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THE ROLE OF PURINES AND PYRIMIDINES IN BOVINE RED BLOOD CELL METABOLISM

by

Michael John Seider

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I hereby recommend that this dissertation prepared under my direction by Michael John Seider

entitled THE ROLE OF PURINES AND PYRIMIDINES IN BOVINE RED BLOOD CELL METABOLISM

be accepted as fulfilling the dissertation requirement for the degree of Doctor of Philosophy

Dissertation Director — Sept. 19, 1977 Date

As members of the Final Examination Committee, we certify that we have read this dissertation and agree that it may be presented for final defense.

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TABLE OF CONTENTS

TABLE OF CONTENTS--Continued

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS--Continued

LIST OF TABLES

ABSTRACT

Cow red cells, under in vitro incubation conditions, exhibit a comparatively low glycolytic rate of 0.56 ± 0.05 μmoles/ml of packed cells/hr (μmoles/ml cells/hr) with a ratio of lactate formed to glucose consumed of 1.58. I have found that this low glycolytic rate can be stimulated 50-60% above the basal level in the presence of a variety of purine and pyrimidine compounds including adenosine, inosine, hypoxanthine, xanthine, and uracil. In contrast, calf red cells, which have a much higher basal glycolytic rate, are affected to a much lesser extent. To ascertain the mechanism by which this stimulation takes place in cow red cells, both glucose transport and glycolytic enzyme activities were determined in the presence of these stimulators. Glucose influx in cow red cells, which exhibits both a low K_m of 117 μM and a V_{max} of 0.38 μmoles/ml cells/minute, was unaltered in the presence of adenosine. On the other hand, hexokinase, which in normal hemolysates of cow red cells has an activity of 0.49 ± .03 μmoles/g Hb/min. was found to be stimulated to 0.73 μmoles/g Hb/min. in the presence of adenine. In contrast, both pyruvate kinase and phosphofructokinase were unaffected by these stimulators. These data suggest that certain purines and pyrimidine compounds may exert their stimulatory effect on hexokinase activity resulting in an augmentation of cow red cell glycolysis.

Cow red cell glycolysis, which can be stimulated by a variety of purines and pyrimidines, was also found to be augmented by its own plasma. Dialyzed or charcoal-treated plasma can no longer stimulate glycolysis, suggesting that the stimulating factors could be purines or pyrimidines. Determination of the purines and pyrimidines in plasma revealed the presence of xanthine (.31 μM), hypoxanthine (.60 μM), and adenosine (.05 μM) as well as two unknown compounds. However, each of the known purines exists in concentrations too low to play the stimulatory role. On the other hand, it would seem plausible that together all the plasma compounds, including the unknowns, could act synergistically to produce the elevated glycolytic rate. In other species, including dog, cat, rabbit, rat, guinea pig, human as well as calf, plasma stimulation of glycolysis was not observed. Moreover, exchanging calf and cow plasmas produced no stimulatory effect on either calf or cow red cell glycolysis, suggesting that: (a) calf red cells lack the cellular components which respond to this stimulator; and (b) only cow plasma contains this specific stimulator.

CHAPTER 1

INTRODUCTION

Although some may consider red blood cells an adaptation restricted to vertebrates, cells responsible for oxygen transport exist in many different phyla (Scott 1966). As animal size increases and its organization becomes more complex, the need arises for a more efficient mode of oxygen transport. Thus, almost all vertebrates use red blood cells, with two notable exceptions. These are the larva of the European eel (Anguilla) and several species of the family Chaenichthyidae, the Antartic ice fish (Ruud 1965), which survive with neither hemoglobin nor red cells. Lacking an obvious mechanism for transporting oxygen does not appear to affect the ice fish, for they grow to lengths of two feet without suffering any apparent harm. This fish may survive by first living in waters cold enough to depress metabolic function, and second, by adapting its morphology to allow increased diffusion of oxygen from the skin and gills to the blood (Ruud 1965).

With the exception of the mammals and the lungless salamander (Batrachoseps) (Emmel 1924), most vertebrate cells are nucleated, ranging in diameter from 3 to 60 μm. In most cases, the hemoglobins contained within these cells have four subunits and four hemes per molecule, although hagfish possess a monomeric form of hemoglobin with one heme per molecule (Scott 1966). Hemoglobins of different species differ in their physical, chemical and biological properties, and can,

in fact, exist as multiple forms in the same animal, as, for example, the transition of fetal hemoglobin to the adult form. Nucleated cells also possess all the machinery necessary for protein synthesis, and thus can synthesize their own hemoglobins as well as necessary enzymes for assembling this protein. Mammalian eyrthrocytes, on the other hand, lack both protein synthetic machinery and mitachondria and thus require that all necessary components be synthesized prior to loss of the nuclear material in the reticulocyte stage.

As stated previously, the one distinguishing feature of the mammalian erythrocyte is its lack of a nucleus. Non-mammalian cells which possess a full complement of nucleus, ribosomes, mitochondria and endoplasmic reticulum have the ability to make all the enzymes necessary for metabolism, and therefore, possess both anerobic and aerobic metabolism for production of cellular energy. The basic carbon source for most mammalian red cells is glucose, although pig red cells are a conspicuous exception since they do not use glucose due to cellular impermeability to this compound (Kim and McManus 1971a). Several nucleated fish red cells also appear to be glucose impermeable (Bolis and Luly 1972; Kim and Isaacks, unpublished), but with a complete set of metabolic enzymes, alternate sources of energy are much easier to postulate than for the pig, whose plasma lacks substantial amounts of any utilizable carbon except glucose.

In the remainder of this review, which will limit itself to the mammalian red cell, attempts will be made to point out differences in the red cells of various animals in terms of their function and metabolism. Many excellent reviews have covered the areas of comparative

erythrocyte metabolism (Kaneko 1974), erythrocyte metabolism (Rapoport 1968), regulation of glycolysis (Rapoport 1974), purine and pyrimidine salvage pathways (Murray 1971), and red cell enzymes (Paniker 1974).

Red Cell Morphology

Mammalian red blood cells, lacking most internal organelles, exist in a discoid shape with the intracellular protein made up primarily of hemoglobin. It was hypothesized that this shape allowed rapid, homogeneous exchange of gas by diffusion in the shortest time (Hartridge 1920; Ponder 1949) primarily because this shape is a compromise between a sphere and an infinitely thin disk. Both Hartridge (1920) and Ponder (1949) suggested that if red cells existed as either spheres or thin disks, then gas diffusing from the surface of either would reach the cell's center at the same time, but neither shape would be adequate because a spherical shape would offer a small surface when compared with its volume, while a thin disk would permit non-uniform gas distribution and would also have to exhibit strengthened cellular rigidity to maintain its shape. When the shape hypothesis was tested both theoretically (Roughton 1952) and experimentally (Carlsen and Comroe 1958), results from both experiments revealed that gaseous exchange between spherical and discoid shape cells were equivalent. Thus, one can conclude that the discoid shape of the human red cell conveys no great advantage insofar as rapidity of gas exchange is concerned. However, the discoid form of the mammalian red cell is advantageous for another reason. When flowing through blood vessels, the flattened form of the cells contribute to their being lined up in

the axial region of the stream, leaving only plasma flowing at the vessel's edge, lowering the effective viscosity of the blood (Ponder 1949). Therefore, maintenance of red cell shape is important for animal survival, because its loss produces premature destruction and anemia for the animal.

The factors determining the shape of the cell have been extensively investigated. The advent of high powered electron microscopy allowed investigators to discover fibrous proteins called microfilaments and microtubules in various structures just beneath cell membranes, and a structural protein called spectrin has been isolated (Marchesi and Steers 1968). It has now been shown that ATP concentrations affect the function of these fibrous proteins (Weed, LaCelle and Merrill 1969), and that destruction of these proteins either chemically by using vinblastine, or physically by heating, causes cellular sphering (Jacob, Amsden and White 1972).

Cellular shape is important in regulation of cellular function. Alteration of shape changes the Na^+ permeability, as well as active Na^+ transport, and changes in Na^+ permeability can alter glycolytic rates (Parker and Hoffman 1967). Since nucleotide concentrations appear to regulate cellular shape, nucleoside and nucleotide metabolism may play an important indirect role (as well as the more obvious roles described below) in red cell survival and functioning.

Carbohydrate Metabolism

The Glycolytic Pathway and Enzymes

Glucose is the predominant carbohydrate metabolized by mammalian red cells (Bartlett and Marlow 1953; Laris 1958). In contrast, the nucleated red cells of non-mammalian species, possessing a relatively complete set of metabolic reactions, including glycolytic and respiratory pathways, can utilize a variety of carbon sources (Brewer 1974). Mammalian red cells utilize anaerobic glycolysis to produce energy and when compared with non-mammalian red cells, tend to consume glucose and produce ATP at slower rates. It has been shown that fish such as mackerel have higher levels of ATP than either mammals or amphibians (Rapoport and Guest 1941; Bartlett and Borgese 1976). It has been suggested that the teleological reason for declining RBC metabolism as a function of animal temperature is that red blood cells with high carbohydrate consumption rates would become an excellent culture medium for parasites at 37°C, and to avoid infection red cells have adapted by depressing their metabolic rate (Brewer 1974).

Mammalian red cells possess neither DNA, RNA or ribosomes (Rapoport 1960). Thus, during adult life span the red cell is incapable of synthesizing any proteins. This has been suggested as a reason why metabolic activity declines with the age of the erythrocyte (Brewer 1974). The loss of enzyme activity, as well as general protein degradation, has been suggested as the primary reason why red cells die after approximately 100 days in circulation, although this hypothesis has never been proven.

The Embden-Meyerhof pathway (EMP) generates three important compounds in most mammalian red cells (Fig. 1). These include adenosine triphosphate (ATP), 2,3-diphosphoglycerate (2,3 DPG) and reduced nicotinamine adenine dinucleotide (NADH). The EMP is the only source of ATP since the mature red cell lacks oxidative phosphorylation and electron transport systems. Glucose is initially phosphorylated through the use of a high-energy phosphate of ATP at the initial step of glycolysis, and a second ATP is used at the phosphofructokinase step, when a phosphate is attached to fructose-6-phosphate. Therefore, two molecules of ATP are utilized in the early part of the EMP. When the fructose 1,6-diphosphate is split into 2 three-carbon moieties (dihydroxyacetone phosphate and glyceraldehyde 3-phosphate), each resulting 3 carbon sugar can generate 2 ATP; one at the phosphoglycerate kinase step and another at the pyruvate kinase reaction. Thus, each molecule of glucose that is metabolized to 2 molecules of lactic acid produces a net increase of 2 ATP (Prankerd 1955).

The second important compound generated by the EMP is 2,3 DPG, which exists in many animal and human red cells (Harkness, Ponce and Grayson 1969). The synthesis of 2,3 DPG was first described by Rapoport and Luebering (1951), who discovered two enzymes, DPG-mutase and DPG-phosphatase. This shunt pathway bypasses an ATP-generating step at pyruvate kinase and therefore does not contribute to the net synthesis of ATP. Initially, the function of this "energy wasting" cycle was a puzzle until studies carried out by Chanutin and Curnish (1967) and Benesch and Benesch (1967) showed that 2,3 DPG accumulation had an

Figure 1. The major pathways of carbohydrate metabolism in mature mammalian red cells.

Circled numbers refer to following enzymes:
1, hexokinase; 2, phosphofructokinase; 3, glyceraldehyde 3-phosphate dehydrogenase; 4, phosphoglycerate kinase; 5, diphosphoglycerate mutase; 6, diphosphoglycerate phosphatase; 7, pyruvate kinase; 8, lactate dehydrogenase; 9, glucose 6-phosphate dehydrogenase.

Abbreviations: GLU-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; FDP, fructose 1,6-diphosphate; DHAP, dihydroxyacetone phosphate; GA-3-P, glyceraldehyde 3-phosphate; 1,3 DPG, 1,3-diphosphoglycerate; 2,3 DPG, 2,3-diphosphoglycerate; 3 PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; 6 PG, 6-phosphogluconate; GSH and GSSG, reduced and oxidized glutathione respectively; NADH and NAD, reduced and oxidized nicotinamide adenine dinucleotide respectively; NADPH and NADP, reduced and oxidized nicotinamide adenine dinucleotide phosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate.

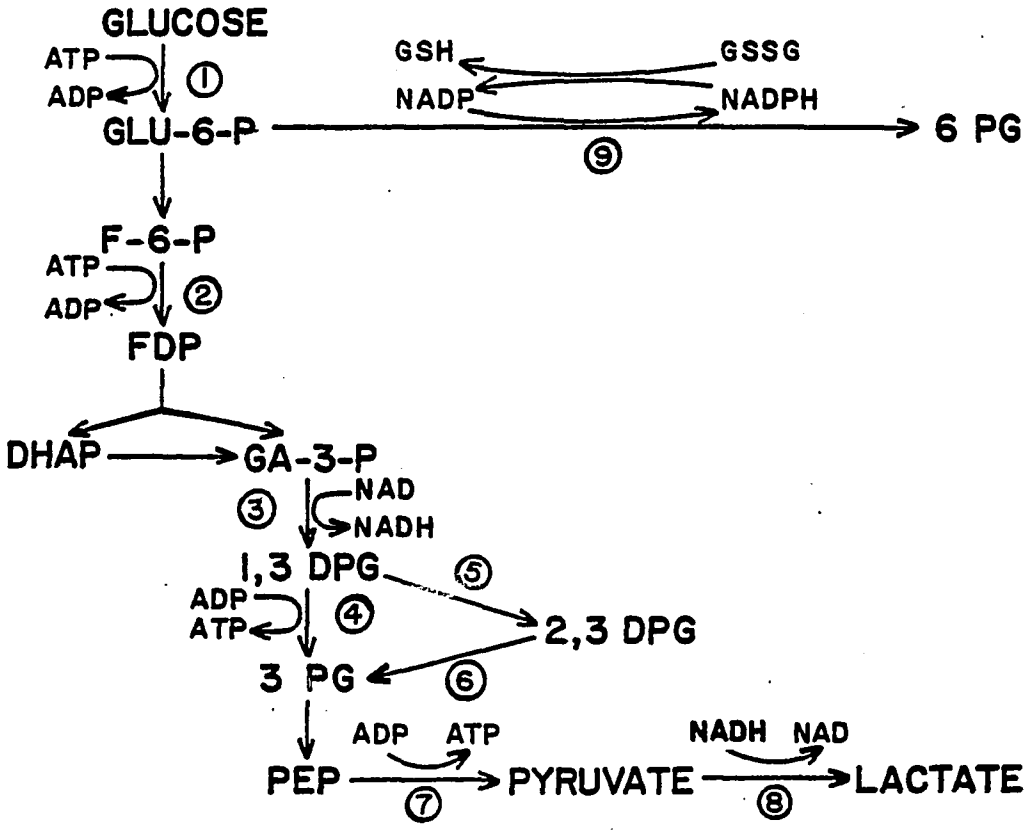

Figure 1. The major pathways of carbohydrate metabolism in mature mammalian red cells.

important role in influencing the release of oxygen from hemoglobin. In some animals, such as cow, cat, sheep, and goat, the levels of 2,3 DPG are practically non-existent (Bartlett 1970), and the probable reason for this is the inactivity of 2,3 DPG mutase (Harkness et al. 1969). Furthermore, O_2-hemoglobin interactions of these animals are affected to a much lesser degree by 2,3 DPG than those animals with high levels of 2,3 DPG (Bunn 1971). Inositol pentaphosphate is to birds and certain teleosts (Isaacks et al. 1976; Isaacks, Kim, Bartlett, Harkness 1977) what 2,3 DPG is to mammals. Of special comparative interest is the finding that during late embryonic development, the erythrocytes of chickens and ducks contains 2,3 DPG as the predominant organic phosphate, which is replaced after hatching with inositol pentaphosphate (Isaacks and Harkness 1975; Bartlett and Borgese 1976).

The third function of the EMP is the reduction of NAD to NADH, which is required as a cofactor in methemoglobin reduction, which is described in detail in a later section of the dissertation. Animal erythrocytes vary in their ability to reduce methemeglobin (MetHb) (Smith and Beutler 1966), with the sheep, cow and goat being the fastest of all animals. It seems paradoxical that species with the fastest MetHb turnover are generally those with the slowest glycolytic rate, except the pig.

It is generally recognized that three enzymes in the glycolytic pathway, hexokinase (HK), pyruvate kinase (PK) and phosphofructokinase (PFK), are responsible for regulating the utilization of glucose in the red blood cell (Rapoport 1968). HK, the first enzyme in the EMP, is non-specific for substrate, being able to use glucose, mannose or

fructose. With a K_m of 200 μM for glucose, this enzyme is always saturated under physiological conditions because the red cell's intracellular glucose concentration ranges from 5.3 mMoles/liter of cell water in humans (Kaneko 1974) to 0.7 mMoles/liter of cell water in cattle (Zinkl and Kaneko 1973). This implies that glucose availability is not a factor in limiting HK activity or glycolytic rate in most mammalian red cells. The V_{max} in human hemolysates is 5 μmoles/ml cells/hour, which is not only the lowest for all EMP enzymes, but is six times lower than the second least active enzyme, aldolase (Rapoport 1968). Even with the abundance of glucose available, it was discovered that the levels of substrate and product were far removed from equilibrium conditions both in human (Yoshikawa and Minikami 1968) and cattle (Zinkl and Kaneko 1973) red cells, thus implying that hexokinase was a rate-limiting step in converting glucose to glucose 6-phosphate. There are numerous compounds which will regulate hexokinase activity. ATP is a substrate which exists in combination with Mg^{2+}. Under normal conditions, the levels of Mg-ATP may be about half saturating for HK, so that ATP concentration may be playing a somewhat limiting role in glycolysis (Rapoport 1968) although others feel that ATP exists in adequate concentrations in the human red cell (Rose 1971). Other possible intracellular regulators of HK activity include glucose 6-phosphate (G6P) and 2,3 DPG (Rose and O'Connell 1964; Dische 1941; Brewer 1969) which are both inhibitory, and Mg^{2+} and inorganic phosphate (Pi), which decrease the inhibitory effects of 2,3 DPG and G6P, respectively (Rose, Warms and O'Connell 1964; Minikami and Yoshikawa 1966).

