold TCA-soluble fraction was hydrolyzed. This was accomplished by using a modification of the technique of Gutteridge and Trigg (1970). TCA was removed from each sample by three extractions with ether. Samples were then made 1 N with respect to HCl, heated at 100 C for 60 minutes, and freeze-dried and resuspended in 0.8 ml of 1 N HCl. Each

sample was divided into five parts (100 ul) and 100 ul of a different carrier (25 mg/ml) (adenine, quanine, cytosine, thymine or uricil) was added to each part. These preparations were subjected to paper chromatography in order to separate the individual bases. Whatman No. 1 filter paper was cut to measure 20 x 50 cm and divided into 5 lanes. Onto each lane, 10 ul of each sample with its specific carrier was spotted. The solvent used to develop the chromatograms consisted of 12 N HCl/2-propanol/ H_2O (16.7/65/18.3; v/v/v). Development times were approximately 20 hours, after which the chromatogram was removed and dried in an atmosphere of NH_3 to neutralize the HCl.

The separated bases could be detected under long wave UV radiation as dark spots against a background of general paper fluorescence. The spots were cut out and assayed for radioactivity as described in section G.

G. Assay of radioactivity

The TCA-soluble fractions and the plasma were prepared for isotope counting in the same manner. The samples (100 ul) were placed in liquid scintillation vials with 1 ml of the secondary solvent, NCS solubilizer (Amersham-Searle Corp., Des Plaines, Ill.) and allowed to digest for 24 hours, after which 15 ml of scintillation fluid was added. The liquid scintillation fluid consisted of 2,5-diphenyloxazole as the primary fluor, 1,4-bis-[2-(phenyloxazole)]-benzene as the secondary fluor, and toluene as the primary solvent.

The spots on the paper chromatograms were cut out, placed in liquid scintillation vials containing 2 ml of NCS solubilizer, and treated as the above samples.

All samples were assayed for levels of radioactivity with a Beckman Liquid Scintillation Spectrometer, Model L S 200. A chemical quench correction curve was used to correct efficiencies.

H. Enzyme assay

In order to extend and supplement the studies on the ^{3}H uridine incorporation into pyrimidines of normal and RBC from *A. marginale*-infected calves, orotidine-5'-monophosphate pyrophosphorylase (OMPpase) was investigated. OMPpase catalyzes the condensation of orotic acid with phosphoribosylpyrophosphate (PRPP) to yield orotidine-5'-monophosphate (OMP). OMPpase assay mixtures contained 1 ml of 1 M - tris buffer which, when diluted 1:10, achieved a pH of 8.8; 0.20 m moles of orotic acid; 0.2 m moles of $MgCl_2$; various amounts (0.05-0.2 ml) of purified *A. marginale* bodies; and 0.4 m moles of PRPP. Controls were also run, eliminating different reactants. The enzyme was then assayed by following the disappearance of orotic acid measured at 295 nm (30 C) with a Beckman DB Spectrophotometer (Beckwith *et al.*, 1963). All biochemical reagents used were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

I. <u>DNA isolation and sucrose density gradient centrifugation</u>

DNA was isolated from RBC of *A. marginale*-infected calves and from RBC of a calf suffering from the phenylhydrazine induced anemia. A phenolic extraction (Thomas *et al.*, 1966) as described by Senitzer (1969) was slightly modified and used in all extraction procedures.

Packed RBC (5 ml) were freed of white cells and plasma by aspirating off the buffy coat. Cells were subjected to freeze-thaw treatment, suspended in 5 ml of 1 x saline sodium citrate (SSC) (0.15 M NaCl, 0.02 M trisodium citrate, 0.0005 M EDTA) and DNA was extracted from this preparation.

The lysed RBC suspension (10 ml) was incubated with pronase (2 mg/ml) and sodium lauryl sulfate (2% final concentration) for 7 hours at 37 C. An equal volume of redistilled phenol saturated with 1 x SSC was added and the suspension rolled (60 rev/minutes) for 30 minutes. The suspension was then chilled to 0 C, centrifuged at 1510 x g for 20 minutes, and the phenol removed with a pipette. Chloroform-isoamyl alchohol deproteinizations were performed and followed by treatment with RNase (20 ug/ml). The solution was then dialyzed against phosphate buffer (pH 7). Following dialysis, 2 volumes of cold 95% ethanol were used to precipitate the DNA which was wound on a glass rod and dissolved with 5 ml of phosphate buffer (pH 7). Figure 2 describes this procedure.

Washed RBC frozen, thawed, and
suspended in 1 x SSC

Pronase (2 mg/ml) and SLS (2%)
added; incubated 7 hours at
37 C with occasional shaking

Equal volumes redistilled phenol,
SSC saturated, was added;
rolled 30 minutes, 60 rev/minutes;
cooled to 0 C
centrifuged 1510 x g

Phenol layer | Aqueous layer

Incubated with 20 ug/ml
RNase 30 minutes at 37 C;
deproteinized with chloroform-
isoamyl alcohol; dialyzed against
phosphate buffer (pH 7)

2 volumes 95% ethanol

DNA | Aqueous supernatant

Spooled onto glass rod
and dissolved in phosphate buffer (pH 7)

Figure 2. Modified scheme for phenolic extraction of DNA from *A. marginale* (Thomas *et al.*, 1966)

DNA isolation from RBC from *Anaplasma*-infected calves was compared to that isolated from RBC of a chemically-induced anemic calf utilizing sucrose density gradient centrifugation. A Buchler gradient maker was used to form 4.5 ml continuous gradients (5-20% sucrose). All sucrose solutions were prepared with phosphate buffer (pH 7). Gradients were carefully layered with 0.5 ml of sample and centrifuged at 32,644 x g for 3.5 hours in a Beckman Model L Ultracentrifuge equipped with an SW-39 rotor. A Buchler manual fraction collector was used to collect approximately 25 fractions (7 drops each) from each gradient. The volume of each fraction was brought to 2 ml with phosphate buffer (pH 7) and measured at 260 nm with a Beckman DB Spectrophotometer.

J. RNA isolation and sucrose density gradient centrifugation

Both cold and hot phenol extractions have proven useful in the isolation of RNA. In this study, the hot phenol extraction described by Larsen and Frederiksen (1972) was used because of its greater extraction efficiency.

Attempts were made to extract RNA from RBC from *A. marginale*-infected calves and from RBC of a chemically-induced anemic calf. Three grams of washed, packed RBC were frozen, thawed, suspended in 20 ml of acetate buffer (pH 5.1), and made 1 mM with respect to EDTA. SLS (2 ml, 10%) was added to this suspension which was then

extracted at 55 C with one volume of phenol saturated with water containing 0.1% 5-hydroxyquinoline. After occasional vigorous shaking during a period of 3 minutes at 55 C the mixture was cooled to 0 C and centrifuged at 2,000 x g for 5 minutes. The aqueous phase was aspirated and kept at 0 C and the phenol phase and the interphase re-extracted with 10 ml buffer at 0 C. The supernatants were pooled and re-extracted with 0.5 volume phenol at 0 C. Two volumes of 95% ethanol containing 0.2% potassium acetate were added to the aqueous phase and the solution was held overnight at 0-5 C. RNA was collected by centrifugation (10,000 x g for 30 minutes) and the pellet dissolved in 4.0 ml of 0.01 M potassium phosphate buffer (pH 5.7) and 400 ul salts mixture (0.14 M NaCl, 1 mM $MgCl_2$, 0.05 M sodium acetate (pH 5.1)). This solution was then dialyzed against the same buffer. A schematic representation of this procedure is given in Figure 3.

The RNA preparations (0.5 ml) were carefully layered onto continuous sucrose density gradients (pH 5.1, 5-20% sucrose) which were formed as previously described. In some cases the RNA samples were incubated with DNase (100 ug/ml) at 37 C for 30 minutes prior to layering on the gradients. This was done to remove any contaminating DNA. The gradients were centrifuged at 36,114 x g for 14 hours using a Beckman Model L Ultracentrifuge. This was followed by fractionation of the gradient as previously

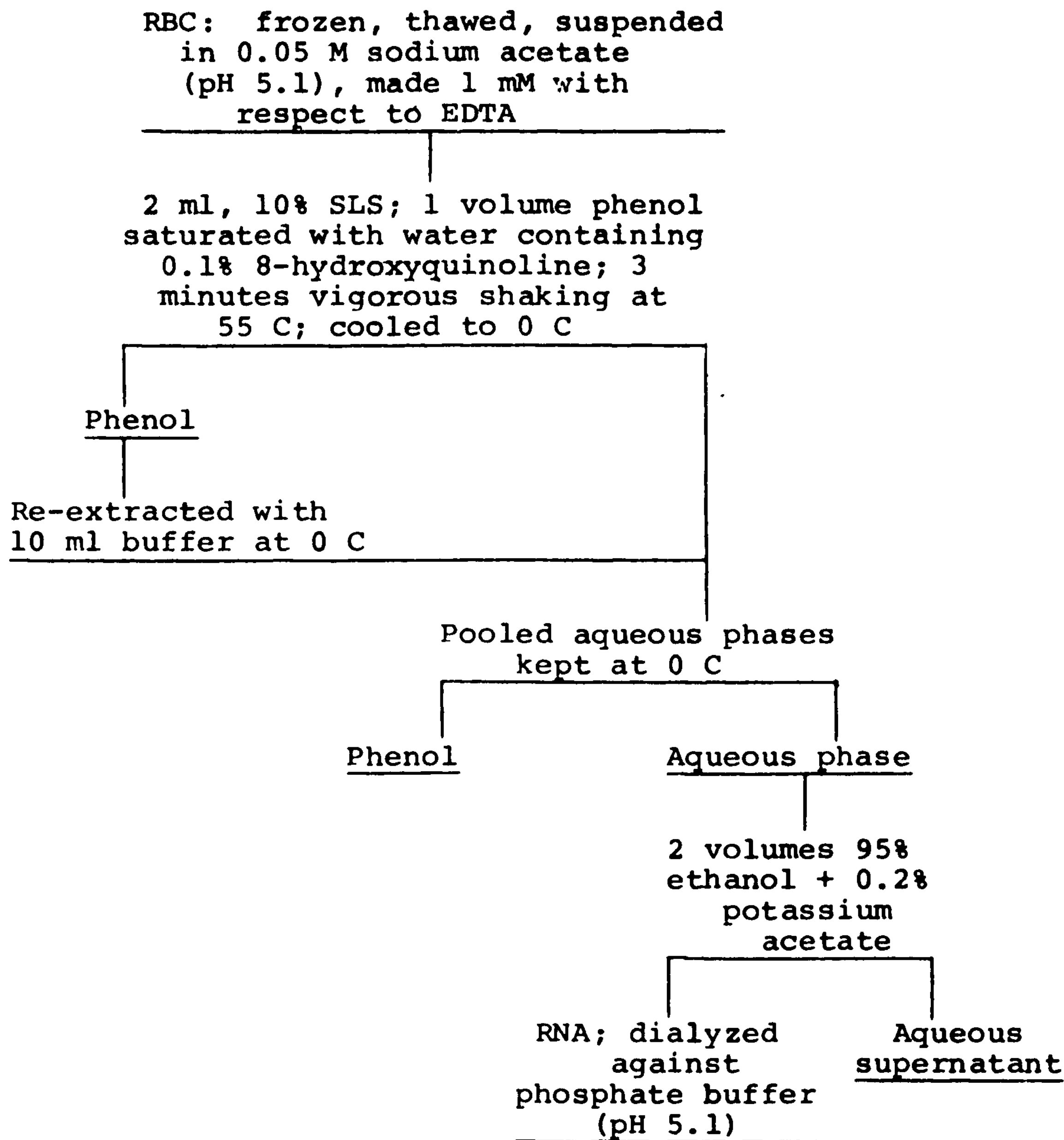


Figure 3. Scheme for hot phenol RNA extraction (Larsen and Frederiksen, 1972)

mentioned. The fractions (5 drops each) were brought to a final volume of 2 ml with phosphate buffer (pH 5.1) and measured at 260 nm with a Beckman DB Spectrophotometer.

K. Electron microscopy

DNA extracted from RBC from *A. marginale*-infected calves (Thomas *et al.*, 1966) was prepared for electron microscopy by a procedure modified from the protein film techniques of Mosharrafa *et al.*, (1970) and Senitzer (1969). Electron microscope grids (copper, 30 mesh) were coated with a parlodion film and coated with a thin layer of carbon. Various concentrations of DNA (15 ug/ml-50 ug/ml) were placed on the grids in the following manner. One volume of DNA in 1 M ammonium acetate (made with glass distilled water) was mixed with 0.1 volume of a 0.1% solution of cytochrome C in 4 M NaCl. Twenty-five ul of the mixture was then drawn into a syringe equipped with a lambda pipette. The solution was carefully deposited on a glass ramp (acid cleaned) set at a 30° angle into a 0.15 M ammonium acetate solution (made with glass-distilled water). The DNA protein mixture was allowed to slowly run down the ramp and spread onto a 5 cm^2 surface of the ammonium acetate. The resulting film was picked up by punching the carbon coated grids through the surface of the ammonium acetate. This was followed by dehydration in ethanol for 30 seconds. The grids were then placed in a vacuum evaporator and shadowed with 50 mg of platinum at an angle of 15° from two perpendicular directions.

Grids were observed in a RCA EMU-e Electron Microscope. All electron micrographs were taken at a magnification of 8,000 x.

Results and Discussion

A. *Introduction*

Histochemical staining procedures using blood smears have been used to demonstrate the presence of DNA and RNA in *A. marginale* (Moulton and Christensen, 1955). Gough (1963) found DNA concentrations in *A. marginale*-infected RBC to be highest at peak infection and RNA concentrations to be highest 3 to 4 days later.

In contrast to the above, when *Anaplasma* bodies were partially-purified Ellender and Dimopoullos (1967) could only isolate DNA. Senitzer (1969) isolated DNA from partially-purified bodies and infected RBC but found no evidence of RNA.

This project was undertaken in an attempt to learn more about the nucleic acid metabolism (RNA metabolism in particular) of *Anaplasma*-infected RBC. Such data obtained would aid in understanding the site and mode of replication of *A. marginale*.

