ATPase Activity of the Erythrocytic Membrane in Anaplasmosis And of *Anaplasma marginale*.* (32079)

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The use of ATPase as an expression of the active transport mechanism has been validated by several investigators(1-4). By definition, active transport refers to those processes in which the accelerated transfer of molecules and ions through membranes occurs against either an electrochemical potential or activity gradient. Furthermore, active transport has been shown to depend upon the availability of high-energy phosphates(5,6) and is subject to interference by inhibitors of glycolysis(7).

Following early reports of alterations in the phospholipid content of erythrocytic stromata during various anemias(8,9) numerous investigations have shown decreased concentrations of phospholipids during anaplasmosis (10-12). These alterations have been related to changes in osmotic fragility of the erythrocyte(13).

Thus, experiments were designed to isolate this active transport from simple diffusion by use of ATPase enzyme activity as a monitor and to determine changes which might occur as a result of infections of *Anaplasma marginale* in calves.

Methods and materials. Experimental animals. Splenectomized calves were maintained as described by Dimopoullos *et al*(14). Experimental anaplasmosis was produced by intravenous inoculation of calves with 50 ml of whole infected blood. Uninoculated, splenectomized calves were maintained similarly and used as controls.

Hematology. Blood samples were collected periodically into tubes containing dipotassium salt of ethylenediamine tetraacetic acid (EDTA) as anticoagulant. Blood smears were prepared from each sample and stained with Giemsa stain to detect the presence of *A. marginale*. Erythrocyte counts and packed cell volumes (PCV) were determined in addition to the percentage of infected erythrocytes.

Preparation of stromata. Approximately 100 ml of blood was collected periodically from each calf in EDTA and centrifuged at 1060 $\times$ *g* for 20 minutes at 4°C to separate the plasma from the erythrocytes. The plasma, buffy coat and the top one-third portion of the packed cells were removed to minimize the effects of increased reticulocyte ATPase activity during the anemic phase of anaplasmosis(15). The remaining erythrocytes were washed 3 times with cold Michaelis' buffer(16) at pH 7.4 and hemolyzed with 10 volumes of cold 1 $\times$ 10^{-4} M EDTA solution (pH 7.4). The resulting hemolysate was centrifuged at 27,000 $\times$ *g* for 30 minutes at 4°C and the sedimented ghosts subsequently washed with cold Michaelis' buffer until free of hemoglobin and finally resuspended in 2 volumes of buffer.

Preparation of marginal bodies. Six liters of blood were obtained by exsanguination of several animals showing a high percentage of infected erythrocytes (>60%). The cells were washed 5 times in cold Michaelis' buffer and suspended in an equal volume of the same buffer. Plasma and buffy coat were discarded. Cells were hemolyzed by sonic vibration using a Model LS75 Branson Sonifier at 7-9 amperes for 90 seconds at 4°C. After the unhemolyzed erythrocytes and remaining cellular debris were removed by slow speed centrifugation (1080 $\times$ *g* for 10 minutes at 4°C), the marginal bodies were collected by centrifugation at 27,000 $\times$ *g* for 30 minutes at 4°C, while the supernatant fluid, which contained some ghosts, was discarded. The crude marginal body preparation was washed

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with cold Michaelis' buffer until free of hemoglobin and quantitated by the gravimetric determination of 1 ml of preparation dried in a desiccator.

One portion of the marginal body preparation was used immediately in an assay of ATPase activity, while another portion was subjected to sucrose density-gradient sedimentation procedures previously described(17). A 3-ml aliquot of the marginal body preparation was carefully layered over the gradient, and the tubes centrifuged at 50,000 $\times$ *g* for 90 minutes at 4°C using a Spinco Model SW-25.1 swinging bucket rotor. The layers were carefully separated, washed free of sucrose solution with cold Michaelis' buffer and used immediately for an assay of ATPase activity. Smears were prepared of all separated layers and examined microscopically after staining with Giemsa stain.

ATPase assay. A 1.5-ml aliquot of the test suspension was added to a flask containing 6.0 ml of the substrate mixture at 37°C. Assay was based on method of Parker and Hoffman(18) in which a 2-ml portion of the incubation mixture was removed at 0, 60 and 120 minutes and placed in an equal volume of 6.0% $HClO_3$ in order to stop the reaction. The PO_4 released was determined by the Fiske-Subbarow(19) method. Units of ATPase activity were uniformly expressed as millimicromoles of inorganic PO_4 produced per hour per milligram of dry stromata.