Phosphofructokinase is the second enzyme responsible for regulation of glycolysis in red cells. Since the levels of substrates and products are far removed from equilibrium (Yoshikawa and Minikami 1968; Minikami and Yoshikawa 1966), this implies that the enzyme limits the phosphorylation of fructose 6-phosphate (F6P). PFK activity is quite sensitive to change in pH, and it has been shown (Rapoport 1968) that increasing pH produces activation (or release of inhibition) of PFK. This pH-related response appears to play an important role in red cell glycolytic regulation, although this enzyme's pH response is in turn influenced by several factors, including substrate and adenine nucleotide concentration. In human red cells, the substrate F6P is not at saturating levels due to the low activity of HK, but binding of F6P to PFK is enhanced by elevated pH, so that more substrate can be phosphorylated. ATP in the form of Mg-ATP is an inhibitor of PFK, and inhibition by ATP is also markedly decreased by elevated pH (Rapoport 1968), as well as by increasing levels of inorganic phosphate (Passonneau and Lowry 1962; Minikami and Yoshikawa 1966). ADP, on the other hand, is an activator of PFK (Mills 1969).

Pyruvate kinase (PK) is the third, rate limiting enzyme in red cell glycolysis. The substrates for PK are phosphoenolypyruvate (PEP) and ADP, and this enzyme also requires Mg^{2+}. The products of the reaction are pyruvate and ATP. This is the second ATP-generating step in glycolysis, with the first being at the phosphoglycerate kinase step. Two net ATP's are generated for each molecule of glucose at this step if none of the 3-carbon substrates have gone through the DPG shunt.

PK is far removed from equilibrium under ordinary circumstances (Yoshikawa and Minikami 1968), and may have an important role in regulating the later part of the glycolytic pathway. Fructose 1,6-diphosphate (FDP) has been shown to be an allosteric activator of PK (Koler and Vanbellinghen 1968), while ATP is an inhibitor. The levels of ADP and PEP are low enough to leave the enzyme unsaturated; thus, ATP inhibition of PK is competitively relieved by elevated levels of PEP (Rapoport 1968). Staal et al. (1971) purified human red cell PK and showed that FDP activation and ATP inhibition were pH and Pi dependent, with enzyme activity increasing with increasing pH or Pi.

In general, cow red cell enzymes are quite similar to human red cell enzymes, although none of the extensive work reported above has been done using cow red cell enzymes. Zinkl and Kaneko (1973) have shown using the equilibrium data of Yoshikawa and Minikami (1968) that the three enzymes discussed above are displaced from equilibrium in cow red cells and that HK is the rate limiting step in cow red cell glycolysis. At the present time, it is unknown whether pH or Pi plays a major role in glycolytic regulation, as it does in humans.

Pentose Phosphate Metabolism

The two most important functions of the pentose pathway in the mature red cell are the reduction of nicotinamide adenine dinucleotide phosphate (NADP to NADPH) by glucose 6-phosphate dehydrogenase, and the production of limited amounts of ribose 5-phosphate, which in turn participates in the salvage pathway for purines (Hershko, Razin and Mager 1969).

NADPH is intimately involved in detoxifying oxidizers such as H_2O_2. As stated above, oxidizers can convert ferrous hemoglobin to the non-functional ferric form called methemeglobin (Jaffe and Neurmann 1972), and the red cell must continually reduce it back to its functional form. This can be accomplished by either a NADH-dependent diaphorase or a NADPH-dependent reductase. Of the two enzyme systems present, the NADH-dependent diaphorase is believed to be more important for most of the physiological reduction of methemeglobin (Scott 1969). In addition, reduced glutathione (GSH), a tripeptide located in the red cell (Majerus, Minnich and Mohler 1970) can protect the globin moiety against oxidative damage and the eventual formation of denatured hemoglobin called Heinz bodies (Allen and Jandl 1961; Harley and Mauer 1961). Besides protecting the hemoglobin directly, glutathione also supplies effective protection against peroxides indirectly through detoxification of H_2O_2 by using glutathione peroxidase (Jacob and Jandl 1966; Hochstein and Utley 1968). Glutathione peroxidase activity is dependent upon the regeneration of adequate amounts of GSH through the glutathione reductase reaction, which in turn requires an adequate supply of NADPH generated from the pentose phosphate pathway (Cohen and Hochstein 1963). The oxidized glutathione (GSSG) formed in the reduction of H_2O_2 (Mills 1959) must either be reduced by the red cell using NADPH and glutathione reductase or extruded (Srivastava and Beutler 1967, 1969). Besides being involved in protecting hemoglobin, glutathione may also play several other roles in red cell metabolism. GSSG was found to inhibit hexokinase (Eldjarn and Bremer 1962) and the ratio of GSSG to GSH

appears to alter the glucose flow through the pentose pathway (Jacob and Jandl 1966).

The second functional role of the pentose phosphate pathway is synthesizing ribose phosphates from glucose (Manohar, Denstedt and Rubinstein 1966), which together with ATP and phosphoribosyl pyrophosphate synthetase (PRPP synthetase) form 5' phosphoribosyl 1-pyrophosphate (PRPP). A detailed discussion of PRPP metabolism is presented in the next section. While 90-95% of the glucose metabolized by the red cell enters the Embden-Meyerhof pathway (EMP) (Bartlett 1970; Harvey and Kaneko 1976), 5-10% is diverted through the pentose pathway and most of the ribose phosphates is synthesized from this source. Another source of ribose phosphates is nucleosides, which are metabolized to the free purine base and ribose 1-phosphate by nucleoside phosphorylase (Gabrio et al. 1955), and blood banking procedures have successfully exploited this pathway in order to maintain red cell viability. To what extent ribose phosphate is generated by this pathway under physiological conditions is not known since certain animal red cells have very low nucleoside phosphorylase levels without any impairment to their metabolism or function (Duhm 1974).

Purine Metabolism

Mammalian red cells, lacking the synthetic machinery necessary for de novo synthesis of purines (Lowy and Williams 1960; Lowy, Williams and London 1962) must rely upon exogenous sources and the salvage of preformed purines and nucleosides (Fig. 2). PRPP is a substrate common to several of the metabolic pathways in purine salvage

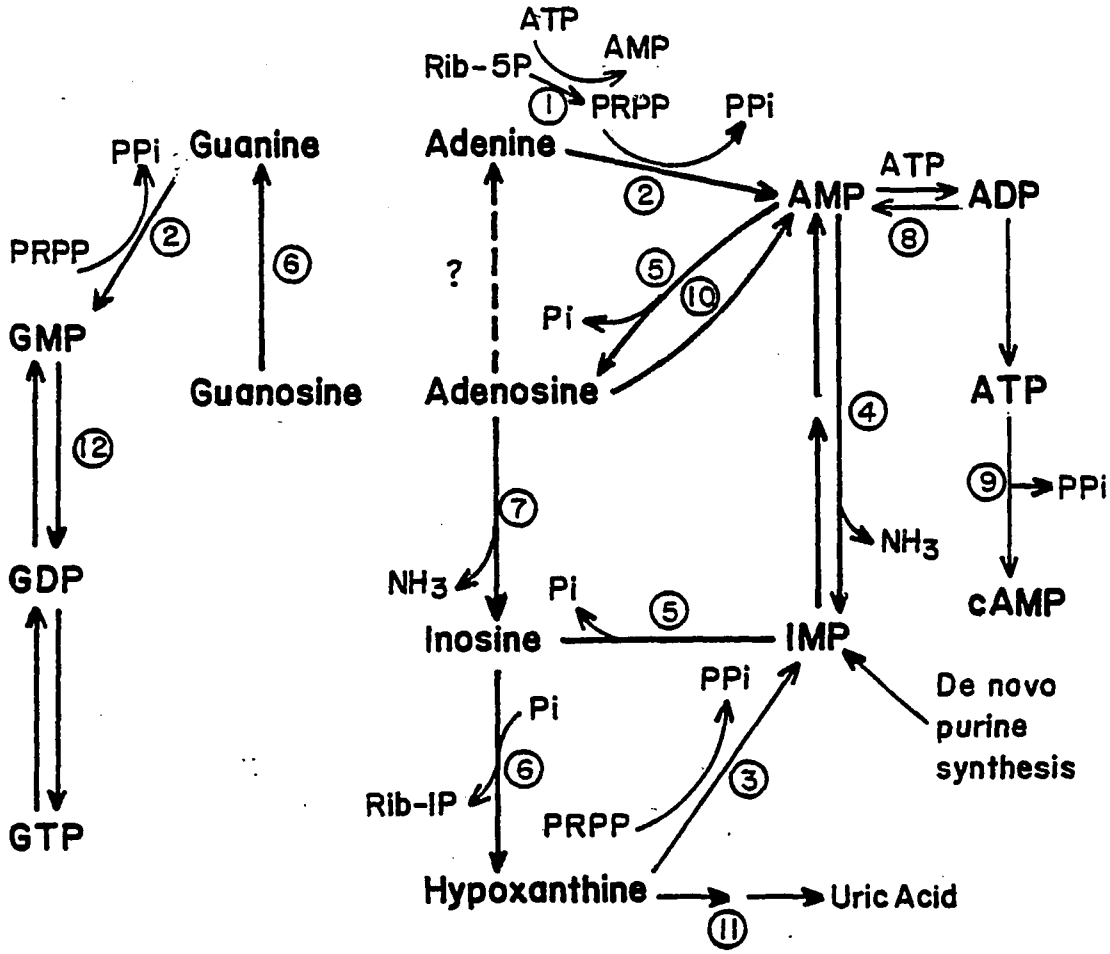

Figure 2. Abbreviated scheme of nucleotide metabolism. -- Enzymes catalyzing the various reactions are: (1) phosphoribosyl pyrophosphate synthetase; (2) purine phosphoribosyl transferase; (3) hypoxanthine phosphoribosyl transferase; (4) adenylate deaminase; (5) 5'-nucleotidase; (6) nucleoside phosphorylase; (7) adenosine deaminase; (8) adenylate kinase; (9) adenyl cyclase; (10) adenosine kinase; (11) xanthine oxidase; (12) guanylate kinase. Source: Mills et al. 1976.

(Fig. 2) and exists in human red cells at a concentration of 1.5 nmoles/ml cells (2.1 μmoles/liter of cell water) (Tax, Veerkamp and Trijbels 1976). PRPP formation from ribose 5-phosphate and ATP is catalyzed by PRPP synthetase, which was first isolated from pigeon liver (Kornberg, Lieberman and Simms 1955). Since the concentration of ribose 5-phosphate is exceptionally low in erythrocytes (Bishop 1964), it was suggested to be the rate limiting step in PRPP synthesis. However, Hershko et al. (1969) found that incubation of red cells in methylene blue, which produced a tenfold increase in ribose 5-phosphate formation, did not increase the rate of PRPP production. Further studies showed PRPP synthetase to be the rate limiting step, with ADP, GDP and 2,3 DPG providing strong feedback inhibition and inorganic phosphate (Pi) at high concentrations relieving this feedback inhibition (Hershko et al. 1969).

The presence of PRPP is vital to the salvage pathway because adenine, hypoxanthine and guanine react with it in the presence of either adenine phosphoribosyl transferase or hypoxanthine-guanine phosphoribosyl transferase to form the purine mononucleotides, including AMP, IMP and GMP (Kong and Parks 1975). Most hypoxanthine produced by the red cell is recovered by the salvage pathway, although some is inevitably converted to xanthine and uric acid by xanthine oxidase. Uric acid is the end-product of purine metabolism, and is not salvageable by red cells in most species. However, in cow and buffalo blood, uric acid can be taken up by the red cell and synthesized to a nucleoside called uric acid riboside in a reaction that requires PRPP

(Hatfield and Forrest 1962). Uric acid riboside is unique in that the ribose moiety is attached at the N-3 position rather than the N-9 position seen in other purine nucleosides (Forrest, Hatfield and Lagowski 1961). While uric acid riboside is not present in fetal calves (Smith and Stricher 1976), it is found to make up a large portion of the UV-absorbing material in the adult (Smith and Stricher 1975). The function of this compound in the cow red cell is unknown.

Much of the information obtained on the purine salvage pathway was initially discovered by scientists looking for ways to prolong the shelf life of stored blood. In the mid-1950's, adenosine, guanosine and inosine were reported to extend the preservation of red cells stored in acid-citrate-dextrose (ACD) (Gabrio and Finch 1954; Gabrio et al. 1955; Gabrio, Finch and Huennekens 1956). While this effect was originally attributed to the production of phosphorylated ribose as an energy substrate within the red cell (Prankerd 1955), it was the studies reported by Nakao and his associates from 1959 to 1962 which shed light on the role of nucleosides in maintaining red cell viability (Nakao et al. 1959, 1960, 1962). They demonstrated that when blood was subsequently incubated with both adenine and inosine, regeneration of ATP and restoration of post-transfusion viability took place. These reports indicated that the purine moiety rather than the ribose sugar played the important role in red cell preservation. Nakao suggested that the mechanism by which adenine and inosine increased ATP levels in nucleotide depleted cells involved adenine supplying the purine skeleton while inosine provided the ribose phosphate for both PRPP synthesis and glycolytic metabolism through the nucleoside phosphorylase

reaction. McManus and Borgese (1961) showed that further metabolism of ribose phosphate through the EMP depended upon NAD availability, so when pyruvate and Pi was added to inosine ACD, enough NAD was made to ensure ATP synthesis, even in cells depleted of ATP and incapable of phosphorylating glucose.

As stated above, adenosine was also superior to inosine in maintaining viability and ATP in stored red cells, and the mechanism of action was explained by Lowy, Ramot and London (1960) and Lowy and Williams (1966), who showed that with high concentrations (3.6mM) of adenosine, some was incorporated into ATP by adenosine kinase (AK), with the rest being rapidly deaminated to inosine by adenosine deaminase (ADA). These two enzymes have been extensively investigated because they play a pivotal role in the red cell's ability to make ATP from adenosine. At physiological levels of substrate ($<1\ \mu M$) phosphorylation is favored by the approximately 15- to 20-fold lower K_m of the kinase, although in human cells, the V_{max} for ADA is significantly greater than that of the kinase (Meyskens and Williams 1971; Miyazaki et al. 1974; Miyazaki, Nambu and Hashimoto 1975; Agarwal, Sagar and Parks 1975). Thus, the relative K_m values for ADA and AK play a crucial role in determining whether adenosine will be salvaged or degraded, although it is possible that allosteric regulation of these enzymes also plays a role in shunting adenosine to inosine or AMP.

Murray (1971) found a total nucleotide turnover in humans of 6 mg of base/kg body weight/24 hours and concluded that considerable quantities of purines enter and leave the red cell's nucleotide pool.

Although adenosine has been found in the plasma of dogs and humans (Rubio, Berne and Katori 1969; Bockman, Berne and Rubio 1976) and human plasma has traces of ATP in it after reactive hyperemia (Forrester 1972), it is unlikely that these low plasma levels can sustain nucleotide levels in red cells. It is therefore likely that the liver, as well as other organs, supply these purines, and investigators have attempted to determine the exact source of purines. Henderson, Frank and LePage (1959) reported the transfer of radioactive adenine from prelabeled rat liver to erythrocytes and from prelabeled mouse erythrocytes to mouse tissue in an experiment involving adenine transfer from liver to erythrocytes in vitro. Pritchard, Chavez-Peon and Berlin (1970) demonstrated the transfer of liver purines to erythrocytes in vivo and suggested that there is a continuous flow of purines in this direction. Lerner and Lowy (1974), using rabbit liver perfused in vitro, measured adenosine and hypoxanthine flux from liver cells to red cells and found that red cells were labeled with liver adenosine and hypoxanthine. All these experiments suggest that a symbiotic relationship exists between the red cells and the organs in terms of purine metabolism.

Purine and Nucleoside Transport

Membrane permeability to glucose does not seem to play a limiting role in glycolysis since most mammalian red cells have a carrier-mediated facilitated transport of glucose which operates at rates 2 to 250 times greater than the metabolic requirement for glycolysis (Widdas 1954; Kim and McManus 1971a). In contrast to glucose, membrane

permeability to purine nucleosides plays a significant role in their metabolism, since permeability of these substances does vary to a large extent depending upon the species. For example, red cells of guinea pigs, dogs and cows cannot utilize inosine due to their exceedingly low membrane permeability to this substrate (Duhm 1974). Other cells, such as humans, pigs and rabbits are freely permeable to this nucleoside. Thus, the ability of the red cell to utilize nucleosides and purines depends to a great extent on the transport mechanisms.

Transfer of nucleosides across the plasma membrane of the human red cell is affected by a carrier-mediated facilitated diffusion process (Oliver and Paterson 1971). Criteria for defining characteristics of facilitated diffusion include: (a) a system that operates only with an existing electrochemical gradient, without the need for any other free energy; (b) a rate of penetration that is not directly proportional to concentration but reaches a limiting value as a function of increasing concentration; (c) a rate of permeation which may be markedly reduced by the presence of inhibitors; (d) the presence of competition by structural analogs; (e) the existence of counter-transport. Counter-transport is a phenomenon in which a permeant species distributed equally across the membrane can be driven outward against its concentration gradient by an influx of another structurally related permeant species diffusing down its concentration gradient. Most of these characteristics with respect to nucleoside transport have been documented and in the following, certain salient features are presented. In studies of red cell nucleoside transport, both uridine and thymidine

were extensively used since these compounds were neither cleaved nor phosphorylated by red cells (Oliver and Paterson 1971). The erythrocyte nucleoside transport system is very non-specific in that both purine and pyrimidine nucleosides can interact with the membrane carrier and be transported across the plasma membrane (Cass and Paterson 1972, 1973). The most potent inhibitors of nucleoside transport in the red cell are nitrobenzylthioguanosine (NBTGR) and nitrobenzylthioinosine (NBTPR), which exhibit approximately 1.5×10^4 binding sites per cell on the human membrane (Cass, Gaudette and Paterson 1974). This is fewer than the 3×10^5 binding sites seen for glucose (Lin and Spudich 1974) and far fewer than the 3×10^5 to 1.3×10^7 sites seen for anions (Cabantchik and Rothstein 1974; Lepke et al. 1976). In a structure-function study using these and other inhibitory nucleoside derivatives, Paul, Chen and Paterson (1975) discovered that effective inhibitors of nucleoside transport were derivatives of 9-β-D-ribofuranosylpurine with S, O or N substituted at the purine 6 position. They suggested that the hydrophobicity of the 6 position substituents appeared to contribute to the inhibitory activity of these compounds. It is worth noting that in human erythrocytes, the transport system for purine nucleosides is not influenced by the corresponding purine bases (Paterson and Oliver 1971), which are also believed to be transported by a facilitated diffusion process. Whittam (1960) reported that uptake of hypoxanthine from the suspending medium was independent of hypoxanthine concentrations in the range of 0.2 to 6.8 mM, while Lassen (1962) discovered mutual inhibition between hypoxanthine and uric acid, thereby implying the presence of a specific transport system common to

several purine derivatives. The hypoxanthine transport system was also described as being a two component system, one part rapidly saturable with a K_m = 0.4 mM and the second, nonsaturable (Lassen 1967). Contrary to nucleosides, counterflow was not observed with respect to hypoxanthine transport.