B. *Incubation of materials*

Since *A. marginale* has not been propagated *in vitro* it was necessary to compare the uptake and accumulation of nucleic acid intermediates and precursors in both normal RBC and RBC from *Anaplasma*-infected calves. The previously

mentioned labeled compounds were used in these experiments. In most cases these materials were incubated with normal or infected cells suspended in normal plasma. In one experiment, tritiated uridine was incubated with normal cells suspended in plasma from infected calves. This was done to determine whether or not plasma from infected animals had an effect on the nucleic acid metabolism of normal RBC.

In this study it was desired to detect only changes in RBC metabolism produced by *A. marginale* infection. For this reason no antibiotics were added to the incubation mixtures. All blood collection and incubation procedures were therefore performed under aseptic conditions.

C. *TCA fraction and assay of radioactivity*

Uptake of radioactive material by normal RBC and RBC from infected calves was measured by following the disappearance of radioactivity from the plasma. However, it was also important to determine the fate of these compounds in the cell. Therefore, cold, and in some cases, hot TCA fractionations were performed on the RBC following incubation.

It has been shown that hypoxanthine is taken up readily by *P. knowlesi* in duck RBC (Gutteridge and Trigg, 1970). It was therefore decided to use ^{14}C-hypoxanthine as one of the labeled precursors in the uptake experiments. Data given in Figure 4 compares the disappearance of

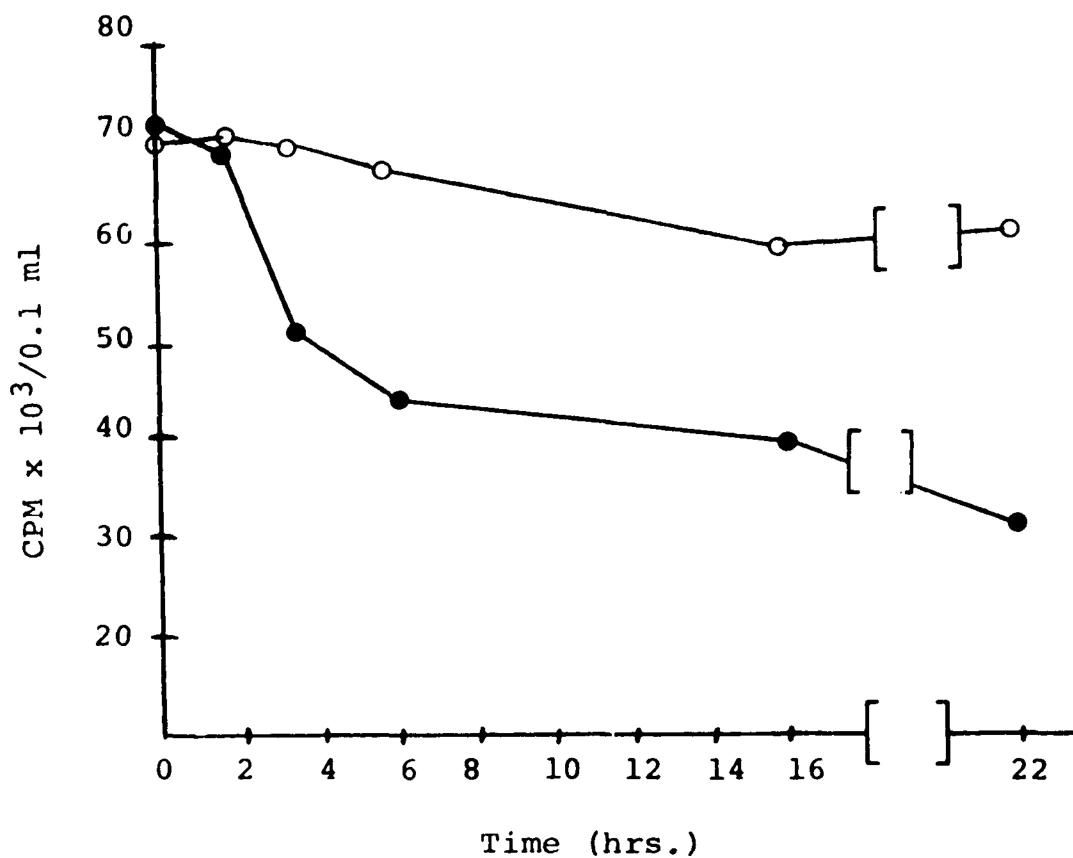


Figure 4. Dissappearance of radioactivity from normal plasma incubated with normal RBC, ●——●; and from normal plasma incubated with RBC from *Anaplasma marginale* infected calves ○——○. RBC suspensions (25% in normal plasma) were incubated *in vitro* with ^{14}C-hypoxanthine. Aliquots of plasma were separated from the cells by centrifugation and prepared for liquid scintillation counting.

radioactive precursor from normal plasma which had been incubated with normal RBC and normal plasma which had been incubated with RBC from infected calves. For the first 2 hours little difference was observed. However, after 2 hours the normal RBC began to take hypoxanthine from the plasma at a rate 36% faster than the RBC from infected calves. Uptake leveled off at round 8 hours in both cases. The amount of hypoxanthine that was fractionated in the cold TCA soluble fraction of normal RBC and RBC from infected calves is shown in Figure 5. In the normal cell, incorporation peaked at 5.5 hours and reached a level twice as high as the level observed in the infected cells. A two-fold increase in activity could be seen in the normal RBC but the apparent slopes of the two curves were similar. According to these data, little variance was observed in the metabolism of hypoxanthine in normal and Anaplasma-infected RBC. The difference appears to be only in the uptake of the compound. These data suggest that infection inhibited the transport of hypoxanthine into the cell.

Sodium formate has been useful in the study of purine synthesis by the avian malaria parasite, P. lophurae (Walsh and Sherman, 1968). In one experiment ^{14}C-sodium formate was incubated with normal RBC and RBC infected from calves suspended in normal plasma. A significant decrease in radioactivity could not be measured in the

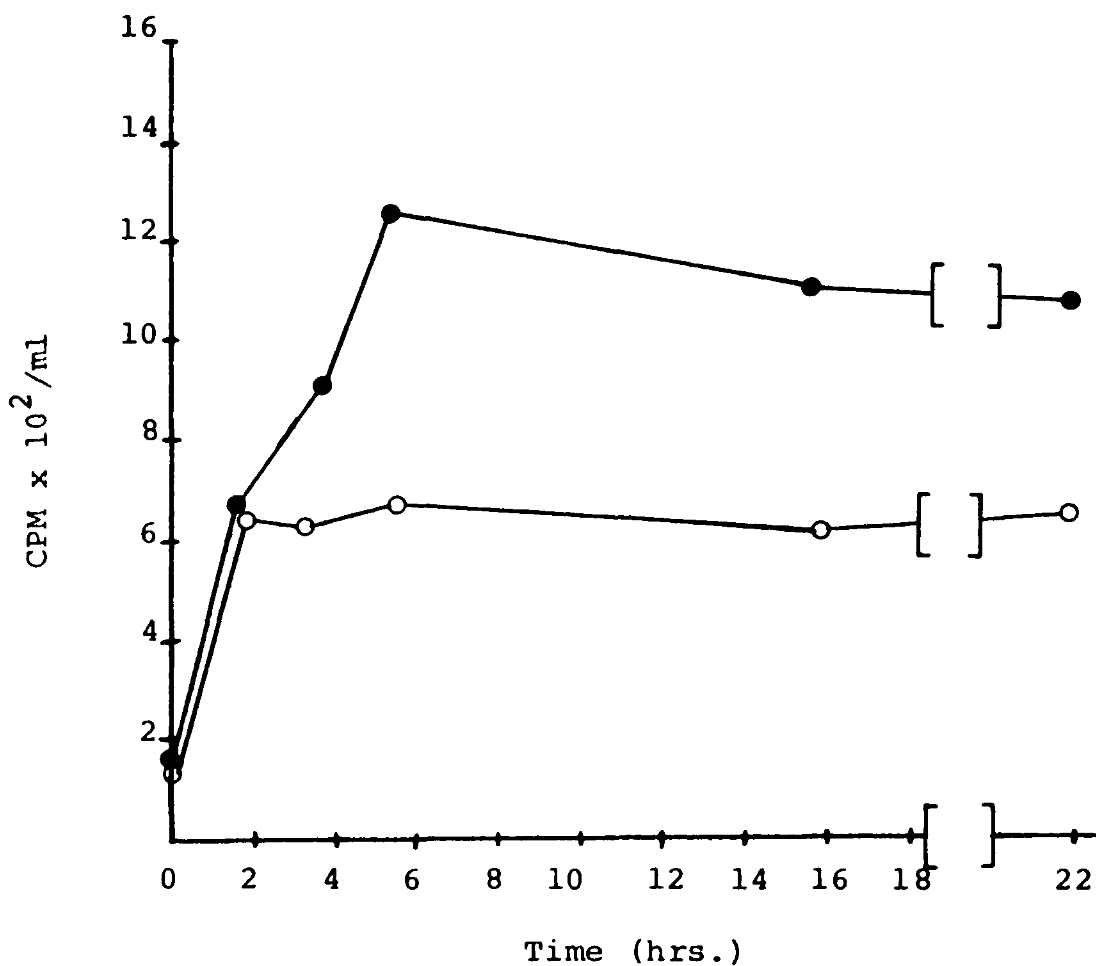


Figure 5. Radioactivity in the cold TCA soluble fraction of RBC from normal, ●——●, and *Anaplasma marginale*-infected calves, ○——○, RBC suspensions (25% in normal plasma) were incubated *in vitro* with ^{14}C-hypoxanthine. Aliquots of cells were washed 3 times with physiological saline, fractionated with cold 5% TCA and prepared for liquid scintillation counting.

plasma of either incubation mixture, even after 6 hours (Figure 6). Also, no increase in radioactivity could be detected in the cold TCA soluble fraction of either normal RBC or RBC from infected calves. This indicates that bovine RBC may lack a specific transport system for sodium formate, at least under in vitro conditions. Therefore, such a compound is of little use in the study of purine synthesis of RBC from Anaplasma-infected calves.

Orotic acid is known to be an important growth factor for many microorganisms. It was found to be the key intermediate in the pathway for pyrimidine synthesis (orotic acid pathway). In this study ^{14}C-orotic acid was incubated with normal RBC and RBC from infected calves suspended in normal plasma. During the first hour of incubation, approximately 10% of the total activity had disappeared from the plasma of the normal cell incubation mixture (Figure 7). During this same period very little activity was lost from the plasma containing the infected cells. However, after 1 hour, the slopes of the curves were identical in both cases. The infection did inhibit transport of orotic acid into the RBC during the first hour, but had no effect after that time. The accumulation of activity into the cold TCA soluble fraction of the RBC from infected calves proceeded somewhat slower than in the normal RBC (Figure 8). Peak incorporation occurred during 5 hours in the infected cells and leveled off from

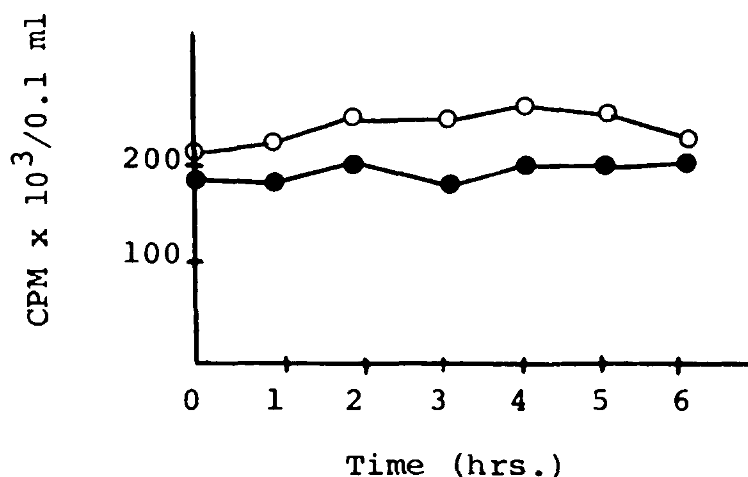


Figure 6. Disappearance of radioactivity from normal plasma incubated with normal RBC,●——●, and from normal plasma incubated with RBC from *Anaplasma marginale*-infected calves,○——○. RBC suspensions (25% in normal plasma) were incubated *in vitro* with ^{14}C-sodium formate. Aliquots of plasma were separated from the cells by centrifugation and prepared for liquid scintillation counting.

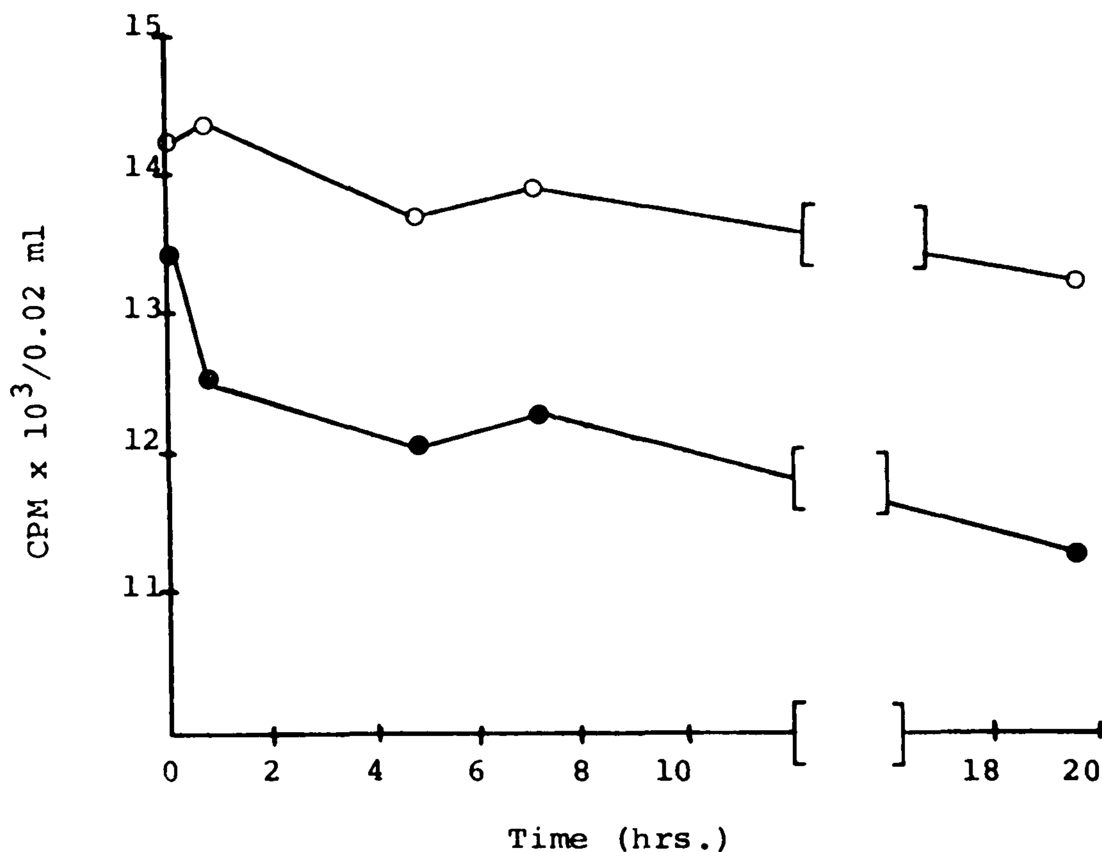


Figure 7. Disappearance of radioactivity from normal plasma incubated with normal RBC, ●——●, and from normal plasma incubated with RBC from *Anaplasma marginale*-infected calves, ○——○. RBC suspensions (25% in normal plasma) were incubated *in vitro* with ^{14}C-orotic acid. Aliquots of plasma were separated from the cells by centrifugation and prepared for liquid scintillation counting.