Results and discussion. After initial investigations had confirmed that bovine plasma possessed no ATPase activity, subsequent studies involved assays of erythrocytic membranes and partially-purified marginal bodies. A comparison of stromatal ATPase activity of 3 *Anaplasma*-infected calves and an uninfected animal is shown in Fig. 1. The 3 animals were inoculated with infected whole blood as previously described on experimental day 7. Days 0 to 6 represent pre-inoculation values. Marginal bodies first appeared in blood cells on day 9 while peak infection was noted on experimental day 18, 11 days after inoculation. Maximum marginal body counts reached 53, 60 and 65% for the 3 infected animals. It is significant that increases in stromatal ATPase activities occurred at the

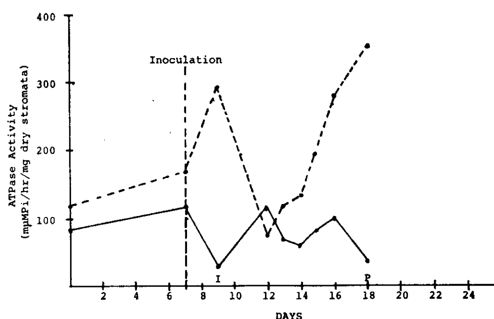

FIG. 1. Comparison of stromatal ATPase activity of uninfected and *Anaplasma*-infected calves. ——— Uninfected animal; - - - - Mean ATPase activity for 3 *Anaplasma*-infected animals; I - Initial appearance of margina bodies; P-Peak infection.

time of initial appearance of marginal bodies in peripheral blood and at the peak of *Anaplasma* infection. The stromatal ATPase activity of the uninfected animals does not show such marked changes on these days. These results would imply that the etiologic agent of anaplasmosis is entirely capable of producing a marked effect on such energy-dependent systems of the cell as active cation transport. However, this is not to rule out the possibility that active transport, in general, and ATPase activity, in particular, are not, in turn, affected by the concentrations of these same cations(20).

In view of the increased ATPase activity noted in the infected erythrocytes, the possibility of enzyme activity in the *Anaplasma* bodies *per se* was investigated. The ATPase activity of the previously described marginal body preparation, as well as the activity of fractions of this preparation obtained by sucrose density-gradient sedimentation is presented in Fig. 2. In addition, the content of

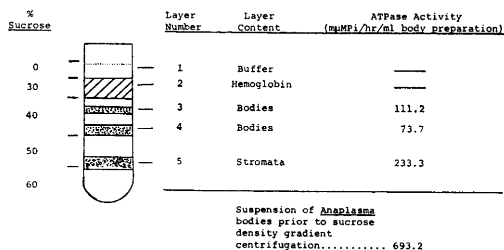

FIG. 2. ATPase activity from fractions of partially-purified *Anaplasma* bodies and erythrocytic components obtained by sucrose density-gradient centrifugation. Enzyme activity reported in millimicromoles of inorganic phosphorus released per hour per milliliter of sample.

each layer as determined by microscopic examination is given. Layers 1 and 2 represent buffer and hemoglobin fractions, respectively, and were free of particulate matter and ATPase activity. Layers 3 and 4 contained purified marginal bodies. These fractions have been shown to contain the highest complement-fixing antigenic activity for *A. marginale* (17), and from data presented here, significant ATPase activity is also noted. Layer 5 was found to contain a very dense suspension of stromatal material remaining throughout the purification procedures. As would be expected of such a dense suspension of erythrocytic membranes, a high level of ATPase activity was found in this fraction(2,21). The apparent loss of ATPase activity from the value shown for the partially-purified preparation used in the density-gadient fractionation (693.2) and the sum of the fractions obtained (418.2) may be attributable to a dilution effect in the fractionation procedure.

These data suggest that the increase obseved in erythrocytic ATPase activity as the infection progresses is due wholly or in part to proliferation of ATPase-containing *Anaplasma* organisms in the cell. The primary physiological function of an active transport system is the protection of the flexible and fragile erythrocytic membrane from the osmotic pressures of the substances which it encloses(22). Furthermore, since ATPase activity has been convincingly linked to this active transport system(2,23-25), disturbances in ATPase activity during anaplasmosis could conceivably impair proper erythrocyte function in the infected animal by rendering it osmotically fragile and less able to adapt to stress.