In this study, I have attempted to ascertain the relationship between glycolysis and purine metabolism in cow and calf red cells, and found that exogenous purines and nucleosides elevate cow, but not calf red cell glycolysis. In addition, possibly physiological roles for this stinulation of glycolysis were explored, and are presented in the remainder of this dissertation.

CHAPTER 2

THE ROLE OF PURINES, PYRIMIDINES AND NUCLEOSIDES ON COW RED CELL GLYCOLYSIS

Introduction and Statement of Problem

Glucose is the predominant carbohydrate metabolized by mammalian red cells (Bartlett and Marlow 1953; Laris 1958). The ATP which is continuously regenerated by glycolysis provides for maintenance of cell shape and active transport of cations across the membrane. Metabolic control of glycolysis has been extensively investigated.

The total metabolic capacities of each glycolytic enzyme have been determined in a number of species (Rapoport 1968; Kaneko 1974). Hexokinase (HK) has been found to possess the lowest capacity of the glycolytic enzymes in all animals tested to date (Kaneko 1974; Harvey and Kaneko 1975). Minikami and Yoshikawa (1966) have shown that HK was displaced from thermodynamic equilibrium, and is stimulated by Mg - ATP and is inhibited by glucose 6-phosphate, Mg - ADP 2,3 DPG and glucose 1,6-phosphate (Rapoport 1968). Several investigators have attempted to correlate enzyme capacity with glycolytic rate, and the best correlation appears to exist when HK is used (Rapoport 1968; Zinkl and Kaneko 1973) with the possible exception of the horse, which has a high hexokinase capacity but a very slow glucose consumption rate (Harvey and Kaneko 1976). Two other enzymes which regulate the glycolytic flow are phosphofructokinase (PFK) and pyruvate kinase (PK).

Both are reported to be inhibited by ATP (Rapoport 1974), while only PFK is inhibited by 2,3 DPG.

Apart from enzymatic control, membrane permeability to substrate is another regulatory parameter in metabolism (Rapoport 1968). However, membrane permeability to glucose does not seem to be limiting in glycolysis since mammalian red cells in general and human red cells in particular have a carrier-mediated facilitated transport of glucose whose rate is 2 to 250 times greater than the metabolic requirement of glycolysis. An exception is the pig, in which the red cell is impermeable to glucose, thereby depriving the cells of the benefits of glycolysis (Kim and McManus 1971a).

Red cells of certain species can also use substrates other than glucose, including free ribose (Jaffe 1959; Kim and McManus 1971a, 1971b; Lachhein et al. 1961), glyceraldeyde and dihydroxyacetone (Brake and Deindoerfer 1973; Beutler and Guinto 1973) for energy production. In addition, the beneficial effects of nucleosides and purines under blood banking conditions have been well documented. In contrast to glycolysis, membrane permeability to purine nucleosides plays a significant role in their metabolism. Thus, red cells of guinea pigs, dogs and cows cannot utilize inosine due to its inability to enter the red cell, although all three of these cells do have purine nucleoside phosphorylase (Duhm 1974). By comparison, the horse cannot use adenosine due to low activity of adenosine deaminase, even though the membrane is permeable to this nucleoside (McManus 1974). Low levels of adenosine have been shown to be preferentially metabolized to AMP via

adenosine kinase (Meyskens and Williams 1971), although as the adenosine concentration increases, the contribution of adenosine deaminase in the nucleoside metabolism becomes greater. While red cells lack the capacity for de novo synthesis of purines, the so-called salvage pathway, which is catalyzed by a series of enzymes including phosphoribosyl pyrophosphate synthetase and purine phosphoribosyl transferase, enables red cells to utilize preformed purines.

There have been extensive studies on the topics of glycolysis and purine salvage pathways. However, few reports have described a connection between the two metabolic pathways. Murphy (1960) noted that when human cells were incubated with inosine and glucose, glucose consumption was less than when cells were incubated in glucose alone. By contrast, Parks and Brown (1973) reported elevated levels of lactate production when human cells were incubated in glucose and a non-metabolized adenosine analog, fluoro-adenosine. Similarly, we have found that purines and nucleosides play a stimulatory role in cow red cell glycolysis.

In this paper, we report firstly, our attempts to investigate this phenomenon in detail and secondly, to elucidate the underlying mechanism for this stimulating effect of purines on glycolysis.

Materials and Methods

Preparation of Blood and Hemolysates

Animal blood was drawn from tail veins of adult Holstein cows, cephalic veins of dogs and cats, jugular veins of sheep, and the vena

cava of adult rats into heparinized tubes and immediately placed on ice. Human blood was drawn from the antecubital vein into heparinized tubes and also placed on ice. Within an hour, the cells were spun down at 1000 x g for 10 minutes in a centrifuge (Sorvall GLC-1) and the red cells were separated from the plasma and buffy coat. The cells were then washed four times by alternate resuspension and centrifugation in ice cold isotonic NaCl.

Hemolysates were prepared by the methods of Beutler (1971, 1975). 0.2 ml of washed, packed fresh red cells were added to 3.8 ml of a hemolyzing solution containing 0.5% β-mercaptoethanol, 10 μMoles of neutralized 10% (0.27 M) EDTA and 38 mMoles of NADP in distilled water. The mixture was placed on ice and vortexed occasionally for 10 minutes. The solution was then spun for 10 minutes at 5000 RPM at 4°C, and the supernatant was removed and placed on ice. The remaining supernatant and ghosts were then frozen, thawed and placed on ice. This freeze-thawed fraction was used for the phosphofructokinase (PFK) assay. Hemoglobins were measured in both fractions using the cyanmethemoglobin method (Drabkin 1950).

Metabolic Studies

Substrates were dissolved in an incubation medium consisting of 150 mM NaCl, 5 mM KCl, and 10 mM sodium phosphate buffer, pH 7.4. Osmotic pressure was determined in all cases by cryogenic osmometer (Advanced Instruments, model 3R) and adjusted by changing the NaCl concentration when necessary. An aliquot of washed red cells was added to the incubation medium, yielding a hematocrit of 10-15%. The

red cell suspensions were kept at 37°C by a heated shaker bath (New Brunswick Scientific, model G 76). Frequent 0.5 ml samples of the cell suspension were taken and extracted with 2 ml of 0.56 M perchloric acid (PCA). The resultant precipitate was spun down at 4°C. 2 ml of the supernatant was removed and neutralized with 5.63 M K_2CO_3. The clear supernatant was stored at 4°C for assays.

Glucose was assayed in the neutralized extracts by the glucose oxidase reaction as described by Saifer and Gerstenfeld (1958), using glucose kits supplied by Worthington Biochemical Corporation. Lactate in the neutralized extracts was measured enzymatically with lactic acid dehydrogenase by the procedure of Lundholm, Mohme-Lundholm and Vamos (1963).

ATP can be quantitatively measured using firefly enzyme luciferin and luciferase. One vial of firefly lanterns (Worthington Biochemical Corporation) was mixed with 5 ml of distilled water, 10 ml of 0.1 M sodium arsenate and 10 ml of 0.04 M $MgCl_2$. Neutralized extract was diluted 1:100 with 0.04 M tris-borate buffer, pH 9.2 and 0.2 ml of this diluted neutralized extract was then added to a 5 x 20 mm test tube. 0.1 ml of firefly extract was then added to the tube and the mixture vortexed and placed in a photometer (Chem-Glow, Aminco Inc.). Twenty-five seconds after addition of firefly extract, the emission of light was quantitated. In all experiments, triplicates were run, as were PCA extracted ATP standards.

Enzyme Assays

Hexokinase (HK), pyruvate kinase (PK) and phosphofructokinase (PFK) were all assayed at 37°C in hemolysates by the methods of Beutler (1971, 1975). All reactions were followed in a Beckman recording spectrophotometer (Model DU) equipped with a Gilford Model 2400 sampling accessory and a Haake constant temperature circulator. Enzymatic activity is expressed in μmoles of reduced or oxidized pyridine nucleotide formed per minute per gram of hemoglobin.

3-O-Methylglucose Flux

3-O-methylglucose flux was measured in cow red cells at 37°C using methods described previously (Kim and Luthra 1976; Zeidler, Lee and Kim 1976). Radioactive 3-O-methylglucose (0.1 μCi/ml) was added to medium containing cold 3-O-methylglucose in concentrations ranging from 10 μM to 40 mM. After addition of red cells, 0.4 ml aliquots were taken at times ranging from 20 seconds to 10 minutes, and the cells were quickly segregated from the medium by centrifugation through 0.8 ml phthalate. Uptake of 3-O-methylglucose was computed from radioactivity determination of cells and specific activity of incubation medium. All intracellular counts were corrected for extracellular trapped counts.

Sources of Materials

All cows and sheep were housed at the University of Arizona Farm, Tucson. Other animals were housed in the Department of Animal Resources at The University of Arizona College of Medicine. All

nucleosides, nucleotides and bases were obtained through Sigma. All enzymes and intermediates were purchased from Boehringer. NADP and NADH were purchased from P-L Biochemicals.

Results

Compared with other species, cow red cells have low levels of intracellular ATP of 0.58 μmoles/ml cells as shown in Figure 3. This level is very close to that found by Kaneko (1974), and appears to strengthen Rapoport's general contention that slow glycolyzing red cells have low intracellular levels of ATP (Rapoport 1968). Figure 3 also shows that adenosine can maintain intracellular levels of ATP, but like glucose, cannot produce net ATP. However, the addition of a carbon source, such as glucose, to incubation medium containing adenosine results in a significantly large net synthesis of ATP ($p < .01$). In this regard, cow and horse red cells are quite similar in their utilization of adenosine since McManus (1974) found the same effect in horse red cells. In keeping with the findings of Duhm (1974), who showed that cow red cells are practically impermeable to inosine, cells suspended in inosine failed to maintain ATP (Fig. 3). The rate of ATP depletion in cells suspended in inosine was the same as in cells suspended in a balanced salt solution without a metabolic substrate. In both cases, they lose ATP with a half time of 60 minutes.

Figure 4 shows lactate production in the presence of these substrates. Even though adenosine can maintain ATP levels, it is sluggishly metabolized by the cow red cells, yielding lactate at the rate of $0.34 \pm .05$ μmoles/ml cells/hr. As expected, inosine produced little

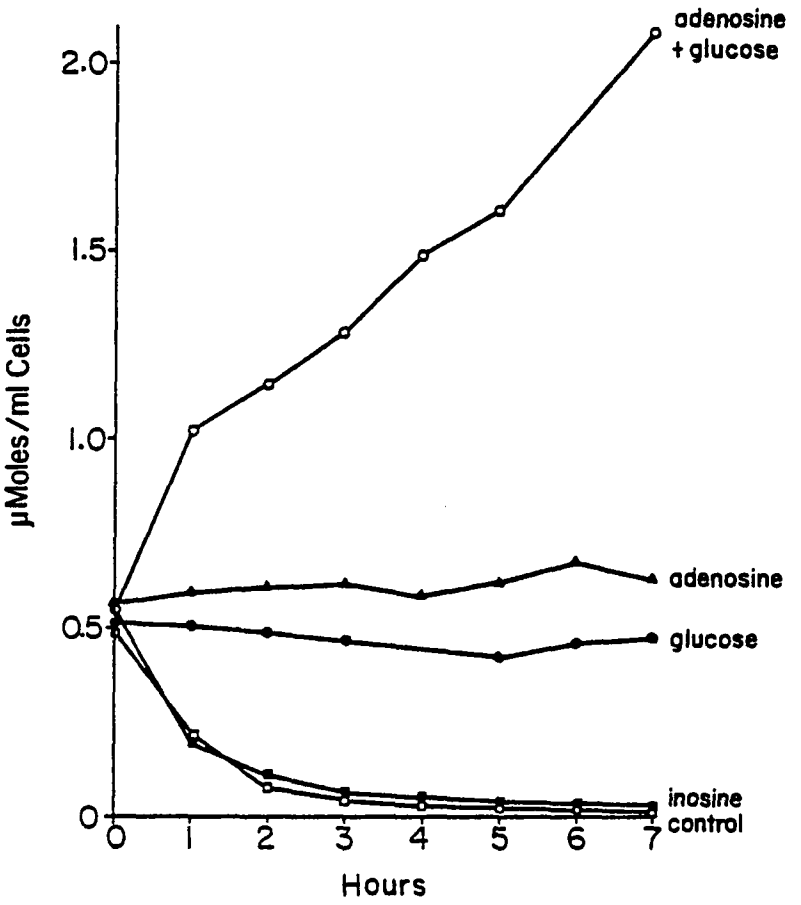

Figure 3. ATP levels in cow red cells suspended in various metabolic substrates. -- Red cells were suspended at a 10-15% hematocrit in: (a) 5 mM glucose (●—●); (b) 10 mM inosine (■—■); (c) 10 mM adenosine (▲—▲); (d) 5 mM glucose + 10 mM adenosine (○—○); (e) no substrate (□—□). All suspending media were composed of a balanced salt solution consisting of 5 mM KCl, 10 mM Na phosphate buffer, pH 7.4 and NaCl to produce an isosmotic solution at 37°C. The figure shows results of one experiment from a series of four.

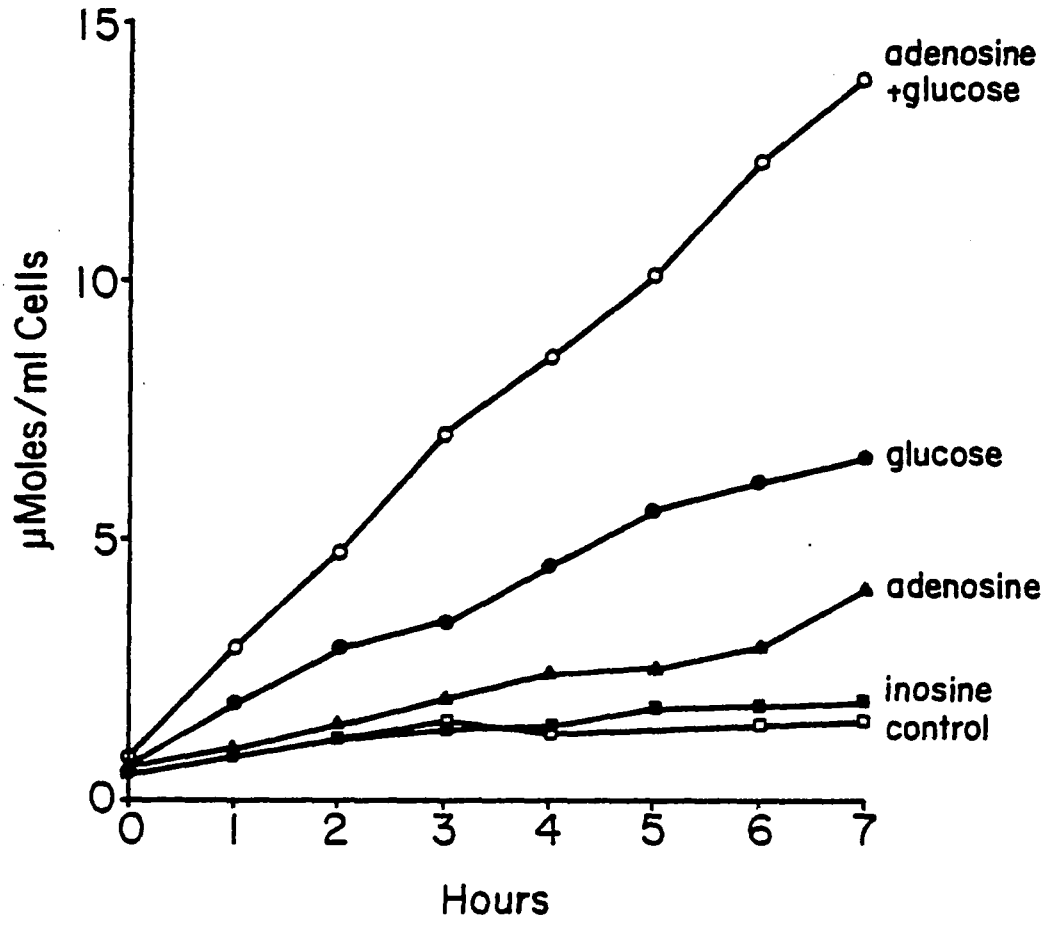

Figure 4. Lactate formation in cow red cells. -- Incubated in: (a) 5 mM glucose (●—●); (b) 10 mM inosine (■—■); (c) 10 mM adenosine (▲—▲); (d) 5 mM glucose + 10 mM adenosine (○—○); (e) no substrate (□—□). The incubation conditions are the same as in Figure 3, with a typical result depicted from a series of five experiments.

or no lactate. Also shown in Figure 4 is lactate production grom glucose either with or without adenosine. Lactate production in cells suspended in glucose plus adenosine (1.66 ± .10 μmoles/ml cells/hr) is significantly ($p < .01$) greater than the sum of lactate production (1.09 ± .05 μmoles/ml cells/hr) from glucose or adenosine alone. These findings suggest that adenosine may have some synergistic effect with glucose on cow red cell glycolysis.