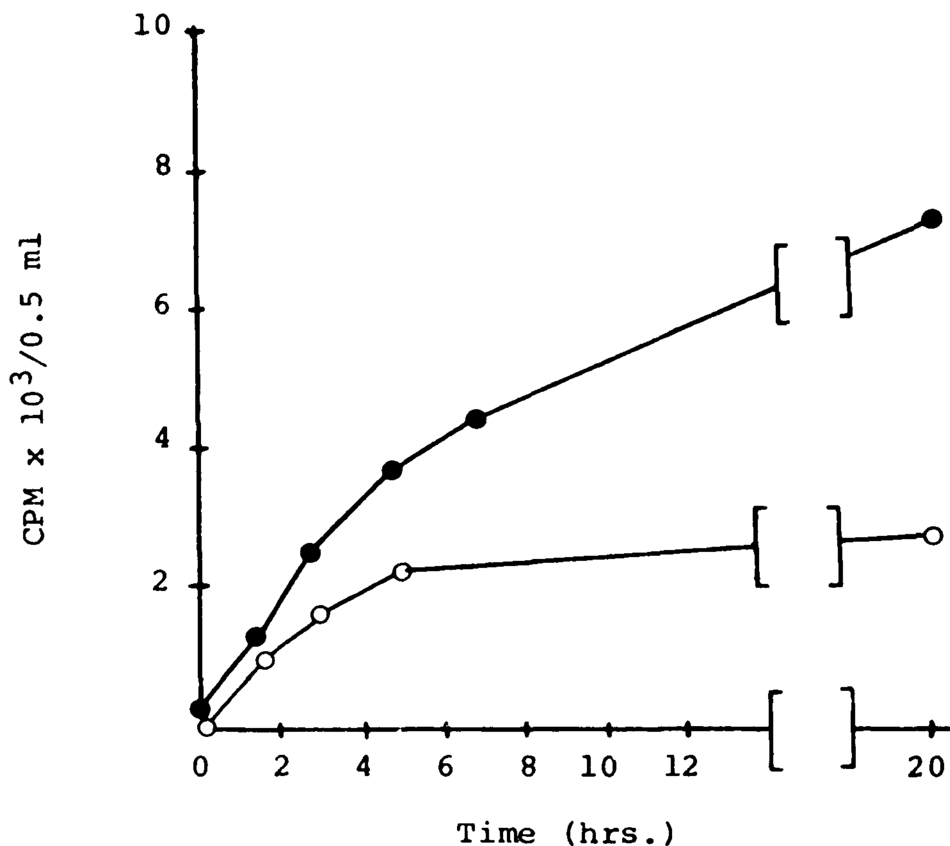


Figure 8. Radioactivity in the cold TCA soluble fraction of RBC from normal, ●——●, and *Anaplasma marginale*-infected calves, ○——○. RBC suspensions (25% in normal plasma) were incubated *in vitro* with ^{14}C-orotic acid. Aliquots of cells were washed 3 times with physiological saline, fractionated with cold 5% TCA and prepared for liquid scintillation counting.

5-20 hours. Peak incorporation had not occurred after 20 hours in normal cells. Activity was incorporated into the hot TCA soluble fraction of infected cells at a rate twice that of normal cells (Figure 9). In both cases peak accumulation occurred at 5 hours, but at a higher level in the infected cells. After 5 hours the slopes were similar. This increase of activity in the nulceic acid fraction of the RBC from infected calves might have been due to nucleic acid synthesis of _A. marginale_. Orotic acid appeared to accumulate in the normal cell and was used for a small amount of nucleic acid synthesis. The amount of nucleic acid synthesis measured by incorporation of ^{14}C-orotic acid into the hot TCA soluble fraction of the normal cells was probably due to the presence of nucleated RBC. Abnormal red cell production, either as a result of active erythrogenesis or of aberrations in maturation of red cells, may cause release of nucleated RBC into the circulation (Schalm, 1970).

A study was also made on the uptake of the pyrimidine nucleoside, uridine. In one experiment ^{3}H-uridine was incubated with normal RBC and RBC from infected calves suspended in normal plasma. The normal RBC removed activity from the plasma rapidly for 2 hours (Figure 10). The activity then began to increase until it approached its original level. Upon initiation of incubation,

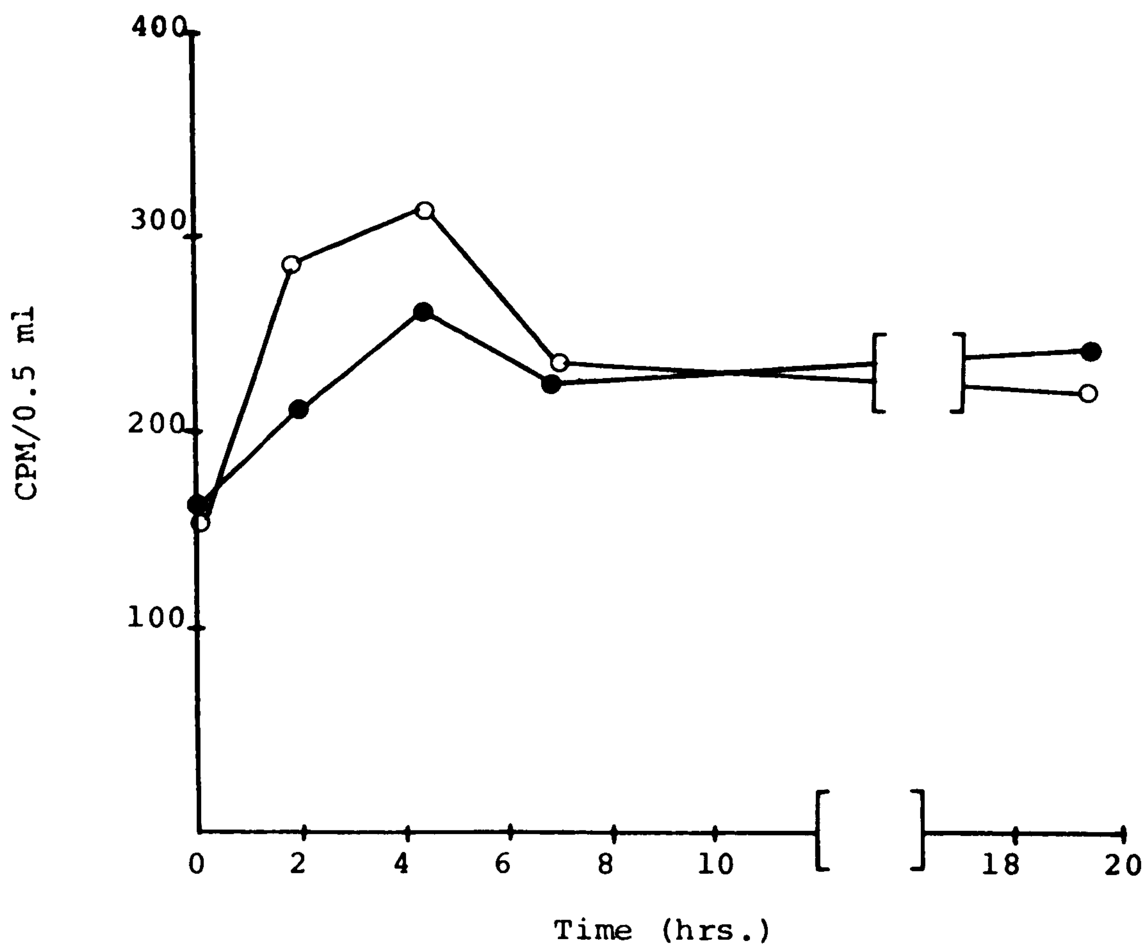


Figure 9. Radioactivity in the hot TCA soluble fraction of RBC from normal, ●——●, and *Anaplasma marginale*-infected calves, ○——○. RBC suspensions (25% in normal plasma) were incubated *in vitro* with ^{14}C-orotic acid. Aliquots of cells were washed 3 times with physiological saline, fractionated with hot 5% TCA and prepared for liquid scintillation counting.

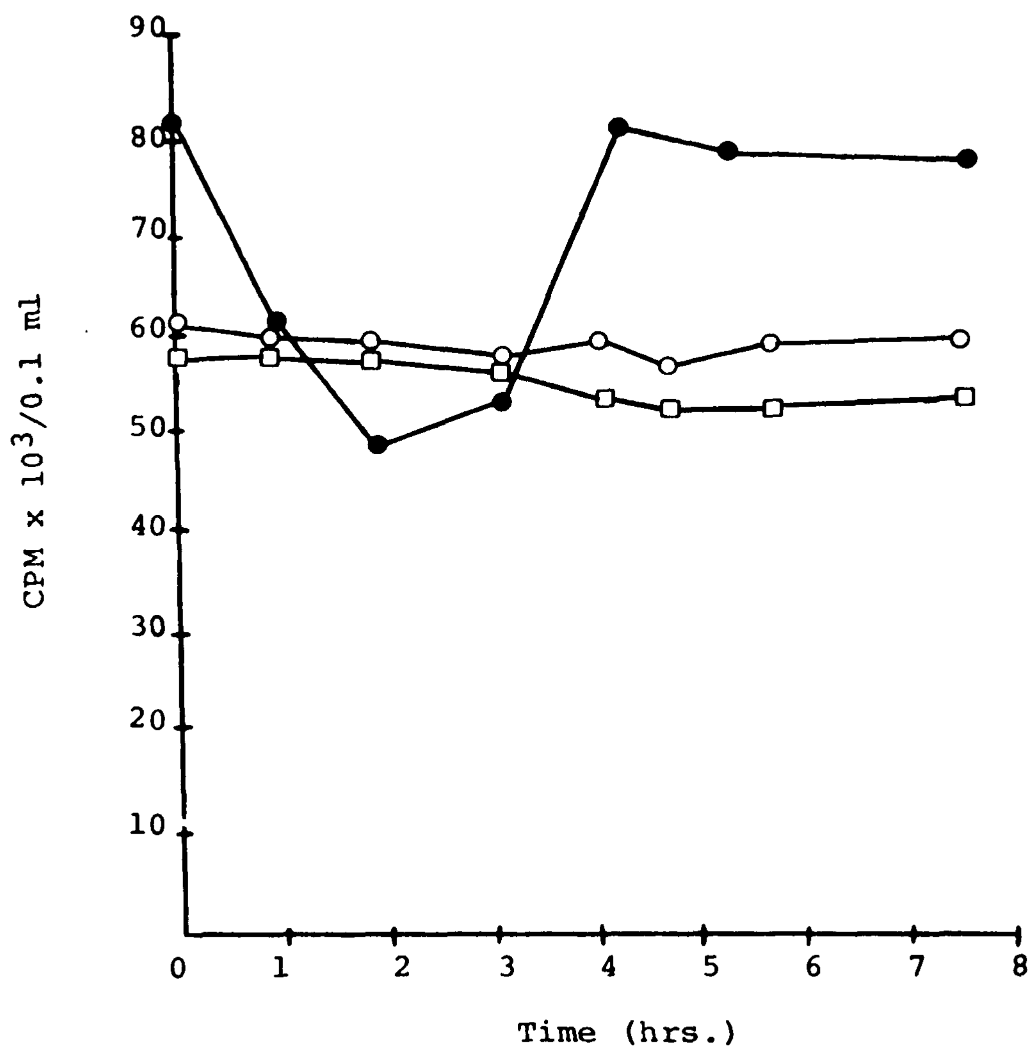


Figure 10. Disappearance of radioactivity from normal plasma incubated with normal RBC, ●——●; from normal plasma incubated with RBC from *Anaplasma marginale*-infected calves, ○——○; and from plasma of infected calves with RBC from normal calves, □——□. RBC suspensions (25% in normal plasma) were incubated *in vitro* with ^{3}H-uridine. Aliquots of plasma were separated from the cells by centrifugation and prepared for liquid scintillation counting.

activity decreased rapidly in the plasma containing the infected RBC. This was followed by a slow removal of activity from the plasma. Activity reached a peak in the cold TCA soluble fraction of the normal RBC after 2 hours incubation and then began to decrease until it leveled off after 3 hours (Figure 11). Activity slowly increased in the cold TCA soluble fraction of the RBC from infected calves and continued to rise during 7 hours of incubation. As shown in Figure 12, peak activity in the hot TCA soluble fraction also occurred at 2 hours in the normal RBC. An insignificant amount of activity was incorporated into this same fraction of the RBC from infected calves. These data indicate that the *Anaplasma* infection inhibited but did not prevent the transport of uridine into the RBC. The same amount of activity was introduced into both normal and infected incubation mixtures. At 0 time, however, 25% of the activity immediately disappeared from the plasma of the infected incubation mixture. It appears that uridine was immediately adsorbed to the membrane and was slowly brought into the cell. Once in the infected cell, uridine accumulated in the pool of metabolic intermediates and probably was not used for nucleic acid synthesis. Whereas in the normal RBC, uridine was rapidly transported into the cell where it accumulated in the pool of metabolic intermediates. Uridine then appeared to be drawn from the pool and used for DNA and RNA synthesis