It seems apparent then from results obtained that *A. marginale* exerts a definite influence upon the metabolism of the erythrocytic membrane and that this influence is manifested in the energy-dependent system of the cell. The data presented in these experiments, however, do not sugggest the manner by which *A. marginale* exerts its effect upon metabolism and energy production. Further study into the metabolism of the isolated organism will be necessary before these and other questions become answerable.

Summary. The erythrocytic ATPase activity of uninfected calves and calves infected with *Anaplasma marginale* was determined. Samples from infected animals showed increased ATPase activity during the initial appearance of marginal bodies in the erythrocytes and at the peak of infection. In view of this increase the possibility of enzyme activity in the *Anaplasma* bodies *per se* was investigated. Marginal bodies were purified and subjected to sucrose density-gradient sedimentation procedures. It was demonstrated that fractions containing purified marginal bodies possessed significant ATPase activity. The possible relationship between this activity and the pathology of anaplasmosis was discussed.

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Maximum Biliary Excretion of Bilirubin and Sulfobromophthalein During Anesthesia-Induced Alteration of Rectal Temperature.* (32080)

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The influence of temperature on various physiological functions such as bile flow, biliary bilirubin concentration and the excretion of dyes such as sulfobromophthalein (BSP) has been recognized for many years(1). Brokaw and Penrod(2) found that BSP uptake from the blood of dogs was diminished during hypothermia. Archdeacon *et al*(3) showed that in the anesthetized rat low temperature exerted a definite inhibitory effect on bile production. Brauer *et al*(4) using intact rats and isolated perfused rat livers have shown that bile flow and the excretion of BSP falls during hypothermia, while there are only rather minor changes in other bile constituents.

The impairment of thermoregulation as a consequence of anesthesia and/or restraint has also been reported(5,6). It would be expected, therefore, that anesthesia-induced hypothermia could reduce the functional capacity of the liver. Anesthesia, as well as restraint, is routinely employed in experiments in which maximum excretion patterns of the liver are determined. The intent of this report is to demonstrate the magnitude of thermoregulatory loss which can occur under conditions employed in maximum biliary excretion (Tm) studies, and to establish the effect of such changes in body temperature on the apparent Tm of bilirubin and BSP.

Materials and methods. Male, Swiss-Webster mice (35-40 g) and male, Sprague-Dawley rats (300-400 g) were used throughout the study. The animals were housed 10 per cage and maintained unrestricted on laboratory diet and tap water until use. Bilirubin (Sigma Chemical Co., St. Louis, Mo.) for infusion in the rat was prepared by dissolving 40 mg of bilirubin in 10 ml of isotonic Na_2CO_3-NaCl solution (0.5 g Na_2CO_3, 0.53 g NaCl per 100 ml). For the mouse 50 mg of bilirubin was dissolved in 10 ml of isotonic solution and the pH adjusted to approximately pH 8 with 5 N HCl. The bilirubin solutions were infused at a rate of about 0.07 and 0.02 ml/min in rats and mice respectively. BSP (Hynson, Wescott and Dunning, Baltimore, Md.), 50 mg/ml, was diluted in sufficient 0.9% NaCl to allow an infusion of about 0.03 ml/min.

Rectal temperature experiments. Rectal temperatures were continuously recorded prior to and following pentobarbital sodium-induced anesthesia (rats, 45 mg/kg, i.p.; mice, 80 mg/kg, i.p.) using a thermistor probe (YSI 401) and a Grass Polygraph recorder. Arterial blood pressure was also measured in these animals using a Statham pressure transducer (P23AA).

Biliary excretion experiments. Rats and mice were anesthetized with pentobarbital sodium (rats, 45 mg/kg, i.p.; mice, 80 mg/kg, i.p.) and restrained in the supine position on a metal surgical board. The bile duct was then cannulated with PE-10 tubing. Infusions were accomplished by cannulation of the femoral vein in the rat (PE-50) and the external jugular vein in the mouse (PE-10). In the rat, bilirubin was infused at a rate of 0.3 mg/min following a priming dose of

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