Figure 5 shows the effect of altering adenosine concentration on cow red cell glycolysis. The upper curve in Figure 5a shows that the basal level of lactate production from glucose alone is 0.85 μmoles/ml cells/hr as indicated by the arrow. Adding increasing amounts of adenosine to the glucose medium causes the lactate formation rate to rapidly rise at first, and then more slowly after the adenosine concentration reaches 1 mM. The lower line in Figure 5a shows the lactate formed when adenosine alone is added to incubation medium. Concentrations of adenosine less than 250 μM produce no lactate. When medium adenosine levels rise above 250 μM, the rate of lactate formation rises, and is dependent upon the medium concentration of adenosine. It is clear in Figure 5a that the sum of lactate formed from glucose alone and increasing levels of adenosine alone is far less than the lactate production seen in mediums containing adenosine and glucose together. The percent stimulation in Figure 5b refers to the magnitude of difference between these two levels. Concentrations of adenosine as low as 10 μM are enough to elevate glycolysis to a small extent. The rate of stimulation rises very rapidly until 1 mM, and then levels off and

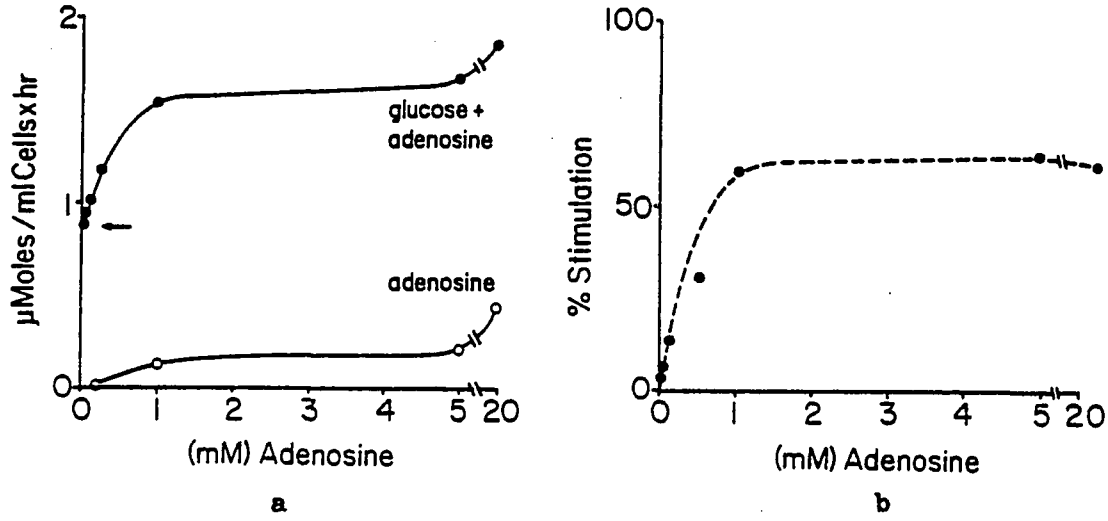

Figure 5. Stimulation of glycolysis in adenosine. -- 5a: Lactate formation from cells suspended in glucose and adenosine. Cow red cells were suspended either in various adenosine concentrations up to 20 mM fortified with 5 mM glucose or various concentrations of adenosine alone. Lactic acid production in glucose alone is indicated by the arrow showing 1 of 3 experiments, all of which yielded similar results. 5b: Effect of adenosine on cow red cell glycolysis. The ordinate is calculated by subtracting the lactate rate seen in adenosine alone from the lactate production rate of cells suspended in glucose + adenosine. The percent stimulation refers to the elevated level of glycolysis seen in glucose and adenosine compared to glucose alone.

remains constant over the range of 1-20 mM adenosine. At these concentrations, adenosine appears to stimulate cow red cell glycolysis almost 60% above the rate seen for glucose alone. Calf red cells, on the other hand, were unaffected by adenosine at any concentration (Fig. A-2, Appendix A). Clearly, these data indicate that cow red cell glycolysis is stimulated by adenosine, and that the concentration necessary for producing maximal stimulation is quite low.

Even though inosine is not metabolized by cow red cells, it was found that inosine, like adenosine, plays a similar role in stimulating glycolysis. The results are shown for both calf and cow red cells in Figure 6. As in adenosine stimulation, the concentration of inosine was varied while the medium glucose concentration was maintained at 5 mM. Osmotic activity of the solution was maintained always at isotonicity by adjusting the NaCl concentration. Glucose consumption was measured over a seven hour period, and the rate was calculated using least squares regression analysis. It is evident that for cow red cells, inosine produces a significant ($p < .02$) maximal stimulation of glycolysis between 10 and 15 mM, elevating the glucose consumption rate from 0.56 to 0.84 μmoles/ml cells/hr, while at either extreme, the stimulatory effect is lost. In calf red cells, on the other hand, no stimulation of glycolysis is seen (lower graph), but instead a decrease in the glucose consumption rate of the calf from 1.81 μmoles/ml cells x hr to 1.55 μmoles/ml cells x hr. Calf cells are permeable to inosine, and probably receive some of their carbon source from metabolizing this compound to ribose 1-phosphate, thereby decreasing the demand for glucose. This was seen by Murphy (1960) in human red cells.

Figure 6. Effects of inosine on glucose consumption in calf and cow red cells.

Incubation conditions are the same as in Figure 3. The glycolytic rates for calf red cells ranged from 1.81 ± 0.11 μmoles/ml cells/hr in glucose alone to 1.55 ± 0.10 μmoles/ml cells/hr in 10 mM inosine and glucose, while the cow red cells consumed glucose at a rate of 0.56 ± .05 μmoles/ml cells/hr in glucose alone versus 0.84 ± .08 μmoles/ml cells/hr in 10 mM inosine and glucose. While the lactate/glucose ratio for calf red cells was 1.70 in glucose and 1.95 in glucose + inosine, cow red cells had a lactate/glucose ratio of 1.58 in glucose and 1.39 in glucose + inosine. The percent change refers to either the increase or decrease in the glycolytic rate of cells suspended in inosine + glucose compared to cells suspended in glucose alone. Inosine was checked for purity and found to be acceptably pure analytical grade using thin layer chromatography. Shown is one typical result from six experiments.

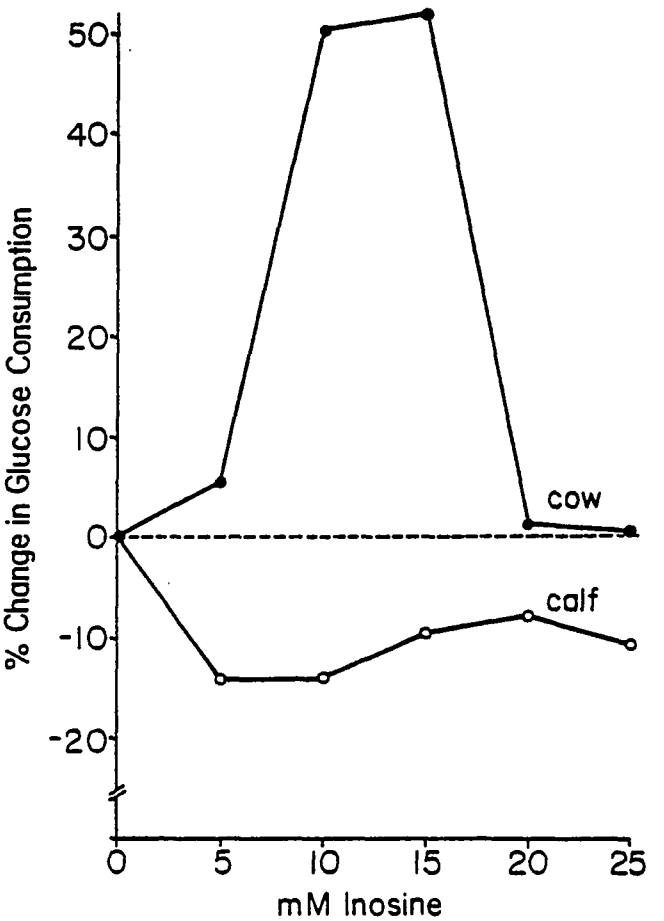

Figure 6. Effects of inosine on glucose consumption in calf and cow red cells.

In view of the external stimulatory role of inosine, it was of interest to determine the active part of this nucleoside molecule responsible for the stimulation. To this end, ribose and the base moieties were tested separately. Results, shown in Table 1, indicate that ribose does not enhance the rate of glycolysis in cow red cells, since the lactate formation rate with either glucose or ribose alone compare favorably with the rate seen in the presence of both compounds. However, when hypoxanthine is added to medium containing glucose, the glycolytic rate is elevated, suggesting that the active portion of the inosine molecule lies in the base, and not in the sugar moiety.

Since hypoxanthine was found to be a stimulatory agent on cow red cell glycolysis, other purines, pyrimidines and nucleosides were tested to discover if any others had similar effects. In all the experiments, the concentration of each compound studied was kept at 1 mM with the exceptions of guanine, guanosine, xanthine and xanthosine, which were made up to only 50 μM since these compounds are extremely insoluble. Figures 7 and 8 depict these results in cow and calf respectively. Four compounds stimulate cow red cell glycolysis, but show little effect in calf red cells. These are adenine, hypoxanthine, uracil, and xanthine. Uridine stimulated in some animals, and was ineffective in others. In cow cells, the others appear to have little if any effect on the rate of glycolysis, including several membrane impermeable nucleotides. In contrast, none of these compounds have any effect on calf red cell glycolysis. Thus, only some purines and pyrimidines stimulate cow but not calf red cell glycolysis.

Table 1. Lactate formation and glucose consumption in cow red blood cells.

	Lactate Formation	Glucose Consumption	Ratio
	(μmoles/ml cells/hr)		
Glucose	0.91	0.60	1.52
Ribose	0.30	-	-
Glucose + Ribose	1.19	0.64	1.86
Hypoxanthine	0	-	-
Hypoxanthine Glucose	1.25	1.07	1.16

Red cells were incubated in medium containing either 5 mM glucose, 3 mM ribose, 5 mM glucose + 3 mM ribose, 1 mM hypoxanthine or 1 mM hypoxanthine and 5 mM glucose. A typical result from a series of three experiments.

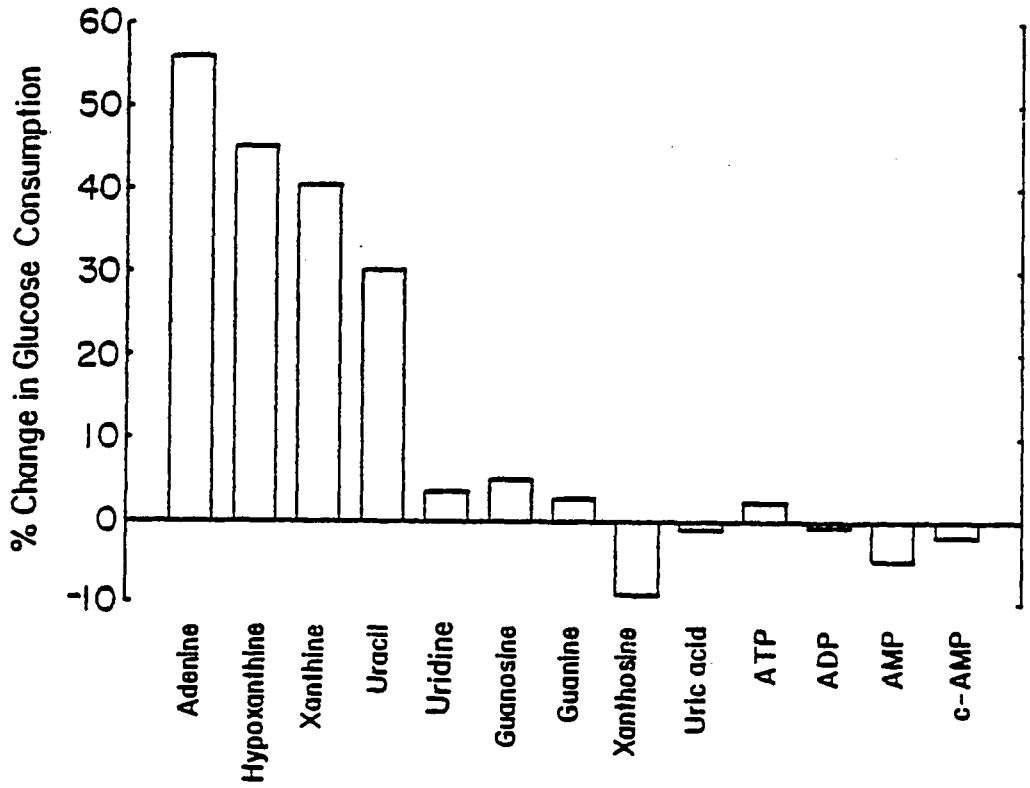

Figure 7. The effects of various nucleotides, nucleosides and purine bases on cow red cell glycolysis. — The percent stimulation refers to the elevated level of glycolysis seen in glucose + purine compared to glucose alone. The results are means from at least three experiments. Increased glucose consumption was significant at $p < .02$ for adenine, hypoxanthine, xanthine and uracil.

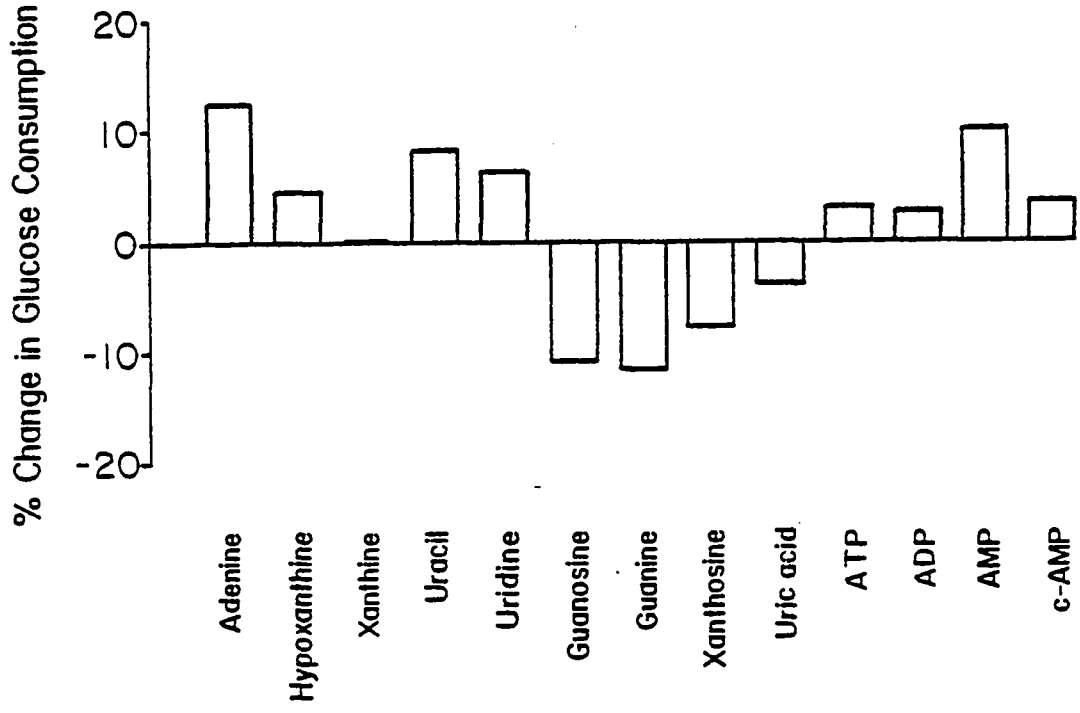

Figure 8. The effects of various nucleotides, nucleosides and purine bases on calf red cell glycolysis. — The experimental conditions are the same as Figure 7.

To ascertain the underlying mechanism for this stimulatory role of purines and pyrimidines, membrane permeability to glucose and the activities of glycolytic enzymes were examined. The flux of 3-O-methylglucose into cow red cells was examined in the presence and absence of adenosine. Radioactive 3-O-methylglucose (0.1 μCi/ml) was added to medium containing cold 3-O-methylglucose in concentrations ranging from 10 μM to 40 mM with or without 5 mM adenosine. The results of the experiment shown in Figure 9 are in agreement with the findings of Hoos, Tarpley and Regen (1972), who found that cow red cells have a very low K_m and V_{max} for 3-O-methylglucose flux. When 5 mM adenosine is added to the medium, the uptake of glucose is unaffected at any 3-O-methylglucose concentration. This result implies that adenosine plays no role in stimulating uptake of 3-O-methylglucose.

The above data indicated that stimulation of glycolysis by purines or nucleosides was probably occurring via some intracellular enzymatic mechanism. Zinkl and Kaneko (1973) have suggested that cow red cell glycolysis is controlled at the hexokinase step. Others have suggested that three enzymes, hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) are the key regulators of mammalian red cell glycolysis (Minikami and Yoshikawa 1966; Rapoport 1968). Stimulation of any one of these three enzymes may be causing the increased glycolytic rate seen in purine and nucleoside stimulation. To this end, HK, PFK and PK were tested with and without the addition of purine or nucleoside. Human red cell enzymes were used as a comparison. The results are summarized in Table 2. Hexokinase in cow red cells has low

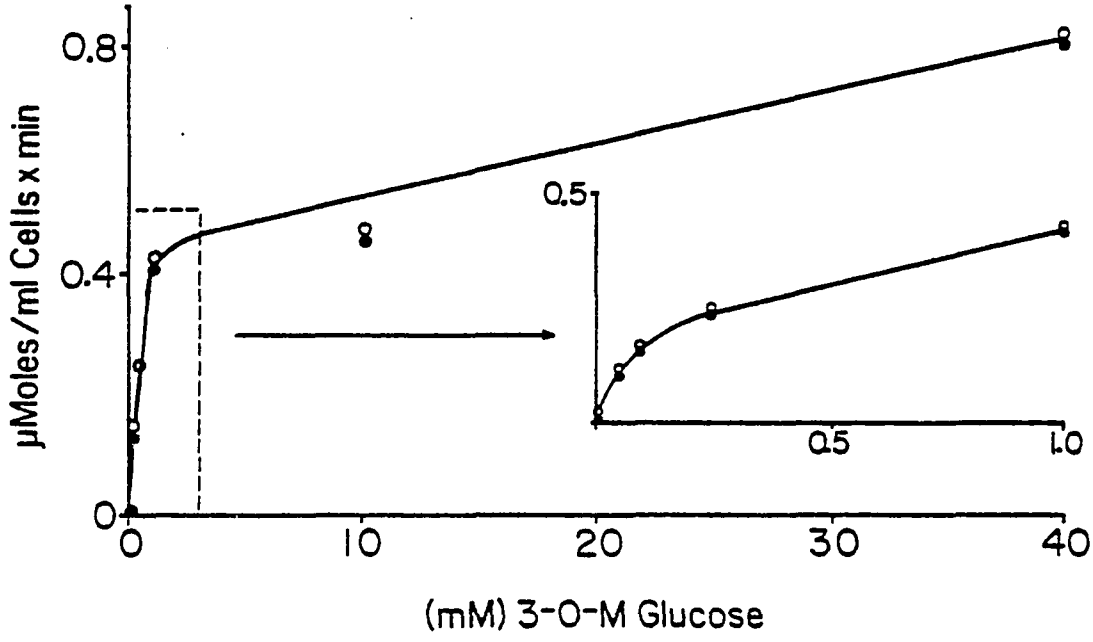

Figure 9. The effect of adenosine on 3-O-methylglucose flux in cow cells. -- Red cells were suspended in media containing 10 µM to 40 mM 3-O-methylglucose augmented with ^{14}C tracer. At frequent intervals, 0.4 ml of the cell suspension was taken out and cells were quickly segregated from medium through 0.8 ml phthalate by centrifugation. Uptake of 3-O-methylglucose was computed from radioactivity determination of cells and specific activity of incubation medium. Uptake with adenosine (●—●); uptake without adenosine (O—O).

Table 2. Effect of several purines on enzymatic activity of cow red blood cells.