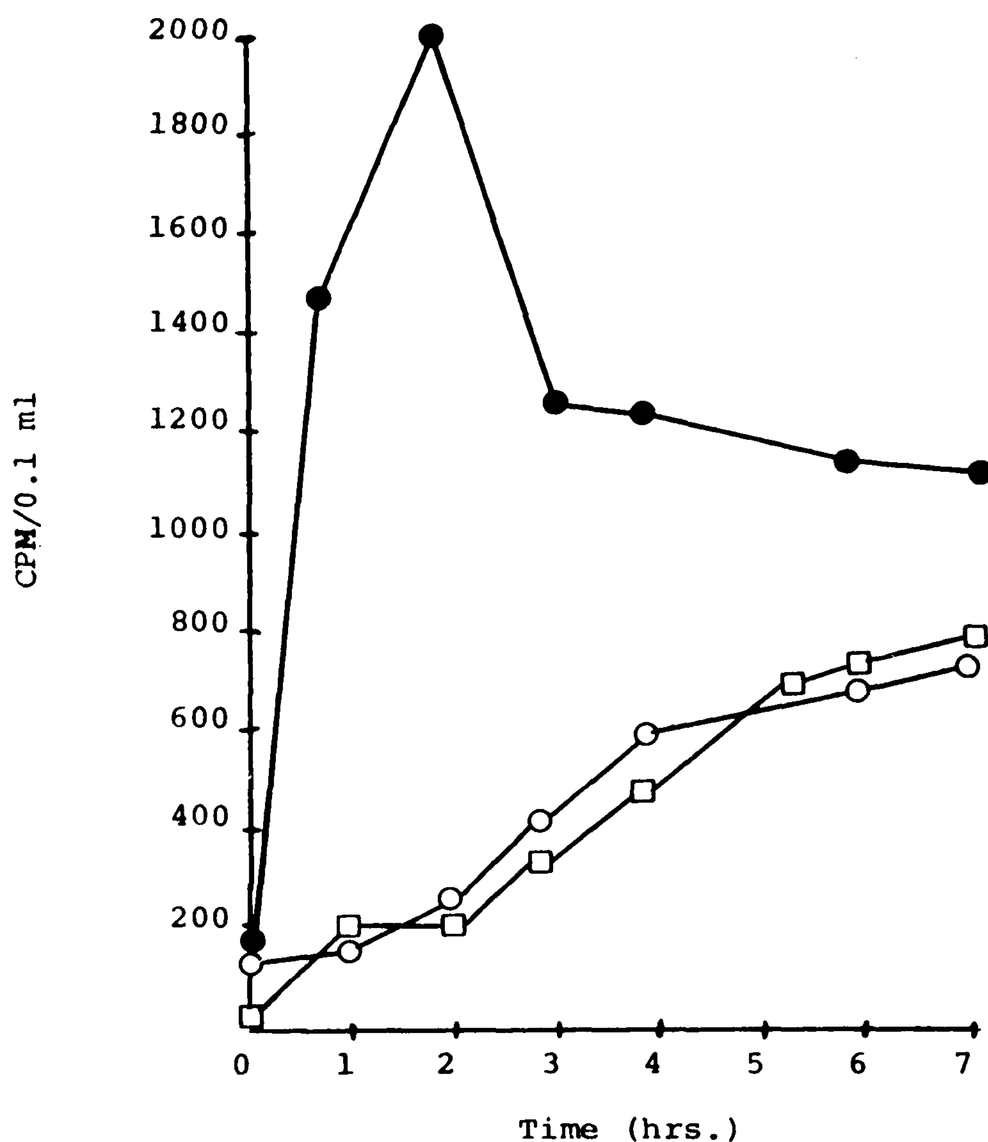


Figure 11. Radioactivity in the cold TCA soluble fraction of RBC from normal, ●——●, and *Anaplasma marginale*-infected calves, ○——○, which were suspended in normal plasma; and from normal RBC, □——□, suspended in plasma from infected calves. RBC suspensions (25% in normal plasma) were incubated *in vitro* with ^{3}H-uridine. Aliquots of cells were washed 3 times with physiological saline, fractionated with cold 5% TCA and prepared for liquid scintillation counting.

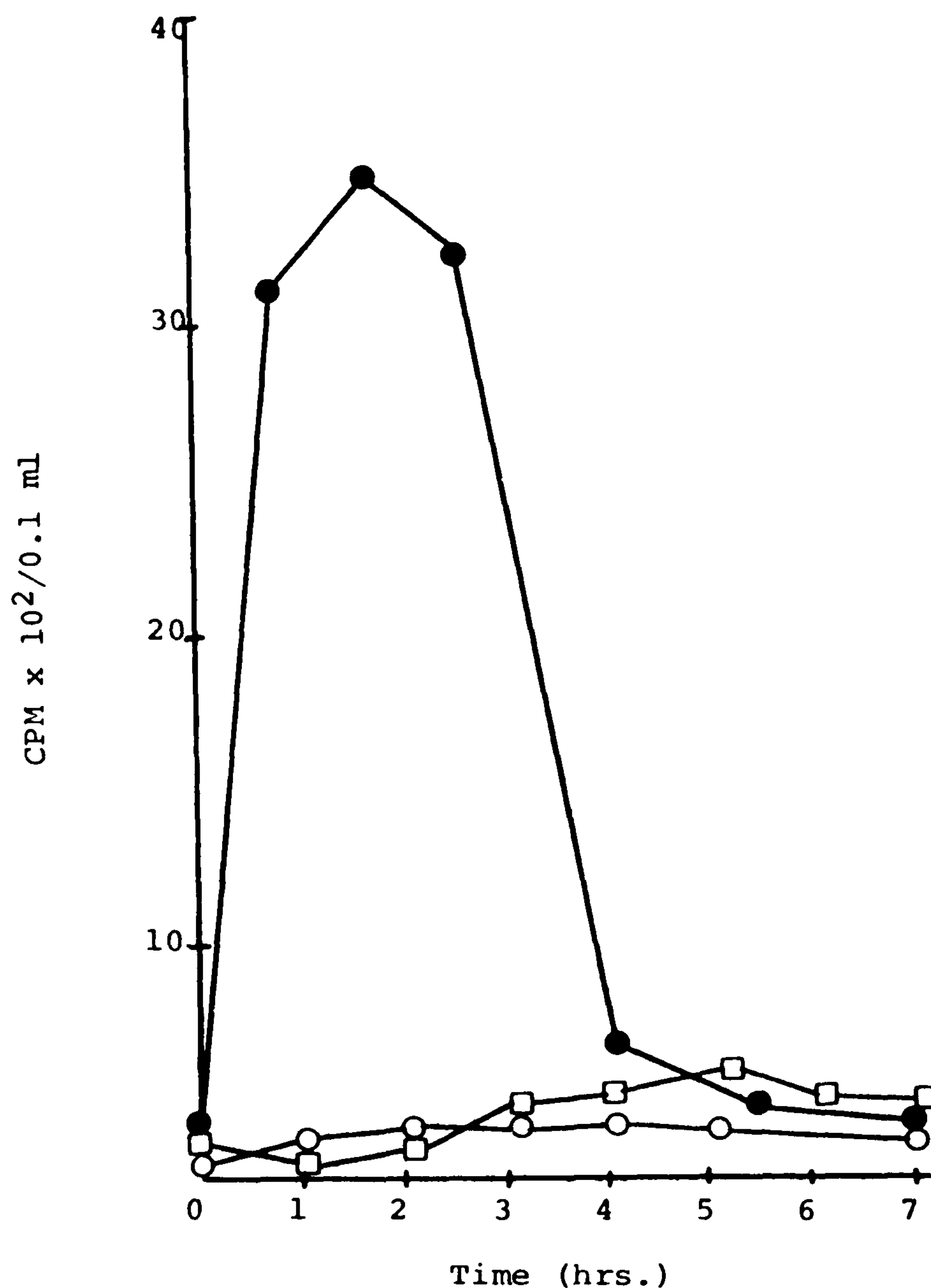


Figure 12. Radioactivity in the hot TCA soluble fraction of RBC from normal, ●——●, and *Anaplasma marginale*-infected calves, ○——○, which were suspended in normal plasma; and from normal RBC, □——□, suspended in plasma from infected calves. RBC suspensions (25% in normal plasma) were incubated *in vitro* with ^3H-uridine. Aliquots of cells were washed 3 times with physiological saline, fractionated with hot 5% TCA and prepared for liquid scintillation counting.

as shown in Figure 12. This nucleic acid synthesis was probably due to the presence of nuclear material in some RBC. After 4 hours there was a rapid decrease of activity from both TCA soluble fractions of the normal RBC. This might be due to the maturation of immature nucleated RBC and subsequent loss of the nucleus and nucleic acid.

A significant difference was observed between the uridine uptake and metabolism of normal RBC and RBC from *A. marginale*-infected calves. It was decided to determine whether or not plasma from infected calves played a role in producing this difference. Normal RBC were suspended in plasma taken from an animal at peak infection and incubated with ^{3}H-uridine as previously described. As can be seen in Figures 9, 10 and 11, the slopes measuring the disappearance of activity from the plasma and the incorporation of activity into the TCA soluble fractions were almost identical to those obtained with the infected cells. A factor probably occurs in the plasma from infected calves that not only inhibits uptake of uridine but also controls its metabolic fate once it enters the RBC. As incubation proceeded, these normal cells became fragile and lysed as easily as RBC from infected calves. Therefore, the normal membrane must be altered to take on the characteristics of a membrane of an RBC from an infected animal. Since the amount of activity that entered the cold and hot TCA soluble fraction also corresponded to

that of a cell from an infected calf, one might postulate that this factor was transported into the normal RBC. Once in the cell it might inhibit or repress enzymes responsible for the metabolism of uridine. One might speculate that this unknown factor is another form of Anaplasma. It might also be the presence of initial bodies in the plasma which caused the RBC transformation. One also cannot rule out the presence of a lytic enzyme which might have caused the normal membrane to take on the fragile properties of an infected membrane.

Sodium bicarbonate has been used successfully in the study of pyrimidine synthesis of P. lophurae (Walsh and Sherman, 1968). In this study ^{14}C-sodium bicarbonate was incubated with normal RBC and RBC from infected calves suspended in normal plasma. The labeled precursor failed to enter both normal RBC and those from calves infected (Figure 13). Both types of RBC appeared to lack a mechanism for transport of this one-carbon compound.

The experiments involving the uptake and metabolism of various nucleic acid precursors and intermediates indicate that infection with A. marginale inhibited the membrane transport of these compounds. In most cases it also appeared to inhibit their metabolism by inactivation of certain enzymes, those associated with orotic acid metabolism being the exception. It appeared that uridine was not used for nucleic acid synthesis in the infected

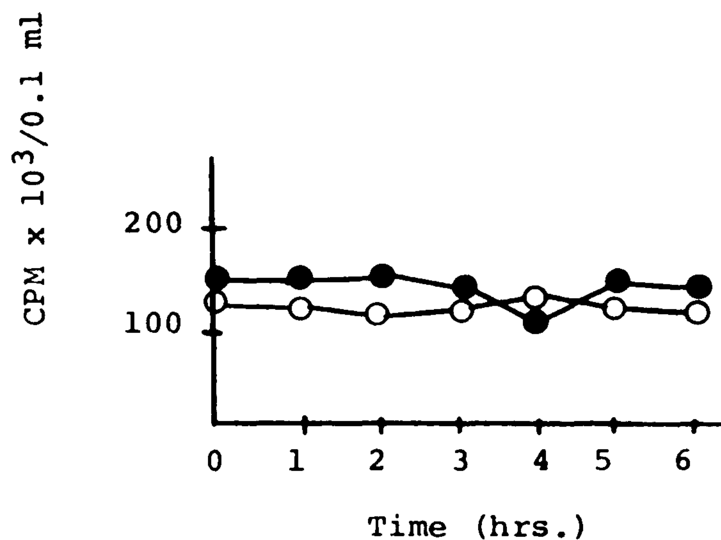


Figure 13. Disappearance of radioactivity from normal plasma incubated with normal RBC,●——●, and from normal plasma incubated with RBC from *Anaplasma marginale*-infected calves,○——○. RBC suspensions (25% in normal plasma) were incubated in vitro with ^{14}C-bicarbonate. Aliquots of plasma were separated from the cells by centrifugation and prepared for liquid scintillation counting.

mixture (Figure 12). Any nucleic acid synthesis in infected RBC can probably be accounted for by _de novo_ synthesis by _A. marginale_.

D. _Hydrolysis and assay of radioactivity_

It was decided to compare the exchange of label between uridine and other nitrogenous bases in normal RBC and RBC from infected calves. This was done by hydrolyzing the cold TCA soluble fraction of the RBC and subjecting the hydrolysates (containing known nitrogenous bases as carriers) to descending paper chromatography. Figure 14 represents a chromatogram showing the relative positions of the bases in the hydrolysate. The bases were detected as spots with ultra-violet radiation. They were cut out and assayed for radioactivity.

Figure 15 illustrates the amount of activity remaining as uracil (U) in normal RBC and those from infected calves. It can be seen that large amounts of activity remained in uracil with peak accumulation at 1.5 hours in the normal RBC and at 4 hours in the RBC from infected calves. This corresponded closely to the total activity found in the cold TCA soluble fractions of these RBC.