	Hexokinase	Pyruvate Kinase (μmoles/min/g Hb)	Phosphofructo-kinase
None	0.489±.028*(16)	12.974±.479 (9)	3.019±.110 (9)
10 μM Adenine	0.620±.045 (4)	13.591±.096 (3)	2.372±.066 (3)
100 μM Adenine	0.726±.107 (6)	13.017±.271 (5)	2.567±.140 (4)
100 μM Inosine	0.788±.028 (4)	13.553±.350 (5)	2.328±.096 (4)
100 μM Hypoxanthine	0.850±.064 (5)	14.357±.337 (5)	2.681±.187 (4)

*$\bar{x}$ ± S.E.M.

( ) = number of determinations

Purines and nucleosides were added directly to the hemolysate.

activity, but in the presence of adenine, hypoxanthine or inosine, the enzymatic activity is found to be significantly accelerated. On the other hand, PFK is little affected by the presence of any of the compounds. This is also true for PK. Human red cell enzyme activity was unaffected by any of these compounds. The values found for enzymatic activities of the three enzymes agree closely with those found by Zinkl and Kaneko (1973) with the exception of pyruvate kinase. The reason why our results are higher is probably due to the way the hemolysate for this assay was prepared. According to the procedure of Beutler (1975), the hemolysate used in the PK assay was dialyzed for four hours prior to use. When undialyzed hemolysate was used, the enzymatic activity was found to be about one-half of that seen in the dialyzed hemolysate.

Figure 10 shows the effect of adenine concentrations on hexokinase activity. Adenine concentrations as low as 10 μM stimulated hexokinase activity by 30%, and 100 μM stimulated hexokinase activity as much as 70%. Thus, while low levels of adenine can stimulate one of the three major regulatory enzymes in the cow red cells, the other two regulatory enzymes were little affected by the addition of nucleosides or purines.

Discussion

The metabolic pathways and fates of purines and nucleosides in red cells have been extensively investigated. We have shown that certain nucleosides, purines and pyrimidines may play an additional role in red cell metabolism other than their usual metabolic role of

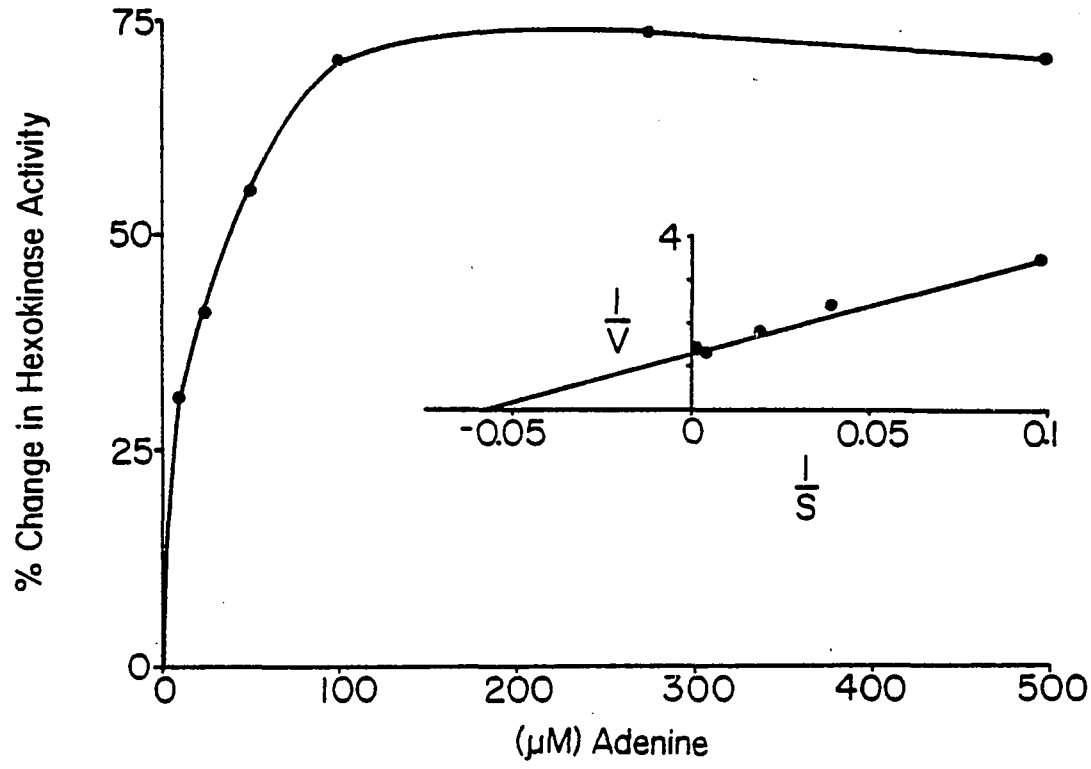

Figure 10. The effect of adenine on cow red cell hexokinase activity. -- Hexokinase was measured in the same manner as described in Table 2. Enzymatic rates were determined for hexokinase in the presence and absence of added adenine. The ordinate is expressed as a percent stimulation of hexokinase activity when hemolysates were incubated with adenine compared with hemolysates without adenine.

supplying carbon skeletons for the synthesis of high energy nucleotides. In the presence of these compounds, the glycolytic rate was elevated from 40 to 80% (Fig. 7) in cow but to a much lesser extent in calf cells. With regard to human red cells, Murphy (1960) reported that addition of inosine to red cells suspended in plasma at a pH of 7.5 produced a depressed glucose consumption but elevated lactate formation. These results implied that when inosine was added to red cells, its metabolism depressed glucose consumption since inosine was providing an additional metabolizable carbon source in the form of pentose phosphate. In cow red cells, inosine itself can neither form lactate nor maintain ATP (Figs. 3 and 4) since the red cell membrane is impermeable to inosine (Duhm 1974). Surprisingly, however, when it is added to glucose-fortified medium, it stimulates glycolysis (Fig. 6). The extracellular effect of inosine in stimulating glycolysis is poorly understood at present. Since it had been reported that extracellular nucleotides could be cleaved on the red cell membrane (Parker and Hoffman 1967) it would seem conceivable that impermeable inosine could be cleaved to ribose and hypoxanthine by the cow red cells. In cow cells exposed to ^{14}C-inosine, we found no evidence for this reaction (data not shown). It is possible that inosine is externally causing the activation of some membrane bound enzyme. It has been shown that hemoglobin-free ghosts contain triose phosphate dehydrogenase (TPD) and phosphoglycerate kinase (PGK) (Ronquist and Agren 1966; Schrier 1966). It is also possible that hexokinase, an enzyme which is stimulated by inosine (Table 2) might be loosely bound to the membrane of the intact cow red cell and be stimulated somehow by external inosine.

With regard to human red cells, Parks and Brown (1973) found an increase in the lactate production in the presence of fluoroadenosine, a nucleoside analog which can be phosphorylated but cannot be deaminated. Their explanation for this phenomenon was that elevating levels of adenine nucleotides would stimulate red cell glycolysis. However, it should be kept in mind that adenine nucleotides can exert both positive and negative effects on the rate-controlling enzymes of the red cell (Rapoport 1968). As with the findings of Parks and Brown (1973), we have also shown that cow red cells elevate their intracellular ATP levels in adenosine and glucose, but not in adenosine alone, implying that these cells require an alternate carbon source in order to synthesize net ATP. Horse red cells have been shown to behave in a similar manner. On the other hand, red cells from animals such as the guinea pig and dog require no additional carbon source (McManus 1974).

It is well known that when no metabolizable substrate is added in incubating red cells, intracellular ATP is depleted at a characteristic rate with half-times from 2 to 8 hours, depending upon the species (Rapoport et al. 1977; Kim and McManus 1971a, 1971b). In cow red cells, the half-time for ATP depletion is less than 1 hour (Fig. 3). This extraordinary depletion rate of ATP is surprising in the light of the finding that the cow red cell lacks ouabain-sensitive membrane Na-K ATPase and has low levels of Ca^{++} and Mg^{++} stimulated ATPase when compared with other animals (Luthra et al. 1976; Gupta, Peterson and Harley 1974).

Unlike the findings of Parks and Brown (1973) on human cells, we have shown that in cow red cells, purine bases and nucleosides exert a direct stimulatory effect on hexokinase (Table 2). It is well known that hexokinase activity is altered by many effectors, including ATP and ADP (Rapoport 1974). We have found that 10 μM adenine in a red cell hemolysate yielded an increase in hexokinase activity (Fig. 10). In order to further confirm that hexokinase was a possible site for purine and nucleoside stimulation, ribose was used in place of glucose in the presence of adenosine. Ribose, which is metabolized to a small extent by cow red cells, avoids the initial glucose phosphorylation step, and we found that adenosine had little effect on the rate or ribose metabolism.

In view of the stimulatory role of purines, a question arises as to the possible physiological significance of this phenomena. It was therefore of interest to determine the levels of purines and nucleosides in cow plasma. We found that the levels are much lower than those used in this study (see Chapter 3). However, when cow red cells are suspended in their own plasma, the glycolytic rate is elevated by 50%, and this effect can be abolished by dialyzing the plasma for 24 hours. These results are presented in the next chapter.

CHAPTER 3

STIMULATION OF COW RED CELL GLYCOLYSIS BY PLASMA

Introduction and Statement of Problem

In earlier reports in which human red cells were suspended in their own plasma, the maintenance of a constant pH was not reinforced (Bartlett and Marlow 1953; Laris 1958) causing elevated glycolytic rates. The stimulated glycolytic rate in plasma under these conditions appears to be accounted for by the pH change in plasma, which tends to undergo alkalinization due to the equilibration of plasma CO_2 with air (Bartlett and Marlow 1953; Murphy 1960). Indeed, Murphy (1960) failed to confirm this plasma stimulation of human red cell glycolysis in strictly pH controlled conditions. In marked contrast, cow red cells exhibited an augmented glycolytic rate in plasma kept at a pH of 7.4. Since it was found that cow red cell glycolysis can be stimulated in the presence of low concentrations of certain purines, pyrimidines and nucleosides (see Chapter 2), it seemed reasonable to suspect the presence of these compounds as the agents responsible for the plasma stimulation. The purpose of this study is to ascertain the underlying mechanism for the stimulation of cow red cell glycolysis by the plasma.

Materials and Methods

Sources and Preparation of Blood and Plasma

Blood was obtained from animals housed at The University of Arizona Farm or the Arizona Health Sciences Center, as described in the last chapter. Red blood cells were spun down in a Sorvell GLC centrifuge at 4°C and the upper two-thirds of the plasma was saved. The white buffy coat, along with the remaining plasma, was aspirated and discarded. Red cells were thoroughly washed in isotonic saline prior to use.

Metabolic Studies

Prior to addition of red blood cells, plasma was allowed to equilibrate with air at 37°C. During incubation, pH was checked every half hour, and readjusted to 7.4 with isotonic NaOH or HCl if a deviation greater than 0.1 units from 7.4 was observed. Cell extraction procedures and determination of glucose as well as lactate were the same as described in the previous chapter.

Ion-exchange and Thin Layer Chromatography of Nucleosides and Purines in Plasma

Plasma purines and nucleosides were determined using ion-exchange chromatography according to the methods of Bartlett (n.d.). After deproteinating the plasma with 10% trichloroacetic acid (TCA), the resultant supernatant was extracted four times with 4 volumes of ether to remove TCA. The extract was loaded onto a Dowex 50 x 8 resin (200-400 mesh), 1 x 20 cm column. The column was eluted with 1000 ml

of a linear gradient of 0 to 4 N HCl. The column eluant was read at 260 mμ, and those fractions showing appreciable absorbance were further analyzed by scanning over the entire ultraviolet range (240-320 mμ) at pH's of 2, 7, and 12, in order to identify the eluted compound. The absorption profile was compared with those of the known standards (Bartlett n.d.).

Plasma levels of nucleosides and purines were also measured, using thin layer chromatography as described by Akaoka, Nishizawa and Nishida (1975) or by Bockman, Berne and Rubio (1976). In the method described by Akaoka et al. (1975), blood was rapidly cooled, centrifuged and the plasma separated from the erythrocytes. The plasma was deproteinized with 5% perchloric acid (PCA) (2:3 v/v) and the supernatant neutralized with 5% KOH. After centrifugation to remove the precipitated salt, the supernatant was lyophilized to dryness, and the dry powder resuspended in 0.6 ml of distilled water. All the supernatant fluid was spotted on the bottom of a cellulose thin layer chromatographic plate (TLC) (Kodak), and the plates were developed in ascending fashion in a closed tank using a solvent consisting of 95% ethanol, 1 M ammonium acetate, pH 7.5 (75:30 v/v). The elution position of standards of hypoxanthine, xanthine and uric acid were identified by visualization under ultraviolet light. In the method described by Bockman et al. (1976), at least 100 ml of blood was collected in heparinized tubes and then immediately poured into equal volumes of ice cold saline. This mixture was then spun down at 1000 x g within 5 minutes and the plasma-saline mixture separated from the red cells.

The plasma proteins were precipitated with enough 5 N perchloric acid (PCA) to give a final concentration of 0.5 N PCA. The samples were then centrifuged at 14,000 x g in an International refrigerated centrifuge (Model B-20) and the supernatant fractions neutralized with 5.63 N K_2CO_3. The resulting precipitate was spun down and the neutralized supernatant was saved. Adenosine, inosine, hypoxanthine and xanthine in this neutralized extract were adsorbed onto Norit charcoal (5 mg for each ml of plasma). After a short centrifugation, the supernatant was discarded and the charcoal was then eluted with two successive 15 ml volumes of 10% pyridine in 50% aqueous ethanol. The eluents were air dried, suspended in 0.4 ml water and spotted onto TLC plates. The plates were developed in 15% ethanol at 4°C, with the necessary standards spotted on one side. In both methods, the plasma compounds were identified under ultraviolet light by their position on the TLC plate which was compared with the standards. To quantitate the amount of compounds in the plasma, areas on the plates corresponding to the known standards were scraped off and eluted with three successive 10 ml volumes of 50% ethanol. The eluents were air dried and resuspended in water. The nucleosides and bases were assayed by conversion to uric acid according to the method of Kalckar (1947). Standards were run to test for recovery in all experiments.

Sources of Materials

All materials were bought from the same vendors as in the previous chapter.

Results

Rapoport (1968) reported that red cells suspended in balanced salt solution decreased their medium pH by 0.2 to 0.5 pH units per hour of incubation at 38°C. In this study when calf or cow red cells were suspended in buffered balanced salt solution containing glucose, the pH slowly decreased as a function of time due to the increasing concentration of lactic acid produced as a by-product of anaerobic metabolism, as shown in Figure 11. In media containing calf and cow red cells, this decrease is about 0.5 and 0.3 pH units per hour respectively, with the more rapid decline in calf medium due to the higher glycolytic rate. When washed, calf or cow red cells were added to their own plasmas, the medium pH rapidly rose to 8.0 for the calf red cell medium, and 8.2 for the cow (Fig. 11). The calf medium rose no further, probably due to extensive hemolysis that occurred by this time. Zeidler and Kim (in press) have shown that elevation of the internal pH of calf red cells, but not cow cells, produces a rapid hemolysis.

Figure 12 shows the effect of pH on cow red cell glycolysis. The desired pH was maintained within 0.1 pH units by titrating with isotonic acid or base during a seven hour incubation. The results are expressed as a percentage of control at pH 7.4, and the numerical values for initial rates at pH 7.4 are given in Table 3. At pH 8.2, the glucose consumption rate is nearly 55% greater than at 7.4, but this is less than several other species, including rats and humans which increase their glycolytic rate 82% and 164%, respectively (Rapoport 1968).

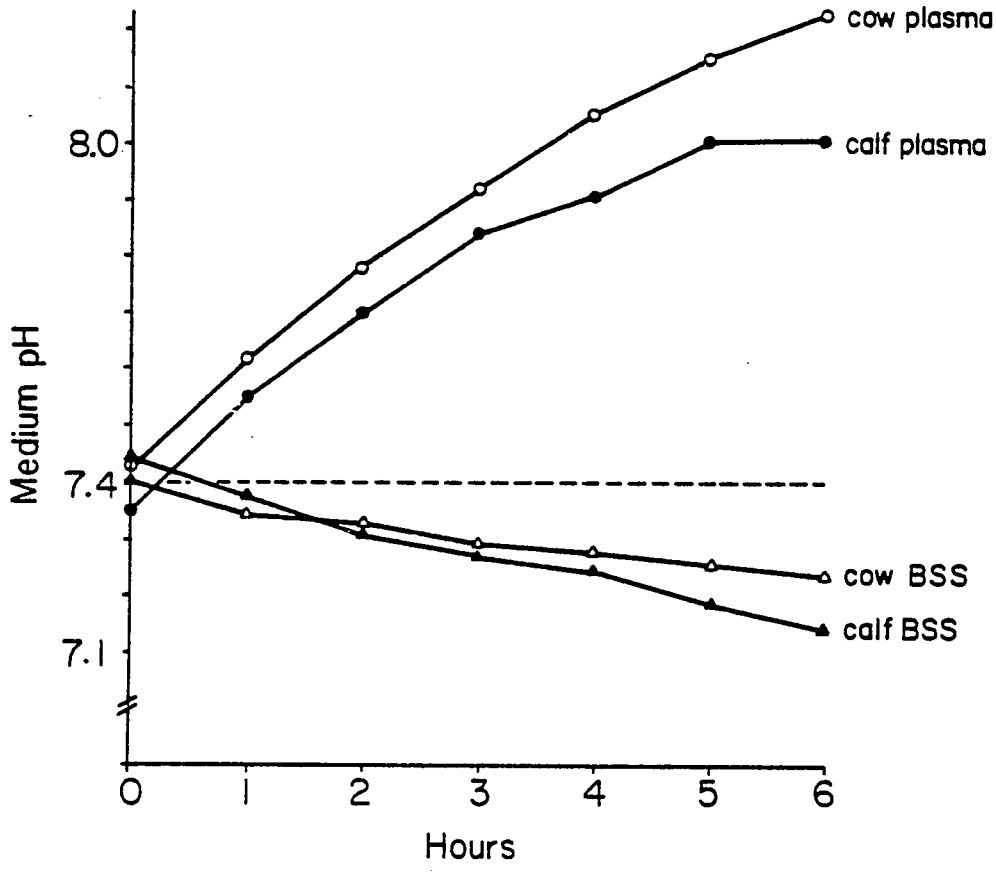

Figure 11. A comparison of the pH change in plasma vs balanced salt solution containing calf or cow red cells. -- Red cells were added to incubation medium consisting of 10 mM KCl, 150 mM NaCl, 10 mM glucose, 10 mM Na phosphate buffer, pH 7.4 or to plasma fortified with additional glucose to give a hematocrit of 10-15%. Calf cells in their own plasma (●—●); calf cells in incubation medium (▲—▲); cow cells in their own plasma (O—O); cow cells in incubation medium (△—△). After 4 hours of incubation in calf plasma, the calf cells began to hemolyze. Equivalent results were seen in two other experiments.

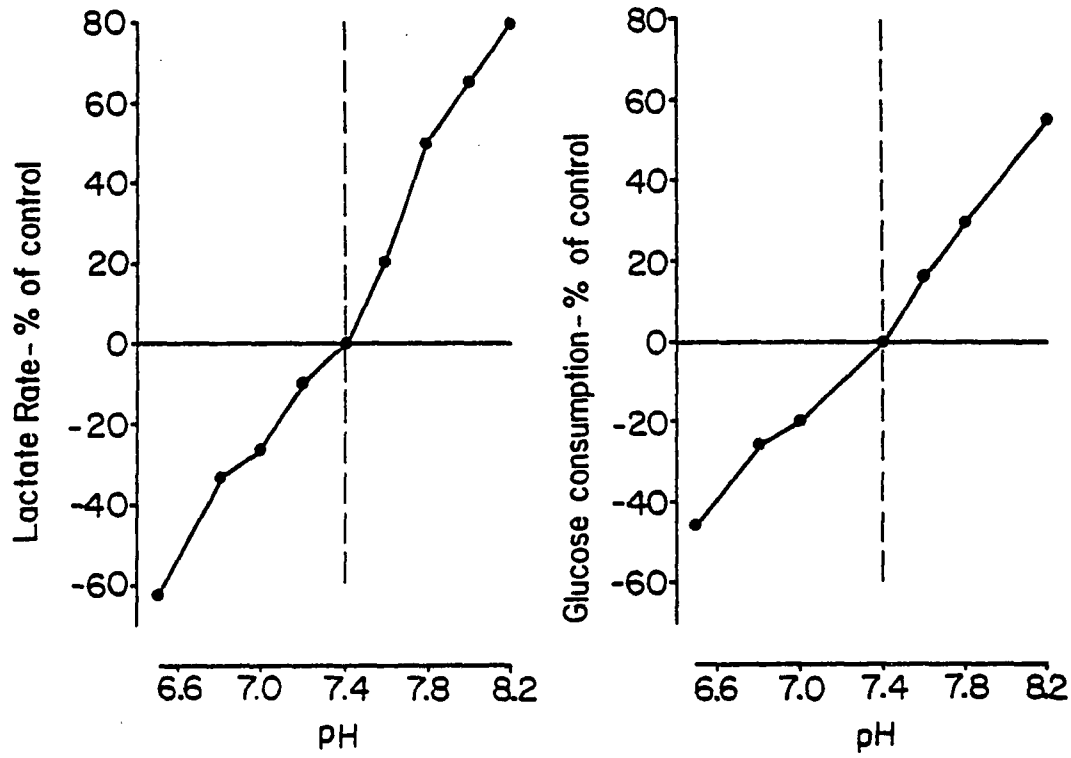

Figure 12. Effect of pH on cow red cell glycolysis. -- The experimental conditions are the same as in Figure 11. Typical results seen from three experiments.

Table 3. Comparison of calf and cow red cell glucose consumption and lactate formation in plasma vs balanced salt solution.

	Calf		Cow	
	BSS	Plasma	BSS	Plasma
	(μMoles/ml cells/hr)			
Glucose Consumption	2.05±.49* (6)	2.17±.51 (6)	0.60±.04 (6)	0.89±.06** (6)
Lactate Formation	3.28±.70 (6)	3.39±.51 (6)	0.87±.05* (6)	1.32±.11** (6)
Ratio (Lactate:Glucose)	1.60	1.56	1.45	1.55

*$\bar{x}$ ± S.E.M.

** ($p < .02$)

( ) = Number of experiments

Balanced Salt Solution: 5 mM KCl, 160 mM NaCl, 10 mM Na Phosphate buffer, pH 7.4, 5 mM glucose.

Hematocrit - 10-15%

Temp. = 37°C

Also shown in Table 3 are the effects of plasma on calf and cow red cell glycolysis. Although each plasma had a glucose concentration between 1 and 3.5 mM, additional glucose was added so that adequate glucose concentrations were maintained over a seven hour period with concomitant maintenance of plasma at pH 7.4. As in other species (Gross and Hurwitz 1958; Lachhein et al. 1961; Kim,McManus and Bartlett 1972), red cells obtained from newborn calf had a much higher glycolytic rate than did cow red cells (Table 3). In contrast to calf red cells, which displayed identical glycolytic rates in both balanced salt solution or plasma, cow red cells significantly ($p < .02$) increased their glycolytic rate nearly 50% when suspended in their own plasma.

To discover the chemical composition of the plasma activator, cow plasma was dialyzed for 4 to 24 hours against isotonic, buffered incubation medium at 4°C, and the dialyzed plasma was fortified with 5 mM glucose in order to replace the glucose lost during dialysis. Figure 13 shows the effect of dialyzed plasma on glycolysis. The levels of lactate in undialyzed cow plasma were quite variable from animal to animal and exceeded the intracellular lactate concentration. Thus, for the purpose of comparison, lactate levels were normalized against the initial cell levels. Figure 13 shows that plasma elevated the lactate formation rate when compared with incubation medium, and that red cells suspended in plasma dialyzed for 24 hours produced lactate no faster than incubation medium. The 4 hour dialyzed plasma (not shown) gave rates which were intermediate to the two extremes shown in Figure 13. These results imply that the stimulatory agent is a small molecule.

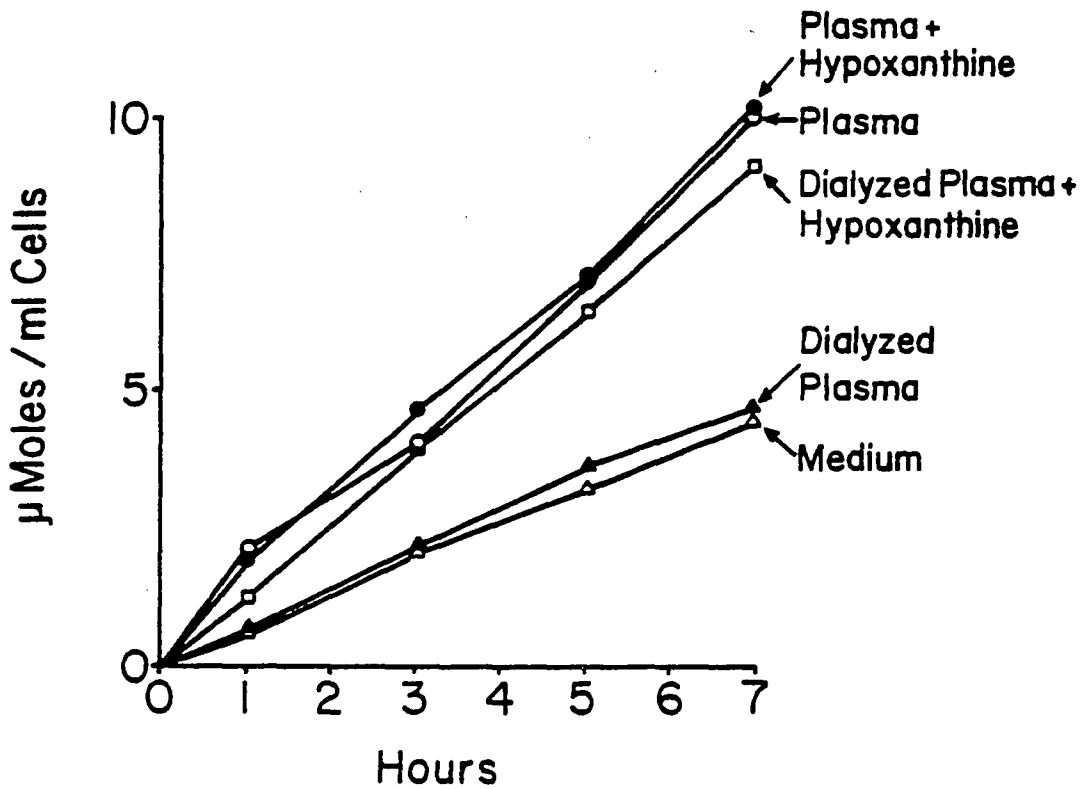

Figure 13. Effect of dialyzed plasma on glycolysis. -- Plasma was dialyzed for 24 hours against buffered incubation medium. After dialysis, 5 mM glucose or 1 mM hypoxanthine was added depending upon the experiment. In the 24 hour dialysis experiment, fresh blood was drawn from the same animal that provided the plasma the day earlier. Dialyzed plasma (▲—▲); undialyzed plasma (○—○); balanced salt medium (△—△); dialyzed plasma + 1 mM hypoxanthine (□—□); undialyzed plasma + 1 mM hypoxanthine (○—○). Equivalent results seen in four other experiments.

If the stimulating factor(s) in plasma were purines, then elevated glycolytic rates should be restored by readding this in vitro stimulator to the dialyzed plasma. The addition of 1 mM hypoxanthine, a non-metabolizable purine, to dialyzed plasma elevated the glycolytic rate to that seen in untreated plasma (Fig. 13). However, when hypoxanthine was added to non-dialyzed plasma, the rate was unaffected, suggesting that the plasma activator operates at its maximal rate.

To determine whether plasma purines play a stimulatory role, plasma was treated with activated Norit charcoal. As shown in Table 4 charcoal-treated plasma failed to stimulate cow red cell glycolysis. As an alternative approach, plasma was first boiled for 30 minutes to denature the proteins which were then spun down and the supernatant fraction was tested for its stimulatory effect. In Table 5, it can be seen that while the boiled plasma has a lower lactate formation rate than that seen in normal plasma, it still elevated glycolysis 33% over the balanced salt solution control. The reason for the drop from the plasma control may be due to loss of activator in the protein precipitate or by heat destruction.

To determine the plasma levels of purines and nucleosides, both ion-exchange and thin layer chromatographic (TLC) procedures were employed. For the ion-exchange column, at least 400 ml of blood was obtained at the local slaughterhouse, and rapidly chilled in small aliquots. The plasma was separated from the cells, deproteinized, loaded on an ion-exchange Dowex column and eluted with a linear gradient of 0 to 4N HCl, according to the method of Bartlett (n.d.).

Table 4. Effect of charcoal extracted plasma on stimulation of glycolysis.

Procedure	Lactate Rate (μMoles/ml cells/hr)	%
BSS* Control	0.79	-
Plasma Control	1.21	53
Charcoal Extracted Plasma	0.79	0

*BSS: Balance salt solution

5 mg of Norit charcoal was added to each ml of plasma, the mixture was stirred for three hours at 4°C and the charcoal was spun out. The plasma supernatant was glucose-fortified and then washed red cells were added to make a final hematocrit of 10-15%. Controls consisted of red cells suspended in glucose fortified incubation medium, or untreated plasma. Rates were calculated using least squares regression analysis, with $r^2 > .95$ in all cases. Typical results from one out of three experiments shown above.

Table 5. Effect of boiled plasma on stimulation of glycolysis.

Procedure	Lactate Rate (μMoles/ml cells/hr)	%
BSS* Control	0.66	-
Plasma Control	0.99	50
Boiled Plasma	0.88	33

*BSS: Balanced salt solution

Cow plasma was boiled for 30 minutes, and the resulting precipitate was removed by centrifugation.

Figure 14 shows that cow plasma had both 0.30 nmoles of xanthine/ml of plasma, .60 nMoles of hypoxanthine/ml of plasma and two unknown peaks which had ultraviolet absorption patterns exhibiting a maxima at 251.5 nm, but did not have any characteristics of known purines or nucleosides. Adenosine was seen in very low concentrations using this method. TLC procedures of Akaoka et al. (1975) and Bockman et al. (1976) allowed a similar type of analysis to be performed on smaller quantities of calf, cow and human plasmas. Since small blood samples could be rapidly chilled and the cells separated from plasma within minutes of being drawn, TLC offered the opportunity to determine whether nucleosides existed in cow plasma. Human plasma was found to have higher concentrations of uric acid, xanthine and hypoxanthine than either calf or cow plasma (data not shown). As in the case of ion-exchange chromatography, TLC's of cow plasma showed a rapidly migrating ultraviolet absorbing peak that was absent in both calf and human plasmas. In dialyzed plasma, all of the ultraviolet absorbing areas were lost, implying that the rapidly migrating compound was not an artifact of the TLC preparative method.

In order to quantitate the amount of purines and nucleosides isolated by thin layer chromatography, the regions corresponding to the standards were scraped off the plate and eluted according to the method of Bockman et al. (1976). Enzymatic assays for hypoxanthine, xanthine, inosine and adenosine were carried out, using the methods of Kalkar (1947), and the results of these experiments are summarized in Table 6. When compared with either human plasma (Mills et al. 1976) or dog plasma (Rubio et al. 1969), the concentrations of purines and

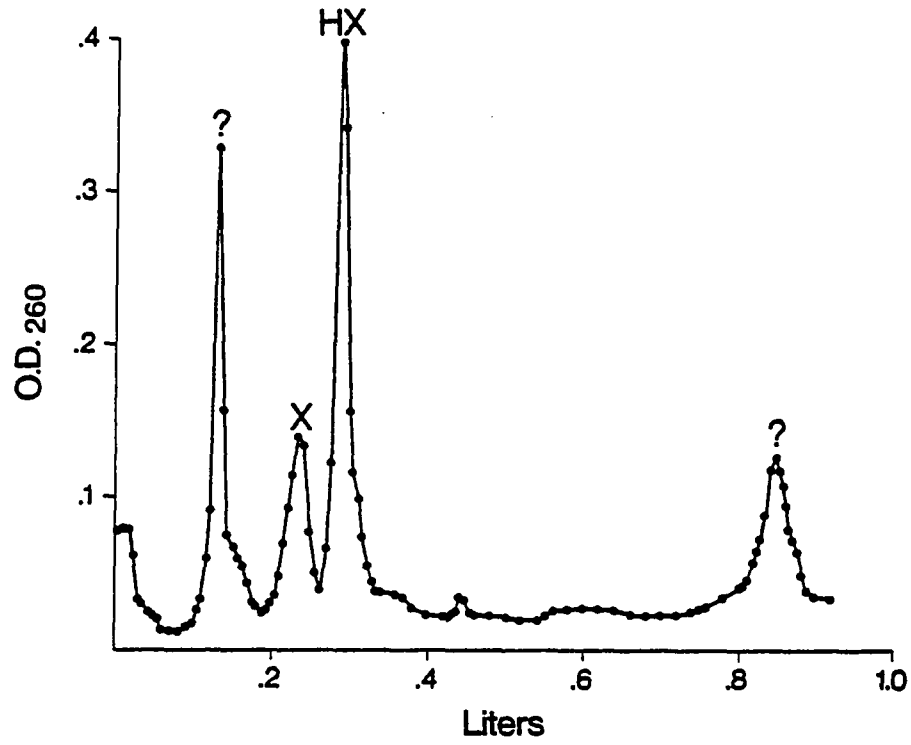

Figure 14. Ion-exchange chromatography of cow plasma. -- 100 ml cow plasma was extracted with trichloroacetic acid (TCA) which in turn was removed by ether. The resulting extract 230 ml was loaded onto a Dowex 50 x 8 resin filled column (1 x 20 cm). The purines were eluted from the column using 1000 ml of a linear gradient of 4 N HCl. The absorbance at 260 mm is plotted against liters through the column. Each compound was identified on the basis of its elution position and its absorption profile in three different pH's over the entire ultraviolet range (200-320 nm). Hypoxanthine = HX; Xanthine = X. Results from one out of six experiments.

Table 6. Levels of nucleosides and bases in calf and cow plasma.

	(nMoles/ml plasma)			
	Xanthine	Hypoxanthine	Inosine	Adenosine
Calf (6)	$0.40 \pm .07$*	$0.60 \pm .07$	$0.14 \pm .04$	$0.09 \pm .01$
Cow (4)	$0.31 \pm .06$	$0.55 \pm .05$	N.D.	$0.05 \pm .02$

*$\bar{x} \pm$ S.E.

( ) = Number of animals

N.D. = not detected

nucleosides in cow and calf plasma are extremely low. These findings imply that none of the identified purines and nucleosides were present in sufficient amount to cause the observed stimulation of glycolysis.

Figure 15 shows that when dog, cat, rabbit, rat, guinea pig and human red cells were suspended in either the balanced salt medium or plasma, the rates of glycolysis were the same, suggesting that other animal plasmas lacked glycolytic stimulators. On the other hand, the stimulators may have been present in all plasmas but the cells were not responsive to these compounds. In order to test this possibility, cross plasma experiments were performed, with the results shown in Figure 16. Apparently, cow red cells can be "turned on" only by their own plasma. Dog plasma hemolyzed cow red cells within minutes, while human plasma hemolyzed these cells at a slower rate. Calf plasma had little effect on cow red cells, implying that calf plasma lacked the stimulator. When calf red cells were suspended in cow plasma, the glycolytic rate was again unstimulated, implying that the calf cells also lacked the internal machinery necessary for producing stimulation of glycolysis. When cat, rat, guinea pig, and dog red cells were added to cow plasma, they all rapidly hemolyzed within 10 minutes.

To determine whether <u>in vitro</u> nucleoside and purine stimulated glycolysis was unique to cow red cells, several other animal red cells were also tested with adenine. Figure 17 summarizes the results. Sheep cells, which are often compared to cow red cells, are unaffected by 1 mM adenine. However, cat cells are stimulated to a small extent both in glucose consumption and lactate production. Thus, stimulation

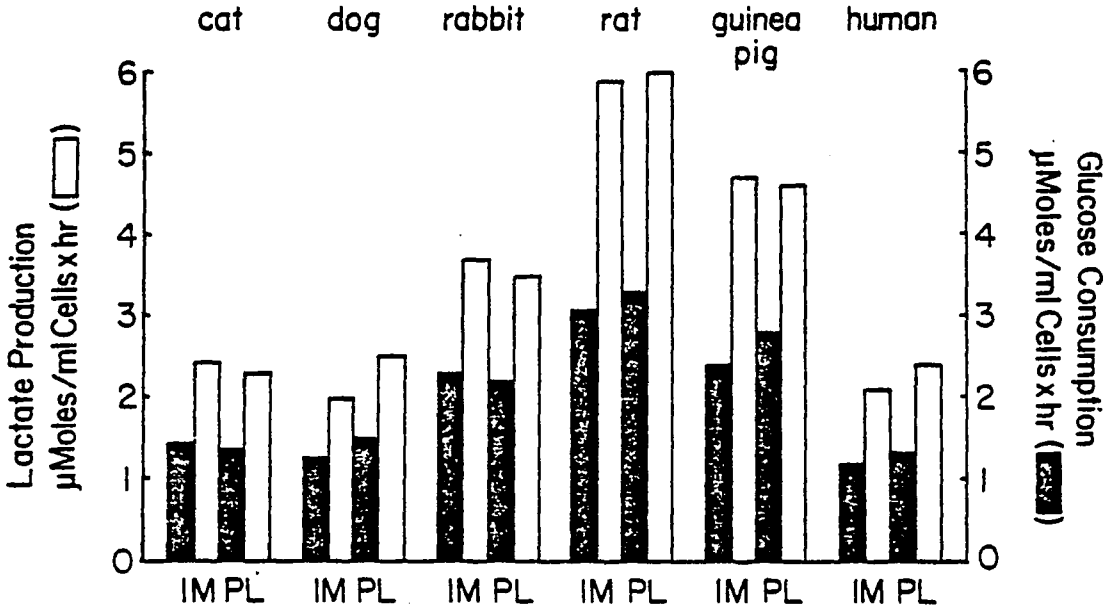

Figure 15. A comparison of glycolysis of red blood cells in the balanced salt medium (IM) versus plasma (PL) from a variety of animals.