The amount of activity going to thymine peaked at 1 hour in the normal RBC and at 2 hours in the infected RBC (Figure 16). The peak activity in the normal RBC reached a level 3 times that reached in the infected cell. This is understandable however, since normal RBC

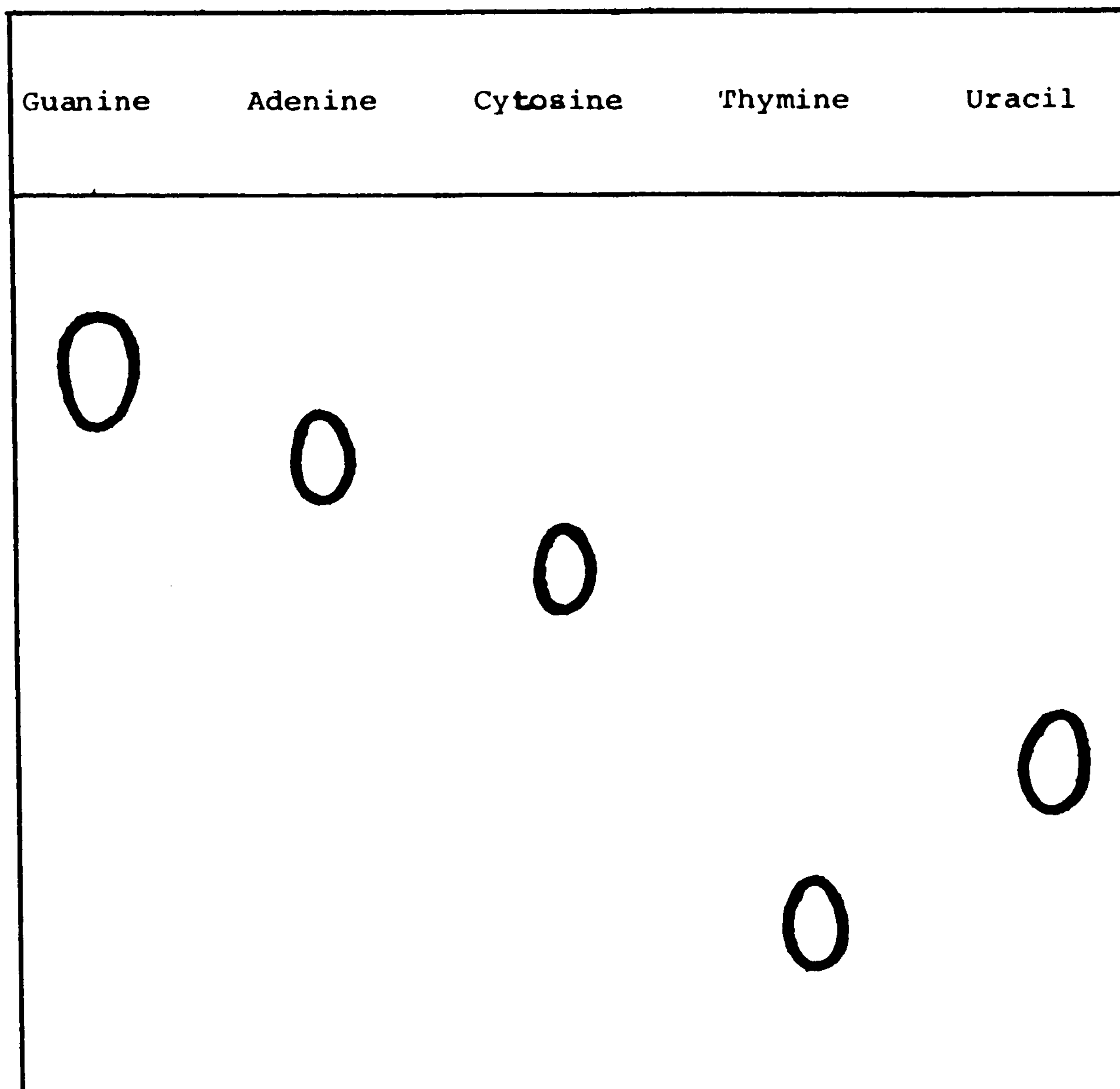


Figure 14. Diagrammatic representation of a chromatogram of a hydrolysate of the cold TCA soluble fraction + known nitrogenous bases as carriers.

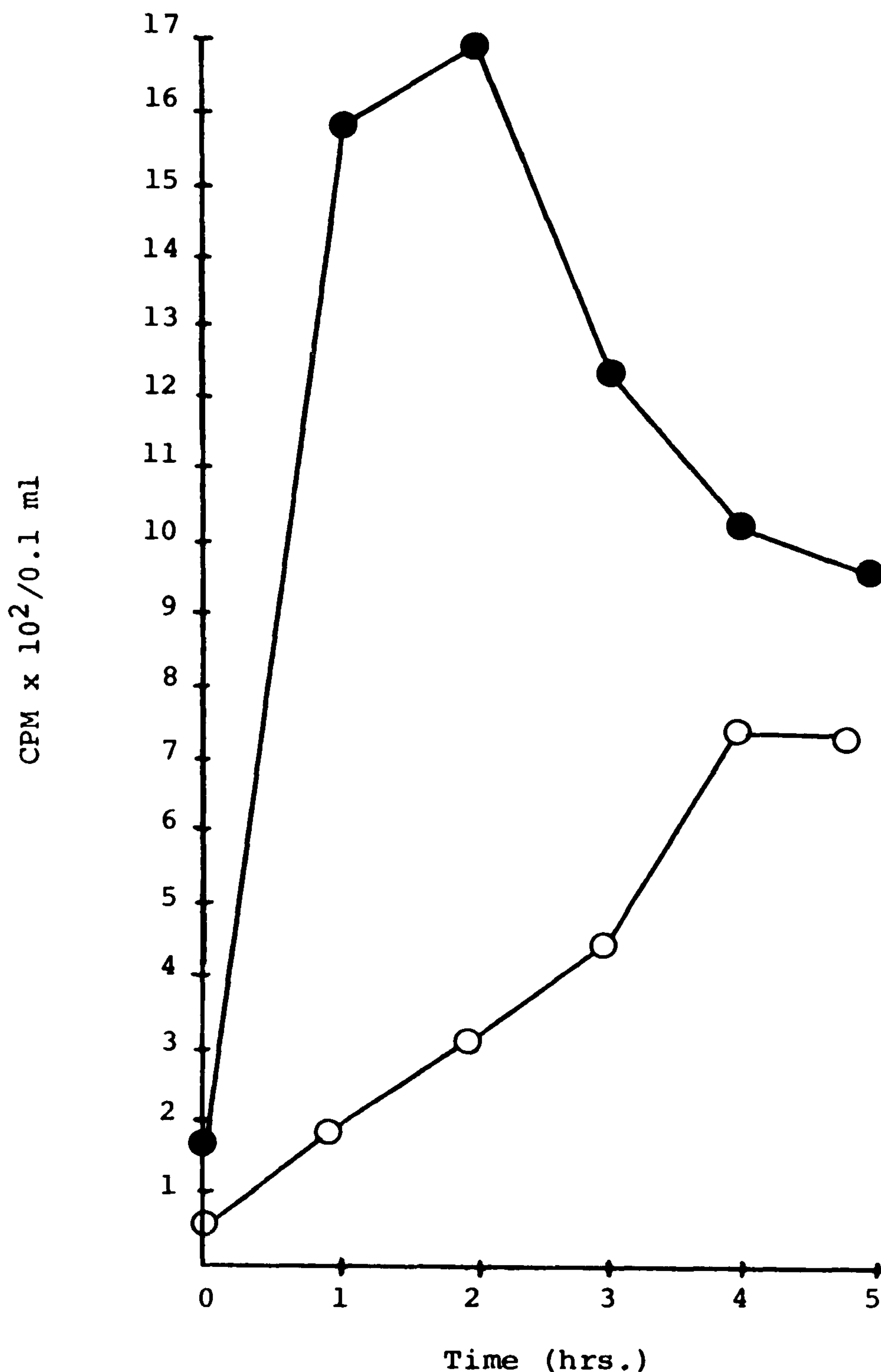


Figure 15. Levels of ^{3}H-uracil in cold TCA soluble fraction of normal RBC,●——●, and infected RBC,○——○, suspended in normal plasma during incubation with ^{3}H-uridine. Aliquots of cells were separated from the plasma and fractionated with TCA. Cold TCA soluble fractions were hydrolyzed and the resulting bases separated by paper chromatography. The areas containing the bases (as revealed by UV) were cut out and prepared for liquid scintillation counting.

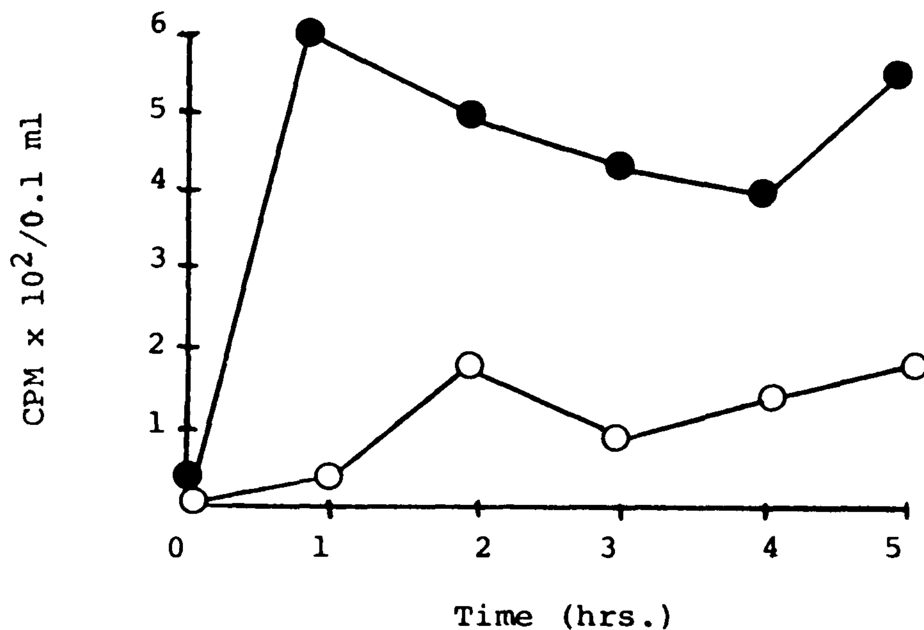


Figure 16. Levels of ^{3}H-thymine in the cold TCA soluble fraction of normal RBC,●——●, and infected RBC,○——○, suspended in normal plasma during incubation with ^{3}H-uridine. Aliquots of cells were separated from the plasma and fractionated with TCA. Cold TCA soluble fractions were separated by paper chromatography. The areas containing the bases (as revealed by UV) were cut out and repared for liquid scintillation counting.

transported more uridine into the cell. Figure 17 shows data comparing the amount of uridine taken up by the normal and infected RBC to the amount of uridine converted to thymine. It can be seen that after two hours of incubation the infected RBC were twice as efficient in converting uridine to thymine (T). The normal RBC reached the same efficiency but not until 5 hours of incubation. This increased conversion in the infected cells could be due to pyrimidine metabolism of *A. marginale* or to an activation of specific host RBC enzymes. In either case, the effect appeared to be temporary since the T:U ratio in the cells from infected calves declined rapidly after 2 hours.

Peak conversion of uridine to cytosine in the normal RBC occurred at 2 hours of incubation (Figure 18). The RBC from infected calves almost completely lacked the ability to convert uridine to cytosine. This loss of ability was also reflected in the C:U ratio in the cells of infected calves (Figure 19). It can be seen that the C:U ratio of the normal RBC was more than twice that of the RBC from infected calves. The responsible enzymes must be almost completely inhibited or repressed.

Both normal and infected RBC lacked the ability to convert pyrimidines to purines. Only small amounts of activity originally found in uridine went to adenine and quanine (Figure 20).

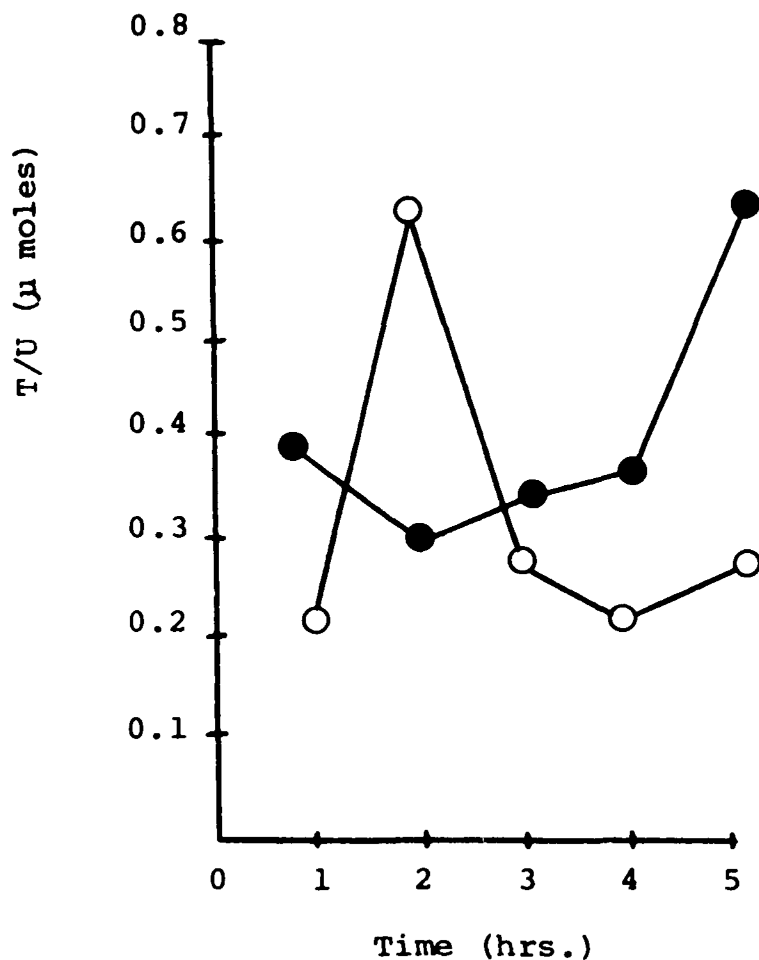


Figure 17. Ratio in μ moles of ^{3}H-thymine and ^{3}H-uracil in the cold TCA soluble fraction of normal RBC,●——●, and infected RBC,○——○, suspended in normal plasma during incubation with ^{3}H-uridine. Aliquots of cells were separated from the plasma and fractionated with TCA. Cold TCA soluble fractions were hydrolyzed and the resulting bases separated by paper chromatography. The areas containing the bases (as revealed by UV) were cut out and prepared for liquid scintillation counting.

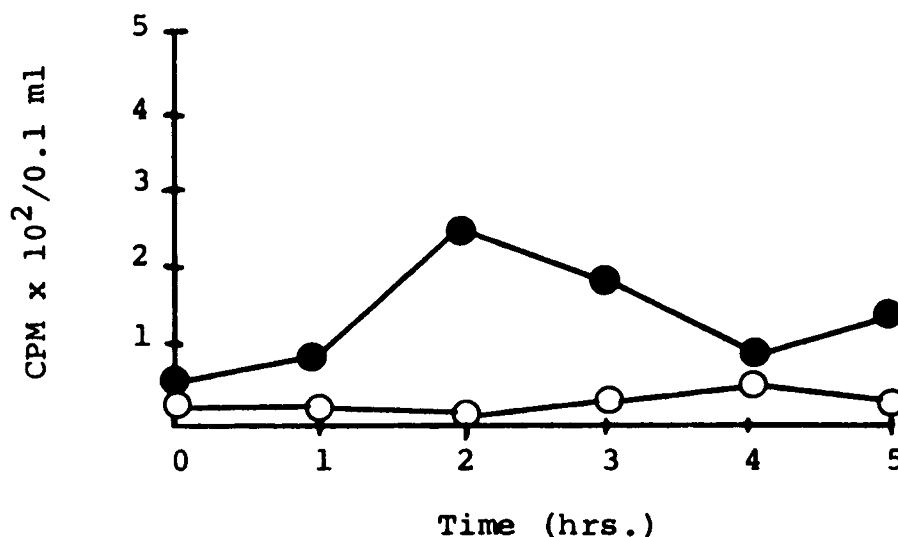


Figure 18. Levels of ^{3}H-cytosine in the cold TCA soluble fraction of normal erythrocytes,●——●, and infected erythrocytes,○——○, suspended in normal plasma during incubation with ^{3}H-uridine. Aliquots of cells were separated from the plasma and fractionated with TCA. Cold TCA soluble fraction were hydrolyzed and the resulting bases separated by paper chromatography. The areas containing the bases (as revealed by UV) were cut out and prepared for liquid scintillation counting.

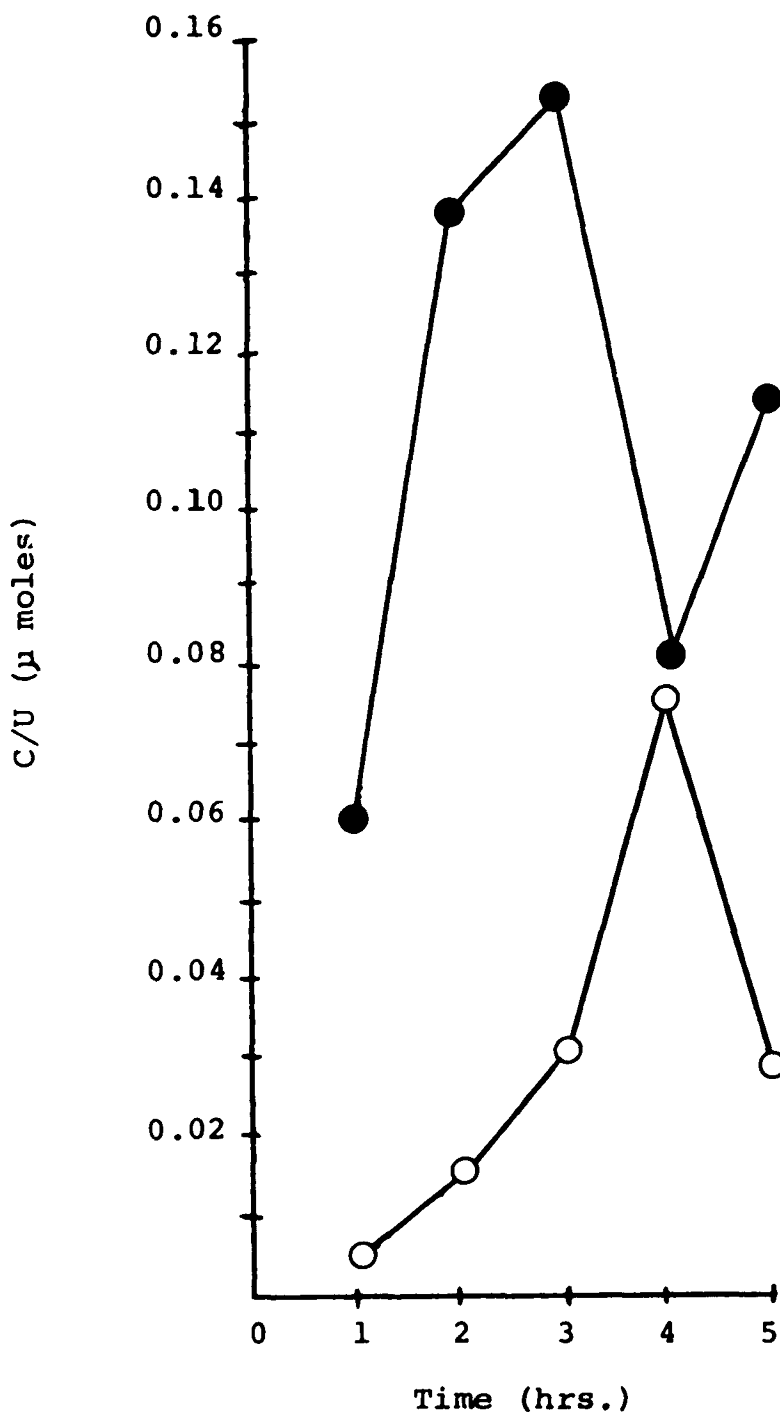
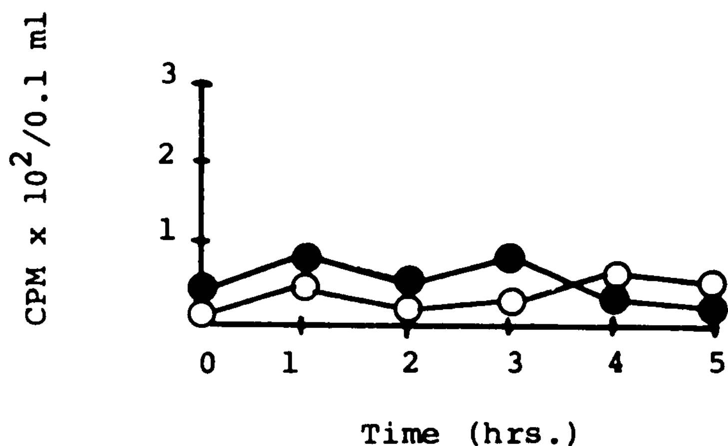


Figure 19. Ratio in µ moles of ^{3}H-cytosine and ^{3}H-uracil in the cold TCA soluble fraction of normal RBC, ●——●, and RBC from infected calves, ○——○, suspended in normal plasma during incubation with ^{3}H-uridine. Aliquots of cells were separated from the plasma and fractionated with TCA. Cold TCA soluble fractions were hydrolyzed and the resulting bases separated by paper chromatography. The areas containing the bases (as revealed by UV) were cut out and prepared for liquid scintillation counting.



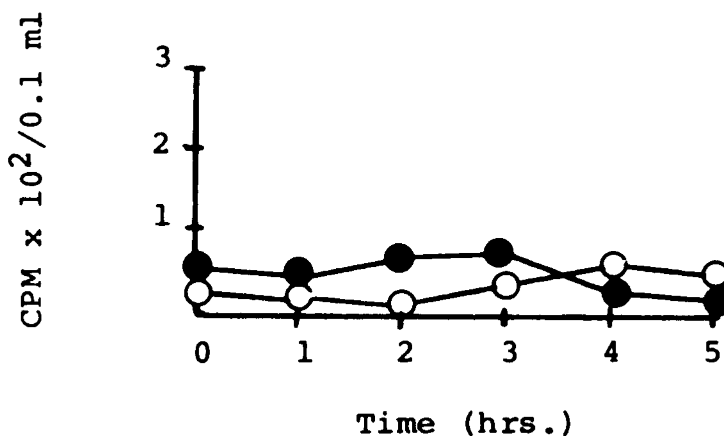


Figure 20. Levels of ^{3}H-guanine (top) and ^{3}H-adenine (bottom) in the cold TCA soluble fraction of normal erythrocytes, ●—●, and infected erythrocytes, ○—○, suspended in normal plasma during incubation with ^{3}H-uridine. Aliquots of cells were separated from the plasma and fractionated with TCA. Cold TCA soluble fractions were hydrolyzed and the resulting bases separated by paper chromatography. The areas containing the bases (as revealed by UV) were cut out and prepared for liquid scintillation counting.

E. Enzyme assay

An important step in pyrimidine biosynthesis is the condensation of orotic acid with phosphoribosylpyrophosphate (PRPP) to form orotidine-5'-monophosphate (OMP). Purified A. marginale bodies were checked for the ability to perform this condensation by assaying them for orotidine-5'-monophosphate pyrophosphorylase (OMPpase) which catalyzes the reaction. This was done by measuring the disappearance of orotic acid from the reaction mixture (orotic acid, $MgCl_2$, PRPP, and partially-purified A. marginale bodies) at 295 nm. The effect of varying amounts of A. marginale bodies on the rate of disappearance of orotic acid measured as the decrease in absorbance at 295 nm can be seen in Figure 21. The rate of decrease was small but it was reproducible and no change was observed in the absence of cell extract. The effect of omissions from the complete reaction mixture was examined (Table 2). The omission of $MgCl_2$ and PRPP reduced activity by 67% whereas the omission of PRPP alone reduced activity by 57%.

The partially-purified Anaplasma bodies apparently possessed the ability to condense orotic acid and PRPP. This is in agreement with earlier data which indicated orotic acid was incorporated into nucleic acids of infected RBC. The presence of the enzyme OMPpase in the partially-purified bodies indicated that at least part of the

Table 2. OMP pyrophosphorylase activity of partially-purified *Anaplasma marginale* bodies. The assay mixture contained 1 ml of 1 M-Tris buffer (pH 8.8); 0.2 m moles orotic acid; 0.2 m moles $MgCl_2$; 0.4 m moles PRPP; and 0.2 ml partially-purified *A. marginale* bodies. All assays were carried out at 30 C.

Experiment Number	*A. marginale* bodies (0.2 ml)	Orotic Acid	$MgCl_2$	PRPP*	% of Total Activity
1	+	+	+	+	100
2	+	+	-	-	33
3	+	+	+	+	43

*Phosphoribosylpyrophosphate

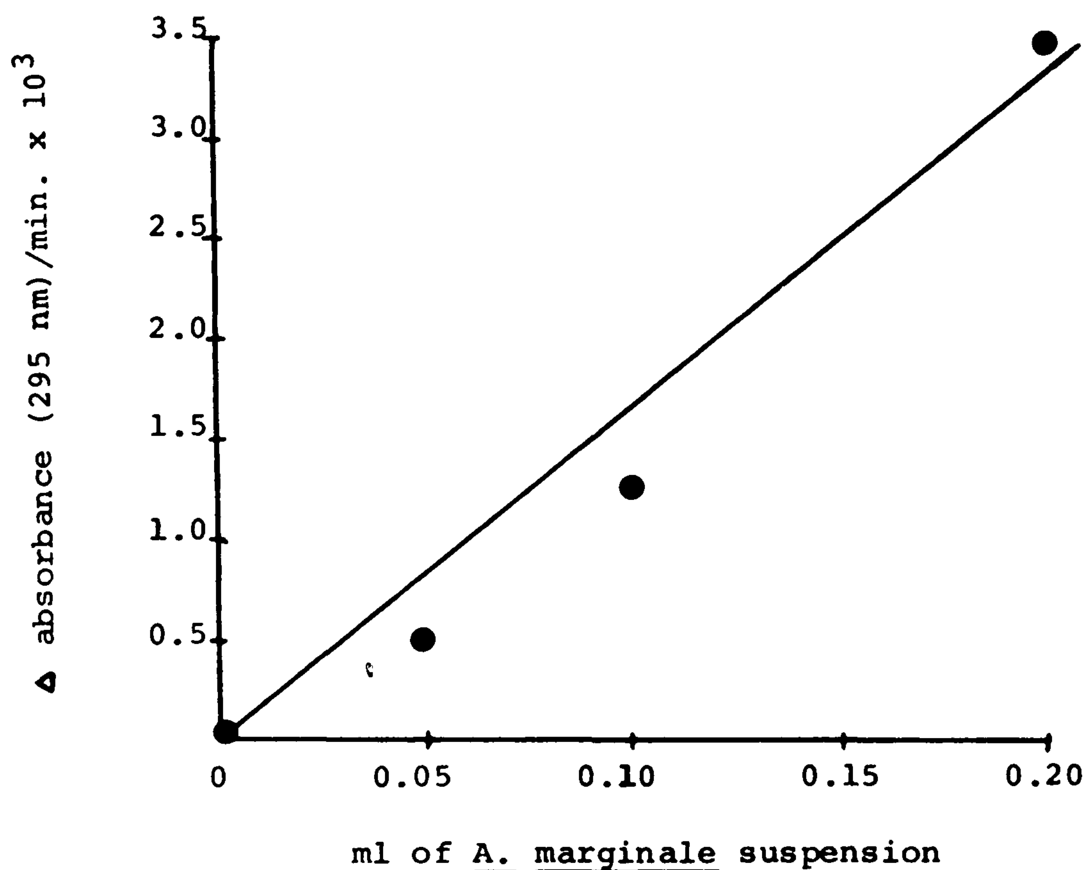


Figure 21. Effect of purified *Anaplasma marginale* bodies on the rate of disappearance of orotic acid measured as the decrease in absorbance at 295 hm. The assay mixture contained 1 ml of 1 M Tris buffer (pH 8.8); 0.2 m moles orotic acid; 0.2 m moles $MgCl_2$; 0.4 m moles PRPP, and varying amounts of partially purified *A. marginale* bodies.