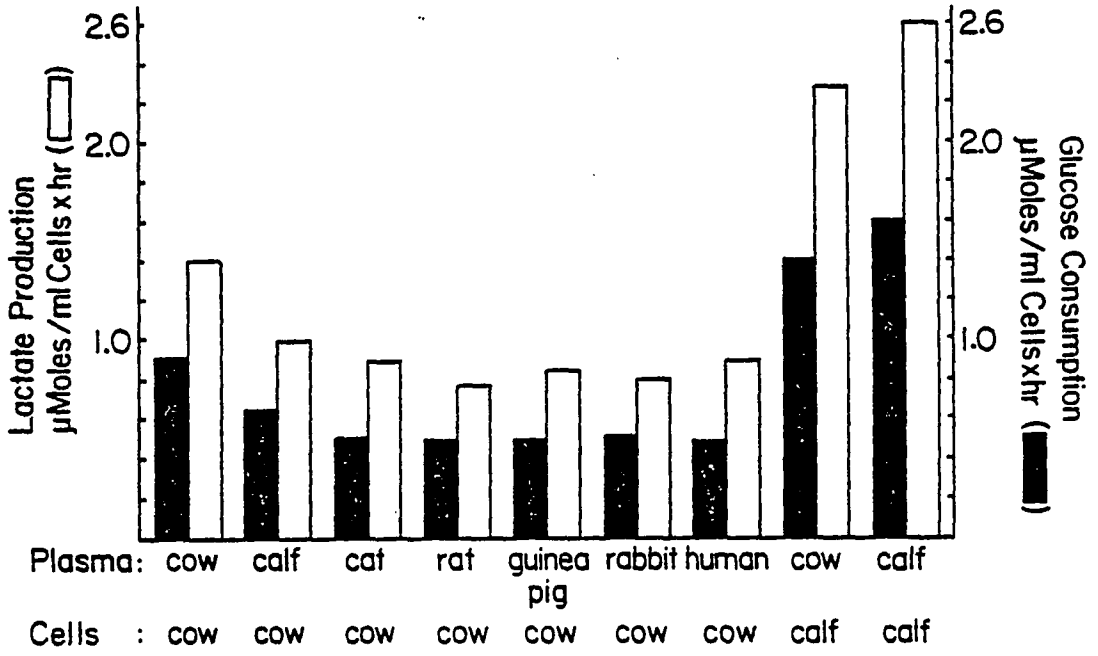

Figure 16. Calf and cow red cell glycolysis in plasmas derived from several different species. — Rapid hemolysis of red cells occurred when dog, cat, rat, rabbit, or guinea pig red cells were suspended in cow plasma.

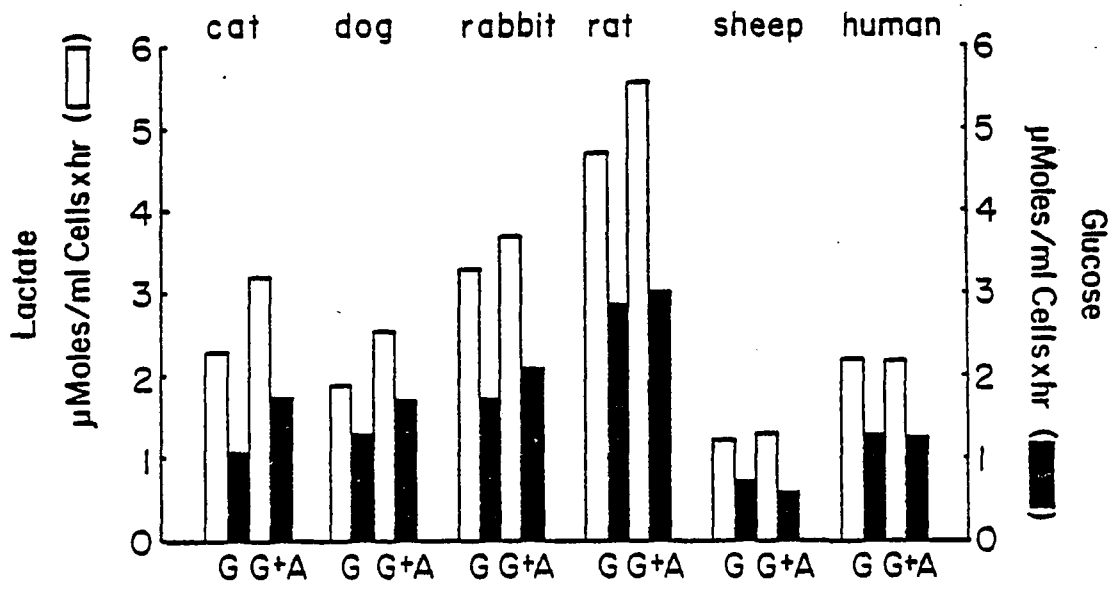

Figure 17. Effect of adenine on glycolysis of red blood cells obtained from a variety of animals. — G refers to glucose fortified balanced salt solution (BSS), and G + A refers to BSS with 1 mM adenine.

of glycolysis is not limited to cow red cells, but appears to occur in other animals as well.

Discussion

In several early glycolytic studies utilizing plasma, the elevated glycolytic rate appears to be accounted for by the increase in plasma pH (Bartlett and Marlow 1953; Laris 1958; Rapoport 1968). In strictly pH controlled plasma, glycolytic rates of human red cells were the same as cells suspended in balanced salt solution (Murphy 1960). In contrast, cow red cell glycolysis was found to be stimulated by its own plasma (Table 3). This stimulatory effect of plasma on cow cells appears to be somewhat unusual, since other species including the dog, cat, rabbit, rat, guinea pig and human did not respond to their own plasma. Dialysis of cow red cells themselves for 6 hours against balanced salt solution still produced the same stimulation upon exposure to plasma, suggesting that no unusual intracellular factors are required. In view of the findings reported in the previous chapter in which cow red cell glycolysis was shown to be augmented by a variety of purines and nucleosides, it seemed reasonable to suspect those purines and nucleosides in the plasma as agents responsible for the plasma stimulation. Indeed, dialysis or charcoal treatment of plasma resulted in the inactivation of this unique stimulation. However, actual quantitation of the purines and nucleosides in the plasma revealed that these compounds are present in 1/100 the concentration needed for maximal stimulation of cow red cell glycolysis in vitro. Moreover, cow red cells suspended in human plasma containing 10 times higher

concentrations of purines and nucleosides failed to stimulate cow red cell glycolysis, suggesting that specific cow plasma compounds other than purines must be participating in this stimulatory phenomena. Unidentified compounds were found by both TLC and liquid chromatographic methods in cow but not calf or human plasmas. Isolation of these compounds was attempted to determine whether these compounds were responsible for stimulation of cow red cell glycolysis. The highly acidic eluent from the first peak of the column was neutralized with a strong base, diluted to isotonic strength, and then added to incubating cow red cells. This peak failed to stimulate the red cell glycolytic rate. However, the possibility for acid inactivation of this compound cannot be ruled out. The second unknown peak was not tested due to excessive acidity. In yet another attempt, the rapidly migrating UV abosrbing spot on the TLC plate was scraped off, extracted and added to BSS. It was found that cow red cells responded to a small extent to this extract, but control extracts from unspotted, developed TLC plates also elevated cow red cell glycolysis, thereby obscuring our initial results (Table A-8, Appendix A). Finally, since charcoal eliminated the stimulatory effect of plasma, extractions of plasma treated charcoal were attempted in order to recover the stimulator. This extract could also stimulate cow red cell glycolysis, but again, when controls were run using BSS treated charcoal, glycolytic rates were also elevated (Table A-9). While it could not be ruled out that together all of these purines and nucleosides including the unknowns could act synergistically to elevate cow red cell glycolysis, there

also exists a possibility that the stimulator may be non-UV absorbing compounds.

It has been shown that nucleosides and purines formed in tissues including the heart (Katori and Berne 1966; Berne 1972), brain (Berne and Curnish 1972), and liver (Lerner and Lowy 1974) may be transported by red cells to other regions of the body (Lerner and Lowy 1974). Adenosine, which is a potent vasodilator, has been found in the coronary venous blood as well (Berne 1972) and is released into the circulation during hypoxia (Liu and Feinberg 1971). If adenosine and other purine bases indeed participate in the stimulatory effect in vivo, one potential metabolic benefit could be the augmented production of ATP by the combined reactions of stimulation of glycolysis and the salvage pathways. It should be recalled that cow red cells undergo an unusually rapid breakdown of ATP when incubated without metabolizable substrate. What factors are responsible for this extraordinarily rapid breakdown in cells almost devoid Na-K and Ca^{2+} ATPase is not known (Luthra et al. 1976).

Calf red cells, which have higher glycolytic rates, did not respond to exogenously added purines or their own plasma. Both the glycolytic rate and intracellular ATP levels fall rapidly in the growing animal. It is not known when the need for the plasma stimulator arises during this neonatal period. The exchange of plasmas from calf and cow failed to stimulate glycolysis in either of these cells, suggesting that: (a) calf red cells lacked the internal machinery necessary for reacting to the plasma stimulator; and (b) calf plasma lacked

the plasma stimulator. Other ruminants have low rates of glycolysis and intracellular ATP concentrations. It is presently unknown whether all ruminants have this particular type of glycolytic stimulation, although sheep are known to be unaffected by exogenously added purines.

APPENDIX A

ADDITIONAL EXPERIMENTS

The additional data collected during the research period for my Ph.D. degree are presented in this appendix. These collateral data are related to the results seen in Chapters 1 and 2 but were not included in them.

The first chapter showed intracellular ATP levels in the presence of various substrates and it was determined that 5 mM glucose or 5 mM adenosine could maintain, but not produce a net synthesis of ATP. Furthermore, after the plasma stimulation of the cow red cell glycolysis waa discovered (third chapter) additional studies were performed to determine if increased glycolytic rates caused by plasma could elevate cellular ATP levels and if higher concentrations of adenosine caused net ATP synthesis. Table A-1 shows the intracellular ATP concentrations of cow red cells in various substrates and the plasma. As seen previously, when incubated in balanced salt solution (BSS) alone, cow red cells undergo a rapid loss of ATP (Table A-1) and addition of glucose prevents this loss. Given an alternative carbon source (5 or 20 mM adenosine + glucose), a net synthesis of ATP occurred. It should be pointed out that 20 mM adenosine alone produces a small amount of net ATP synthesis, whereas 5 mM adenosine could not. McManus (1974) has shown that horse erythrocytes are incapable of synthesizing net ATP when 5 mM adenosine is present in BSS, and he attributes this to low adenosine kinase activity. Apparently, in the cow the adenosine

Table A-1. ATP levels in cow red cells suspended in various metabolic substrates.

Substrate	ATP Concentration (μmoles/ml cells) Incubation Time (Hours)		
	0	3	7
Control	.496	.138	0
Glucose (5 mM)	.443	.520	.417
Glucose + Adenosine (20 mM)	.314	.658	2.118
Adenosine (5 mM)	.395	.341	.394
Adenosine (20 mM)	.329	.544	1.073
Plasma + Hypoxanthine (1 mM)	.386	.308	.350

All suspending media were composed of a balanced salt solution consisting of 5 mM KCl, 10 mM Na phosphate buffer, pH 7.4 and NaCl to produce an isoosmotic solution of 37°C. Hematocrits were between 10-15%. Plasma was dialyzed for 24 hours against BSS at 4°C prior to use, and 5 mM glucose was added to replace that which is lost during dialysis.

permeability limits ATP synthesis, rather than this enzyme activity. As expected, plasma fortified with hypoxanthine fails to increase cellular ATP levels, implying that: (1) both plasma and hypoxanthine are unable to support a net ATP synthesis; and (2) that cow red cells are unable to convert IMP to AMP.

ATP data for calf cells were not previously shown. Table A-2 shows calf red cell ATP production in a variety of substrates. Calf red cells consume ATP less rapidly than do cow cells, and like cow cells, are not capable of synthesizing ATP from purines or nucleosides other than adenine or adenosine. They also appear to convert adenosine to ATP with relative ease, either with or without an alternate carbon source, and metabolize adenine to ATP with marked difficulty.

Although glucose maintains calf and cow ATP levels, it was of interest to see if there was a loss of ADP and AMP from the intracellular pool. Calf and cow red cells incubated in BSS fortified with 5 mM glucose maintained their ATP (Table A-3), but both showed a substantial drop in ADP and AMP levels over the first 3 hours. However, since these compounds make up 1/5 and 1/50 of the total nucleotide pool respectively, the drop in the total nucleotide concentration is small. These results imply that cow red cells are efficient in salvaging and maintaining adenine nucleotide moieties.

Cow red cells are impermeable to inosine (Duhm 1974). Table A-4 shows that inosine alone is not metabolized by cow red blood cells, but when added to glucose-fortified BSS, it causes a significant elevation in glycolysis. Since it is impermeable, these data suggest that

Table A-2. ATP levels in calf red cells suspended in various metabolic substrates.

Substrate	ATP Concentration (μmoles/ml cells) Incubation Time (Hours) 0	3	7
Control	.585	.456	.259
Glucose (5 mM)	.591	.697	.633
Glucose (5 mM) + Adenosine (10 mM)	.648	1.914	2.309
Glucose (5 mM) + Inosine (10 mM)	.639	.705	.730
Adenosine (10 mM)	.679	1.140	1.053
Inosine (10 mM)	.629	.483	.327
Ribose (3 mM) + Adenosine (10 mM)	.604	1.546	1.423
Glucose (5 mM) + Adenine (1 mM)	.513	.569	.580

Methods same as Table A-1.

Table A-3. Concentration of adenine nucleotides in cow and calf red cells suspended in glucose-fortified balanced salt solution.

Incubation Time (Hours)	Calf (μmoles/ml cells)			Cow (μmoles/ml cells)		
	0	3	7	0	3	7
AMP	.023	.017	.010	.011	.004	.003
ADP	.128	.059	.052	.064	.039	.036
ATP	.530	.510	.487	.452	.463	.464

ADP and AMP were measured fluorometrically using the methods of Beutler (1975).

Table A-4. Inosine stimulation of cow red cell glycolysis.

	Lactate	Glucose	Ratio
	(μmoles/ml cells x hr)		
Glucose (5 mM)	$0.85 \pm .05$*(6)	$0.56 \pm .05$ (3)	1.52
Inosine (10 mM)	0	-	-
Glucose (5 mM) + Inosine (10 mM)	$1.14 \pm .05$ (6)	$0.82 \pm .20$ (3)	1.39

*$\bar{x} \pm$ S.E.

( ) = no. experiments

Glucose consumption and lactate production were measured over a seven hour period, and the rate of glycolysis was calculated using linear regression analysis with an $r^2 > .95$ in all cases. Ratio refers to the lactate:glucose ratio.

inosine acts at an extracellular site and it is possible that other permeable stimulators such as adenine or hypoxanthine may also act at the cell membrane externally. Figure A-1 shows that human red cells, which are permeable to inosine, have glycolytic rates that are not elevated except for the small increase in lactate formation due to the metabolism of inosine to hypoxanthine and ribose 1-phosphate, which enters the pentose cycle and glycolytic pathways. Since inosine appeared to act at an extracellular site to stimulate cow red cell glycolysis, it is possible that partial digestion of the red cell membrane would eliminate this effect. To accomplish this the proteolytic enzyme pronase was used on both human and cow cells. The cells were pretreated with pronase, an enzyme which is known to hydrolyze the so-called band 3 proteins expanding across the entire thickness of the membrane. The results are shown in Table A-5. Although overall glycolytic rates for both cow and human red cells were lowered, there was no change in the stimulation of cow red cell glycolysis caused by purines and nucleosides. Human cells again failed to be stimulated by purines under these conditions. More work will need to ascertain the membrane sites involved in this stimulation.

As shown previously, adenosine is a potent stimulator of cow red cell glycolysis. Adenosine also elevated the glycolytic rate of calf red cells (upper curve of Fig. A-2) but this increase can be entirely ascribed to the lactate produced from adenosine (lower curve, Fig. A-2).

Purine compounds may increase glycolysis by elevating the intracellular concentration of glucose. If glucose availability is a

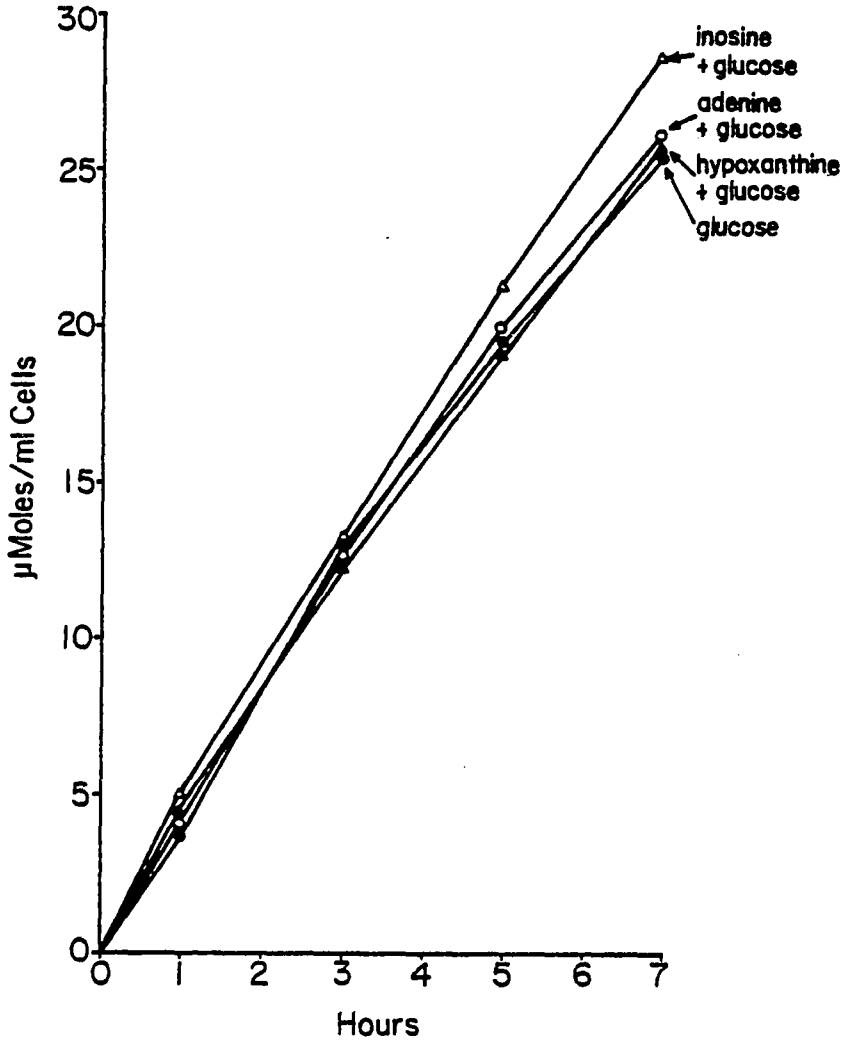

Figure A-1. Lactate production in human red cells suspended at a hematocrit of 10-15% in BSS fortified with 5 mM glucose and various stimulators of cow red cell glycolysis. -- The composition of BSS is given in Table A-1.