Table 2. OMP pyrophosphorylase activity of partially-purified *Anaplasma marginale* bodies. The assay mixture contained 1 ml of 1 M-Tris buffer (pH 8.8); 0.2 m moles orotic acid; 0.2 m moles $MgCl_2$; 0.4 m moles PRPP; and 0.2 ml partially-purified *A. marginale* bodies. All assays were carried out at 30 C.

Experiment Number	*A. marginale* bodies (0.2 ml)	Orotic Acid	$MgCl_2$	PRPP*	% of Total Activity
1	+	+	+	+	100
2	+	+	-	-	33
3	+	+	+	+	43

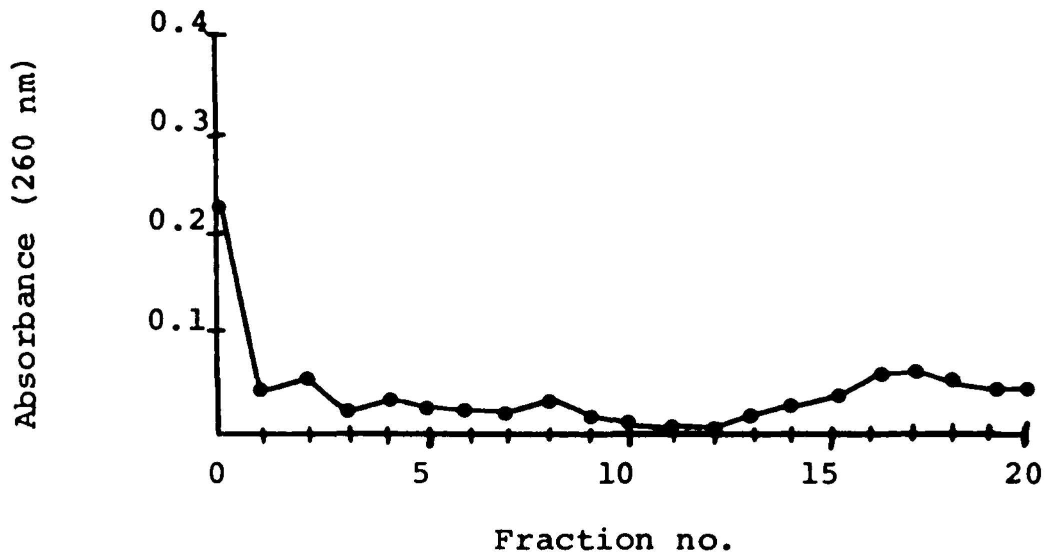
*Phosphoribosylpyrophosphate

enzymatic capabilities for pyrimidine synthesis resided in the parasite itself. However, one could speculate that contaminating erythrocytic membrane may be contributing to this activity.

F. DNA isolation and sucrose density gradient centrifugation

Using ultracentrifugation on CsCl density gradients, Senitzer (1969) found two species of DNA associated with *A. marginale* infected RBC. One had a buoyant density of 1.700 and the other a buoyant density of 1.715. Ultracentrifugation of DNA isolated from bovine RBC at 0% infection resulted in the banding of only one species of DNA. It corresponded to a density of 1.700 and was concluded to be bovine in origin. The more dense DNA found in the infected RBC appeared to be that of *A. marginale*.

In this study a comparison was made of DNA species of RBC from *A. marginale*-infected calves and RBC from chemically-induced anemic calves. DNA was isolated from the RBC using the phenolic extraction procedure of Thomas *et al*. (1966) previously described in Materials and Methods. DNA preparations were centrifuged on 5-20% neutral sucrose gradients at 32,644 x g for 3.5 hours. The gradients were fractionated and each fraction measured at 260 nm. The results of this procedure can be seen in Figure 22. Centrifugation of a DNA preparation from RBC infected calves resulted in two nucleic acid species. One



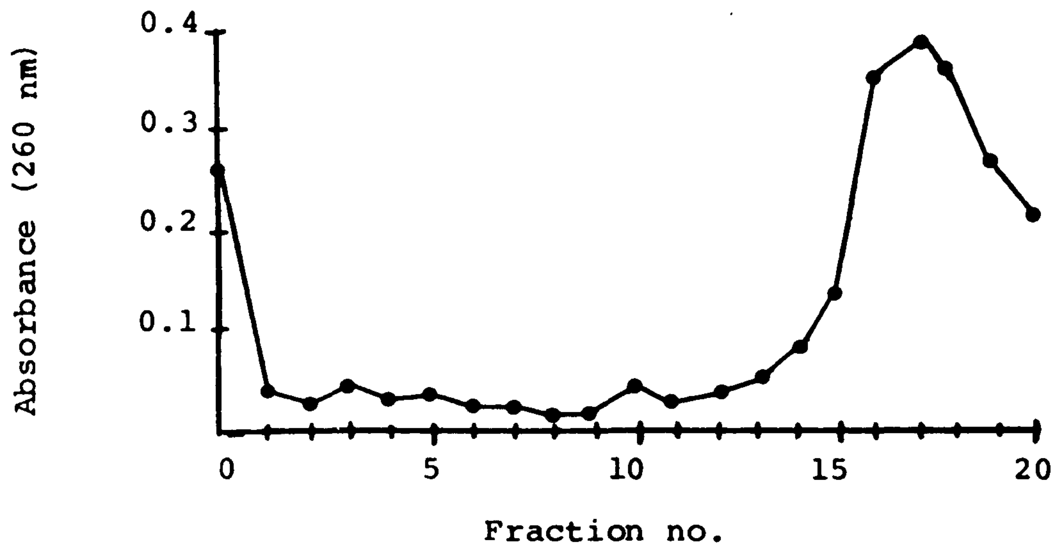


Figure 22. Sucrose gradient centrifugation of DNA from *A. marginale* infected RBC (bottom) and DNA from RBC of a chemically induced anemic calf (top). The 5-20% sucrose density gradients were centrifuged at 20,000 rpm in an SW-39 rotor (av 32644 x g).

was sedimented and the other was located near the top of the gradient. One of these DNA species was probably bovine in origin. The second could be due to the presence of DNA in A. marginale or possibly DNA from nuclear material found in certain RBC associated with the anemia condition. In order to clarify this, DNA was extracted from RBC of a chemically-induced anemic calf and subjected to the same sucrose gradient centrifugation procedure. The results of this experiment can also be seen in Figure 22. Approximately the same amount of DNA was sedimented but, only a small amount was present near the top of the gradient. DNA species found in the upper band of the sucrose density gradient appeared to be associated with the Anaplasma infection whereas the sedimented species probably came from contaminating white blood cells or nucleated immature RBC associated with the anemic condition.

G. RNA isolation and sucrose gradient centrifugation

There have been conflicting reports on the presence of RNA associated with A. marginale. Histochemical procedures were used to demonstrate the presence of RNA in A. marginale (Moulton and Christensen, 1955). Gough (1963) reported evidence of RNA in infected RBC but Ellender and Dimopoullos (1967) and Senitzer (1969) could find none in marginal bodies.

In this work a hot phenol extraction procedure was used in an attempt to isolate RNA associated with *A. marginale*-infected RBC. This method was reported by Larsen and Frederiksen (1972) as being useful in extracting a large percentage (including small molecular weight RNA) of the total cellular RNA. RNA was extracted from RBC of *Anaplasma*-infected calves and from RBC of a chemically-induced anemic animal. The extractions were centrifuged on 5-20% sucrose gradients at 36,114 x g for 14 hours. After fractionation each sample was measured at 260 nm. The results of subjecting RNA preparations from RBC at peak infection to this procedure is shown in Figure 23. One large peak was found at the bottom of the gradient, a smaller peak was found near the top and several minute peaks in the center of the gradient. The same RNA preparations were also incubated with DNase and subjected to the same procedure. This resulted in the disappearance of the minute bands in the center of the gradient (Figure 23). The peaks at the bottom and top of the gradient were probably RNA. At this point it would be impossible to say whether or not this RNA is associated with the *Anaplasma* infection.

RNA preparations from RBC of a chemically-induced anemic calf were subjected to the same centrifugation and extraction procedure. The results of this procedure are illustrated in Figure 24. Peaks were found at the bottom

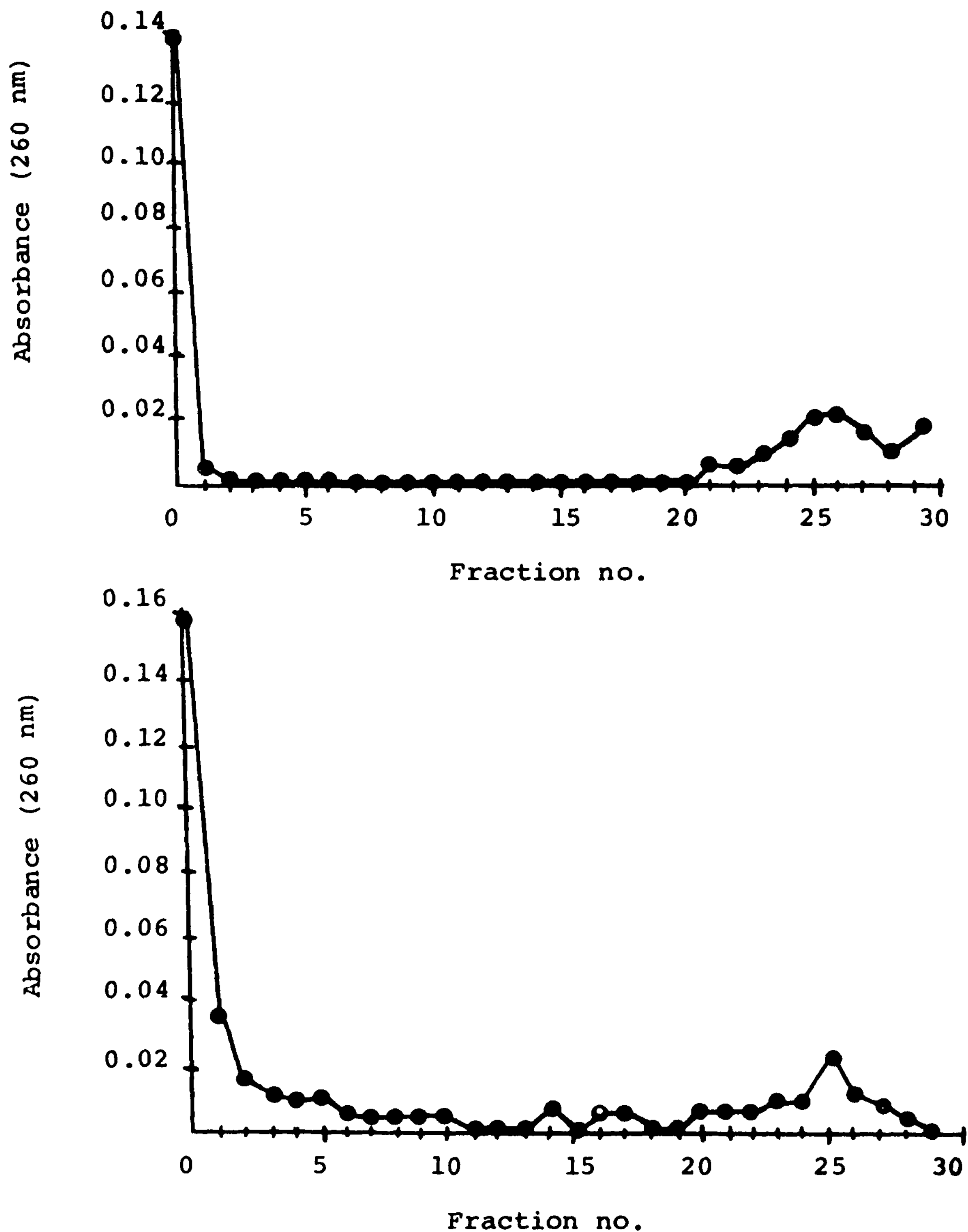


Figure 23. Sucrose gradient centrifugation of RNA, before (bottom) and after (top) incubation with DNase, from *A. marginale* infected RBC. The 5-20% sucrose density gradients were centrifuged at 21,000 rpm in an SW-39 rotor (av 36114 x g).