Table A-5. The effect of pronase on human and cow red cell glycolysis.

Substrate		Glucose Consumption (μmoles/ml cells x hr) W/O+	Glucose Consumption (μmoles/ml cells x hr) W+	Lactate Production (μmoles/ml cells x hr) W/O	Lactate Production (μmoles/ml cells x hr) W	Ratio W/O	Ratio W
Control	H*	2.16	1.71	3.76	3.08	1.74	1.80
	C*	0.45	0.36	0.68	0.53	1.51	1.47
Adenine (1 mM)	H	2.14	1.70	3.79	3.17	1.77	1.86
	C	0.59	0.52	0.88	0.75	1.49	1.44
Inosine (10 mM)	H	2.01	2.09	4.06	4.09	2.02	1.96
	C	0.50	0.48	0.79	0.66	1.58	1.32
Hypoxanthine (1 mM)	H	2.10	1.67	3.76	3.28	1.79	1.97
	C	0.53	0.44	0.78	0.68	1.47	1.55

*H = human cells, C = cow cells.
+W/O = without pronase treatment, W = with pronase treatment

Enough red cells were added to a medium containing 150 mM NaCl, 5 mM KCl, 10 mM phosphate buffer, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$ and 1 mg/ml pronase to make a 20% hematocrit. This mixture was incubated at 37°C until initiation of hemolysis, and the reaction was stopped with 0.10% bovine serum albumin in cold saline. For cow red cells, the pronase concentration was increased to 5 mg/ml medium. After completion of incubation, the cells were well washed prior to being added to various substrates. The methods of measuring lactate and glucose are the same as Table 1.

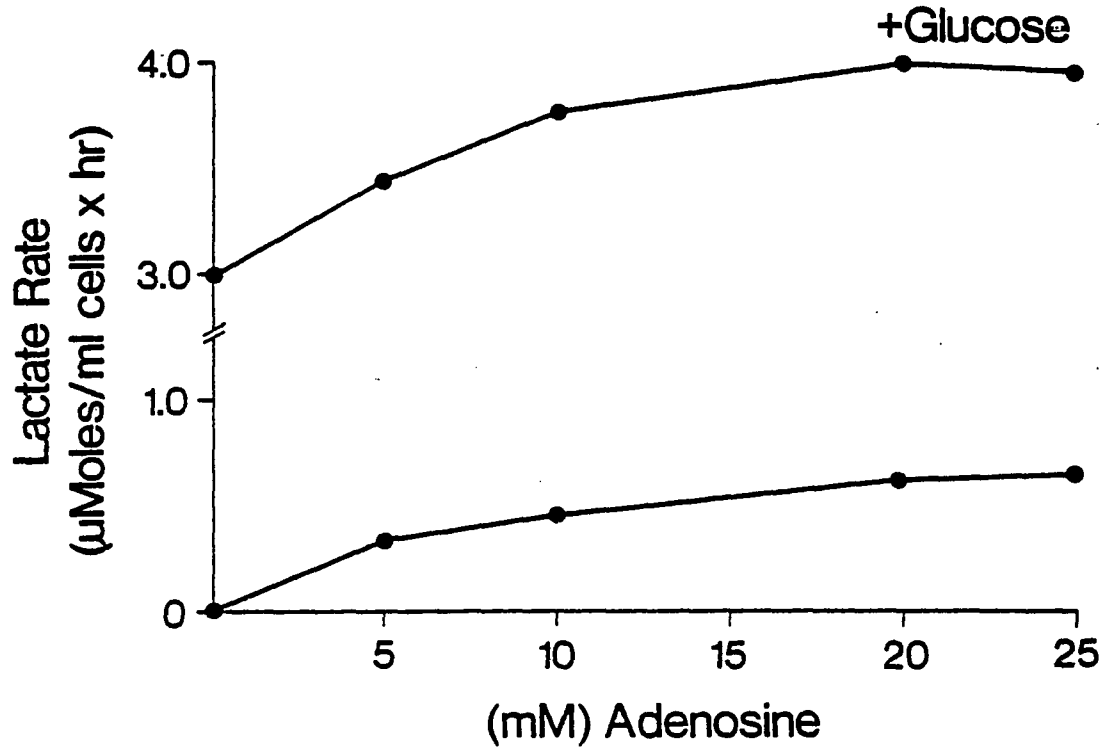

Figure A-2. Lactate formation as a function of adenosine concentration. -- Calf red cells were suspended either in 5 mM glucose (upper line) and various adenosine concentrations up to 25 mM, or various concentrations of adenosine alone (lower line).

rate limiting step in cow red cell glycolysis, then increasing the extracellular concentration of glucose should cause an elevation of the glycolytic rate. Figure A-3 shows that the rate of cow red cell glycolysis is unaltered by the extracellular glucose concentration (open circles). Furthermore, when 10 mM inosine is added to BSS containing varying quantities of glucose, the amount of stimulation caused by inosine is also unchanged (filled circles). These data confirm previous data showing 3-O-methylglucose flux in cow cells which is unaltered in the presence or absence of adenosine. Another possible site of purine stimulation is at the level of hexokinase, since previous data have shown that cow hexokinase is stimulated by adenine, hypoxanthine, and inosine. Calf and human cells do not respond to exogenous purines, nor does their hexokinase (Table A-6).

As described previously, when cow red cells are incubated in their own plasma an augmented glycolytic rate is seen. It is possible that cellular factors released into the plasma are partially responsible for this effect. Table A-7 gives the results of an experiment in which red cells were pre-incubated for 4 hours in BSS prior to commencing the experiment. Pre-incubated red cells have glycolytic rates that are no different from controls.

In an attempt to isolate the unknown compound described previously, thin layer chromatograms were run on cow plasmas (Fig. A-4). The rapidly migrating unknown spot seen in the middle chromatogram was scraped off, extracted with 50% ethanol, and air dried. Similar spots from unspotted, developed plates were treated similarly. After

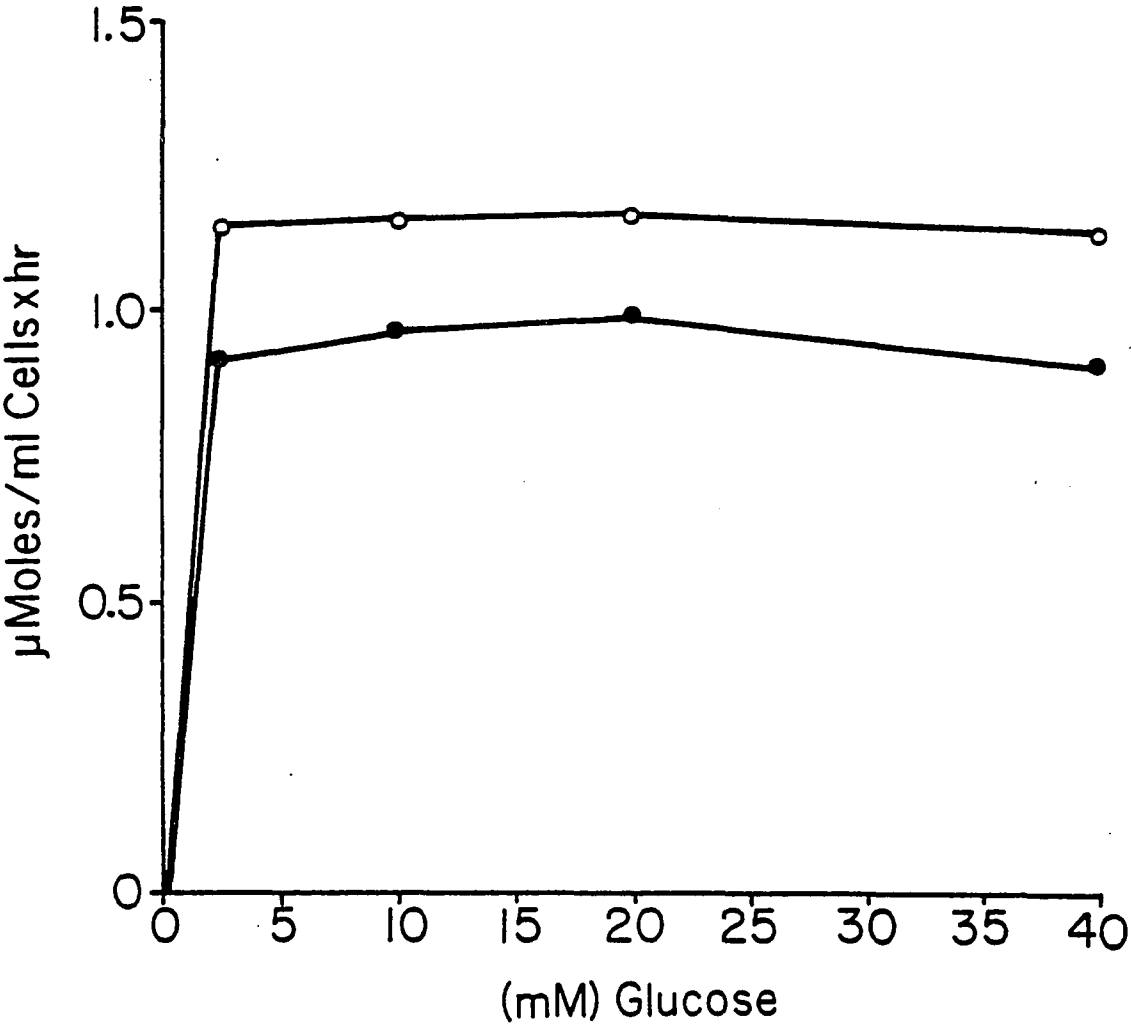

Figure A-3. Lactate formation as a function of glucose concentration in the presence (filled circles) and absence (open circles) of 10 mM inosine.

Table A-6. Effects of purines and nucleosides on calf and human hexokinase activity.

	Calf	Human
	Hexokinase Activity (IU/min/g Hb)	
Control	1.477±.027*(3)	1.247±.040 (3)
Adenine (100 μM)	1.460±.010 (2)	1.240±.058 (3)
Inosine (100 μM)	1.367±.055 (2)	1.115±.012 (3)
Hypoxanthine (100 μM)	1.495±.078 (2)	1.143±.081 (3)

*$\bar{x}$ ± S.E.M.

( ) = no. experiments

Table A-7. The effect of pre-incubating cow red cells in NaCl prior to commencing incubation in various substrates.

Substrate	Red Cell Treatment	Lactate Rate (μmoles/ml cells/hr)
Glucose (5 mM)	non-incubated	1.05±.03*(3)
Glucose (5 mM)	pre-incubated	.95±.05 (3)
Plasma	non-incubated	1.76±.07 (3)
Plasma	pre-incubated	1.69±.05 (3)

*$\bar{x}$ ± S.E.

( ) = no. experiments

Cow red cells were either suspended at 4°C in BSS for 4 hours, or else used right after saline washing.

Figure A-4. Thin layer chromatography of cow, calf and human plasma.

A. Three plasmas were treated according to the methods of Akaoka et al. (1975) with standard spotted to one side.
B. Cow plasma was treated according to the method of Bockman et al. (1976).
C. Dialyzed cow plasma treated in the same manner as B.

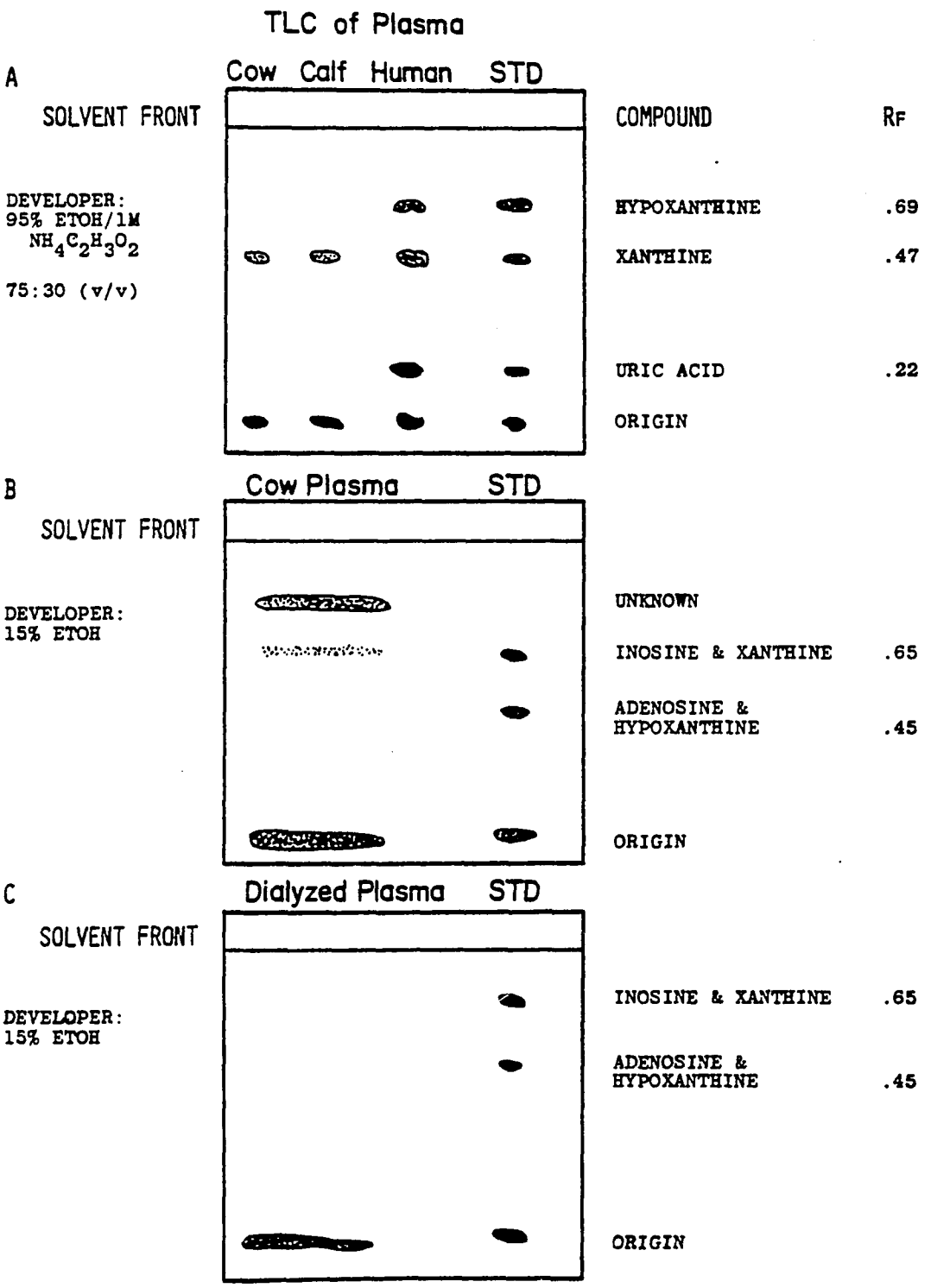

Figure A-4. Thin layer chromatography of cow, calf and human plasma.

resuspending the powder in glucose fortified BSS, red cells were added and the incubation begun. Table A-8 gives the result of this experiment. As expected, plasma stimulated red cell glycolysis, but glycolytic rates from extracts of control and UV absorbing plates are identical, indicating the presence of some interfering compound either located on the plate or in the chemicals used to extract the TLC plates. The plasma was incubated with charcoal, and the charcoal was extracted with pyridine. After being dried, the powder was resuspended in glucose-fortified BSS. As a control, non-plasma-incubated charcoal was also extracted with pyridine and treated as above. Table A-9 shows these results. Again glycolytic rates in both plasma and BSS incubated charcoal are elevated, obscuring the stimulator. Thus, while the stimulatory activity of plasma can be eliminated by incubating the plasma in charcoal prior to use, the stimulator cannot be eluted from the charcoal without some interfering substances.

Table A-8. The effects of the rapidly migrating plasma compounds from TLC plates on glycolysis.

Substrate	Lactate Formation (μmoles/ml cells/hr) experiment number 1	2
Plasma	.941	1.204
IM* + Glucose (5 mM)	.656	.791
Control	.788	1.187
U.V. Abs.	.755	1.191

*IM = Incubation Medium

The cellulose containing the UV absorbing peak was scraped off one TLC plate, and extracted with 50% ethanol, dried and suspended in glucose fortified BSS. As a control, an unspotted, but developed TLC plate also had a portion of its cellulose scraped off and extracted as above. Control refers to the unspotted TLC plate. U.V. Abs. refers to the unknown UV compound on the TLC plate. Lactate rates were then determined using methods described in Table A-4.

Table A-9. The glycolytic effects of the rapidly migrating compounds in plasma isolated by charcoal.

Substrate	Lactate Formation (μmoles/ml cells/hr)
Plasma	1.204
IM* + Glucose (5 mM)	.791
Control	1.217
Plasma Charcoal	1.122

*IM = Incubation Medium

Attempts to extract the stimulator from charcoal. Charcoal was incubated either in plasma or BSS, for 4 hours at 4°C, and then extracted with 10% pyridine solution. The pyridine was air-dried and resuspended in glucose fortified BSS. Lactate rates were determined as in Table A-4. Control refers to charcoal incubated in BSS rather than plasma prior to extraction.

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