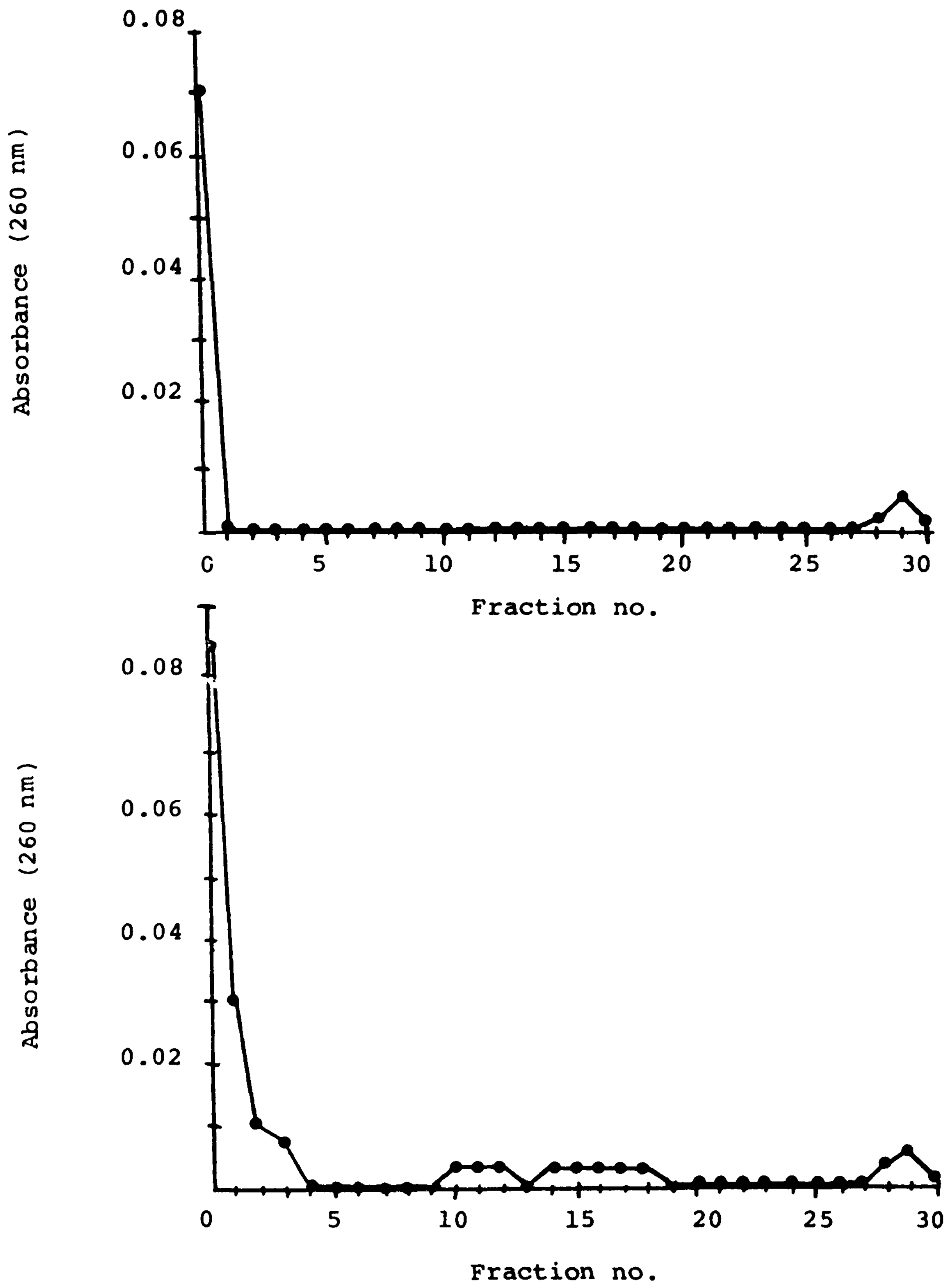


Figure 24. Sucrose gradient centrifugation of RNA from RBC of chemically induced anemic calf before (bottom) and after (top) incubation with DNase. The 5-20% sucrose density gradients were centrifuged at 21,000 rpm in an SW-39 rotor (av 36114 x g).

and top of the gradients and several in the middle. The middle peaks disappeared upon treatment with DNase (Figure 24). These results are identical to the ones obtained with RBC from A. marginale-infected calves.

The middle bands must have been due to contaminating bovine DNA. The remaining peaks appeared to be RNA but the question still arises, "Are they associated with the Anaplasma infection?" Both peaks were reduced by 50% in RNA preparations from the RBC of the chemically-induced anemic calf, but they appeared at the same position in the gradients. It, therefore, appeared that the majority of the RNA isolated from infected RBC by this procedure was bovine in origin. A vegetative organism should contain large quantities of RNA (rRNA, mRNA, and tRNA). These data point to the fact that the A. marginale bodies found in mature erythrocytes might not be vegetative forms but a resting form. Perhaps the actual replicating form resides in the immature RBC and cannot be observed with conventional staining techniques. One also cannot rule out the possibility that the vegetative body is located in the bone marrow and is never present in circulating RBC. Data presented earlier (Results and Discussion, Section C) tended to give plasma from infected calves a role in active infection which cannot be overlooked.

H. Electron microscopy

Senitzer (1969) extracted DNA from partially-purified *A. marginale* bodies and observed the nucleic acid molecules with electron microscopy. In this study DNA was extracted from RBC of *Anaplasma*-infected calves and electron micrographs were taken of the molecules utilizing a modification of the technique of Mosharrafa *et al*. (1970). An attempt was made to locate two species of DNA (bovine and *A. marginale*) in the electron micrographs. In the majority of the micrographs numerous strands of linear DNA could be seen (Figure 25). However, there appeared in a few micrographs small molecules of circular DNA (Figure 26). The micrographs did not reveal any information as to whether the molecules were single- or double-stranded.

The circular DNA observed corresponded to the circular DNA Senitzer (1969) found associated with partially-purified *A. marginale* bodies. Since only two species of DNA (*A. marginale* and bovine) have been reported in *A. marginale*-infected RBC, it was assumed that the linear molecules were bovine in nature.

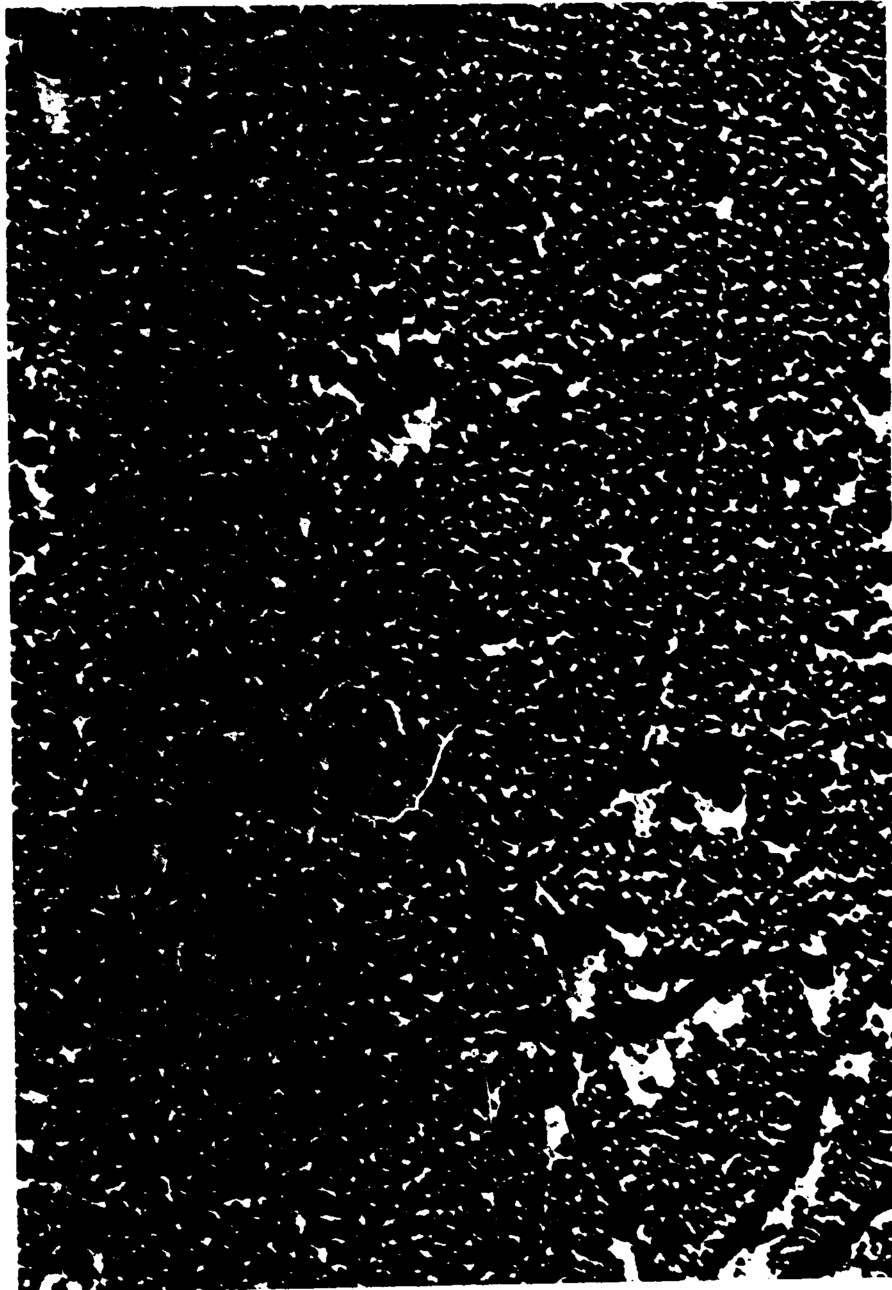


Figure 25. Electronmicrograph of linear DNA isolated from A. marginale-infected RBC.

Figure 26. Electronmicrograph of circular DNA isolated from *A. marginale*-infected RBC.

Summary and Conclusions

A. *In vitro uptake of labeled precursors*

In most experiments packed RBC from normal and infected calves were suspended in cell free normal plasma. In one experiment normal cells were resuspended in infected plasma. The appropriate radioactive precursor, ^{14}C-hypoxanthine, ^{14}C-orotic acid, ^{14}C-sodium bicarbonate, ^{14}C-sodium formate, or ^{3}H-uridine, was added. Uptake of precursor was followed by measuring the disappearance of activity from the plasma. RBC were also fractioned using cold and hot TCA fractionation. In all cases disappearance of activity from the plasma was inhibited when RBC from infected calves were being incubated. When incubated with plasma from infected calves, normal RBC remove ^{3}H-uridine from the plasma in a manner similar to RBC from infected calves. Sodium bicarbonate and sodium formate were not taken up by either normal RBC or RBC from infected calves. With all precursors taken up, levels of activity reached a higher peak in the normal RBC than in the RBC from infected calves. Uridine was readily incorporated into the hot TCA soluble fraction of the normal RBC. No uridine was incorporated into this fraction of either the RBC from infected calves or normal RBC which

were suspended in infected plasma. Normal and infected RBC accumulated radioactivity from orotic acid in the hot TCA soluble fraction. However, the RBC from the infected calves accumulated a larger amount of the orotic acid than the normal cells.

These data indicate that the *Anaplasma marginale* infection inhibited the transport of most of the compounds surveyed into the RBC. Normal RBC and those from infected calves apparently lacked a transport mechanism for sodium bicarbonate and sodium formate.

Because of the incorporation of radioactivity into the hot TCA soluble fraction, the nucleated RBC of normal RBC suspensions apparently used uridine in the synthesis of nucleic acids. The RBC from infected calves showed a greater ability to use orotic acid for nucleic acid synthesis. Plasma from infected animals demonstrated the ability to transform normal RBC into RBC with properties similar to infected cells. This could have been due to an unknown form of *A. marginale* or some other unknown factor present in the plasma.

B. *In vitro incorporation of label into individual nitrogenous bases in RBC from normal and infected calves*

Following incubation of the normal RBC and those from infected calves with ^{3}H-uridine, the cold TCA soluble fraction was hydrolized with hot HCl. More

activity was found associated with thymine in the normal RBC than in those from infected calves. However, when the total amount of activity entering the RBC was compared to the amount of activity going to thymine, it appeared that the RBC from infected calves were more efficient than the normal RBC. More activity was found associated with cytosine in the normal RBC than in the RBC from infected calves. The ratio of ^{3}H-uracil and ^{3}H-cytosine in the RBC also showed the normal RBC to be more effective in transferring activity from uridine to cytosine. No activity was found associated with adenine and guanine in RBC from normal and infected calves.

These data indicate that RBC from infected calves were more efficient in converting uridine to thymine than normal RBC. But, RBC appeared to completely lose the ability to form cytosine from uridine once infected with *Anaplasma*.

C. *In vitro enzyme assay*

Orotidine-5'-monophosphate pyrophosphorylase (OMPpase) activity in partially-purified bodies was assayed for by measuring the disappearance of orotic acid from the incubation mixture at 295 nm with a spectrophotometer. The decrease in optical density with time was small but could be repeated.

These data indicate that some OMPpase activity was associated with *A. marginale*. It therefore appeared that

Anaplasma had at least part of the enzymatic capabilities to synthesize pyrimidines.

D. DNA and RNA isolation

DNA was isolated from RBC of *A. marginale*-infected calves and from RBC of a chemically-induced anemic calf. These DNA preparations were centrifuged on sucrose density gradients. Preparations from RBC of *A. marginale*-infected calves revealed two bands of DNA on the sucrose gradients whereas preparations from the chemically-induced anemic RBC revealed only one band of DNA. The lightest band was assumed to be associated with *A. marginale* and not associated with immature RBC present because of the anemic condition.

Hot phenol was used in an attempt to isolate RNA from *Anaplasma*-infected RBC and from RBC of a chemically-induced anemic calf. All RNA isolated from infected-RBC using this procedure was probably bovine in origin.

The lack of RNA associated with *A. marginale* indicated that the organism was not replicating during the *in vitro* incubation.

E. Electron microscopy

DNA was isolated from RBC of *A. marginale*-infected calves and prepared for electron microscopic observation. Two species of DNA, one circular and one linear were revealed.

Two species of DNA have been reported in *Anaplasma*-infected RBC (*A. marginale* and bovine). Circular DNA has been isolated from partially purified bodies. The linear DNA seen in this experiment was therefore assumed to be bovine. The circular forms were probably from *A. marginale*.

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Vita

Michael Edward Billups was born in Lake Charles, Louisiana, on September 26, 1944. He graduated from LaGrange Senior High School in 1962, entered McNeese State College, and received his Bachelor of Science degree in Chemistry in May, 1968.

In September, 1968, he entered the Graduate School of McNeese State College and received the Master of Science degree in Microbiology in August, 1970. He entered the Graduate School of Louisiana State University in September, 1970, and was appointed on a National Institutes of Health Research Grant. His major field of study was Microbiology with a minor in Biochemistry. He is presently a candidate for the Doctor of Philosophy degree.

EXAMINATION AND THESIS REPORT

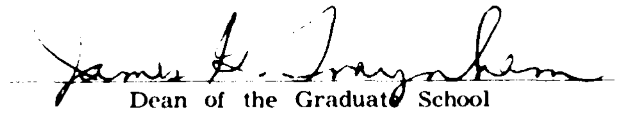
Candidate: Michael E. Billups

Major Field: Microbiology

Title of Thesis: A Comparison of In Vitro Nucleic Acid Metabolism of Normal Bovine RBC and RBC from Calves Infected with Anaplasma marginale.

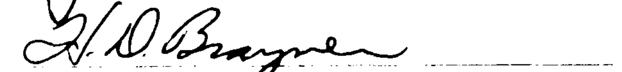
Approved:

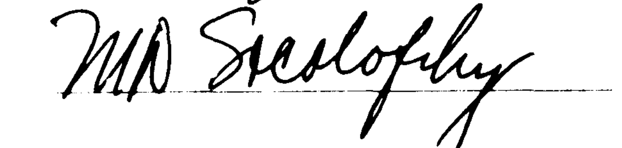
Major Professor and Chairman



Dean of the Graduate School

EXAMINING COMMITTEE:





Date of Examination:

September 28, 1973