NUCLEOTIDE METABOLISM IN ERYTHROCYTES

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by

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Ph.D. ABSTRACT BIOCHEMISTRY

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The role of adenine and adenosine in elevating cellular ATP concentration is investigated. Incubation of erythrocytes with inosine, glucose and adenine; or adenosine in low concentrations, doubles the fresh cell concentration of ATP. The mechanism by which adenine elevates adenine nucleotide concentration is known, however the mechanism by which adenosine acts has not been elucidated The findings of this study suggest that adenosine is directly incorporated by an erythrocyte adenosine kinase. Evidence for the existence of this enzyme in human erythrocytes along with some of its enzymatic properties are presented.

Under optimal conditions the maximum concentration of ATP synthesized was twice that present in fresh cells. It is suggested that ATP itself, controls the rate of adenine nucleotide synthesis as well as the final intracellular ATP concentration.

High concentrations of adenosine inhibit glucose and adenine elevation of ATP synthesis. The most likely site of adenosine action is on PRPP synthetase.

PREFACE

The study described in this thesis is a continuation of a research program on erythrocyte metabolism which was initiated by Dr. O. F. Denstedt in 1939 and now is under the direction of Dr. David Rubinstein. The research has dealt extensively with behavior of erythrocytes during storage. Initially, the main attention was focused on the changes that occur in the cells during storage in the cold. Subsequently, interest was centered successively on the glycolytic activity, nucleotide metabolism and maintenance of high energy phosphate. The present investigation is concerned with the role of adenosine and adenine as purine precursors in the elevation of ATP concentration in both fresh and preserved erythrocytes.

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LIST OF ABBREVIATIONS

ACD	Acid-Citrate-Dextrose medium of blood preservation
CPD	Citrate-Phosphate-Dextrose medium of blood preservation
AMP, ADP, ATP	Adenosine mono-, di-, tri-phosphate
GMP, GDP, GTP	Guanosine mono-, di-, tri-phosphate
IMP, IDP, ITP	Inosine mono-, di-, tri-phosphate
UDP, UTP	Uridine di-, tri-phosphate
XMP	Xanthosine monophosphate
UDPG	Uridine diphosphate glucose
NAD	Nicotinamide Adenine Dinucleotide
NADH	Reduced Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
EDTA	Ethylenediamintetraacetic acid
DHAP	Dihydroxyacetone phosphate
R-5-P	Ribose-5-phosphate
Rib-5-P	Ribulose-5-phosphate
R-1-P	Ribose-1-phosphate
PRPP	Phosphoribosylpyrophosphate
2,3-DPG	2,3-Diphosphoglyceric acid
1,3-DPG	1,3-Diphosphoglyceric acid
F-1,6-diP	Fructose-1,6-diphosphate
G-6-P	Glucose-6-phosphate

F-6-P	Fructose-6-phosphate
Xu-5-P	Xylulose-5-phosphate
G-3-P	Glyceraldehyde-3-phosphate
Pi	Inorganic phosphate
P~P	Pyrophosphate
S-AMP	Adenylosuccinic acid
PFK	Phosphofructokinase
NDP	Nucleoside Diphosphokinase
HG-PRT	Hypoxanthine-Guanine Phosphoribosyl Pyrophosphate Transferase
R.B.C.	Red Blood Cells
Δ F	Change in free energy
CO_2	Carbon dioxide
HMP	Hexose Monophosphate

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INTRODUCTION

Structure and Composition of the Human Erythrocyte

The erythrocyte is a nonnucleated, biconcave disk with a diameter varying from 6 to 9 μ and a thickness of about 1 μ at the center increasing to 2 to 2.5 μ toward the periphery (1).

The nucleated precursor forms of the erythrocyte have complete aerobic oxidative energy-yielding systems, but these disappear as this cell differentiates through the reticulocyte stage into the mature red blood cell. The earliest nucleated forms contain all the subcellular components and the enzyme systems necessary for replication, maturation and differentiation (2). The immediate precursor of the mature red cell, which is considered to be the reticulocyte, has no nucleus and cannot divide. However, the reticulocyte carries strands of ribonucleic acids by virtue of which it retains a vestige of the capability of protein synthesis. This capability, however, is limited primarily to the synthesis of globin which in turn is used for the synthesis of hemoglobin (3). The reticulocyte possesses all the enzymes of the tricarboxylic acid cycle as well as the cytochrome system (4).

The mature erythrocyte lacks a nucleus, RNA, and no longer has the capacity to synthesize hemoglobin. In contrast to the reticulocyte, the mature red blood cell is incapable of respiratory activity. The cytochrome system is absent (4) and the enzymes of the tricarboxylic acid cycle are but incompletely represented by remnant activities of isocitrate dehydrogenase, fumarase and malate dehydrogenase.

The prime function of the erythrocyte is the transport of oxygen and carbon dioxide. This is accomplished by the cell serving as a conveyor of a 34 per cent solution of hemoglobin. The interior of the erythrocyte is the most concentrated protein solution in the body, thereby permitting the movement of about 16 g of hemoglobin per 100 ml of whole blood without the high viscosity which would attend the presence of an equivalent amount of protein in free solution. The transport of oxygen and carbon dioxide does not involve the expenditure of energy. The energy required by the erythrocyte is used mainly to maintain the cell in a functional state.

In the absence of glucose, its ordinary source of energy, the erythrocyte changes its shape, from that of a biconcave disc to a more spherical form, and the intracellular sodium concentration is increased while that of potassium is correspondingly decreased. Finally the cell undergoes lysis.

Utilization of glucose by way of the Embden-Meyerhof system is now recognized as virtually the only energy-yielding process in the mature erythrocyte(6). The pentose phosphate pathway accounts for only a small amount of the total glucose utilization. The pentose pathway, however, is responsible for the reduction of NADP to NADPH, which may serve in the reduction of oxidized glutathione(7,8) or of methemoglobin (9). The potential energy of ATP is available for translation into work performed to maintain the erythrocyte in a functional state.

The chain of events maintaining the functional state of the human erythrocyte can be summarized as follows:

1. Sodium expulsion and potassium uptake to maintain osmotic and electrical equilibrium.

2. Adenosine triphosphatase (ATP'ase) activity to maintain equilibrium.

3. Glycolysis to maintain ATP levels.

4. Lipid exchange to maintain the physical status quo of the cell membrane.

5. Hemoglobin reduction to prevent membrane disruption by oxidation (10).

Carbohydrate Metabolism of the Erythrocyte

Glucose is normally the only substrate used by the erythrocyte for the production of energy. The rate of glucose utilization is several times less in the normocyte than in the reticulocyte precursor (11) and somatic cells (12).

The only functioning pathways for glucose utilization in the erythrocyte are glycolysis and the hexose monophosphate shunt (13). Several investigators have attempted to determine the relative contribution of glycolysis and the pentose phosphate shunt to the metabolism of glucose by erythrocytes. Murphy (6) has demonstrated that the relative amounts of glucose metabolized via the two pathways varied significantly with pH and partial pressureof oxygen. A high oxygen atmosphere favored the shunt (aerobic) pathway, which varied little with pH while, anaerobic glycolysis increased by 43% when the pH was shifted from 7.4 to 7.8. At pH 7.5 in an air atmosphere, approximately 11% of the glucose is metabolized through the hexose monophosphate pathway. Szeinberg and Marks (14) observed that the presence of cysteine, ascorbic acid,

or pyruvic acid caused a considerable increase in the percentage of glucose oxidized to CO_2 without a concomitant increased rate of glucose utilization or lactic acid formation. Furthermore, methylene blue, primaquine, acetylphenylhydrazine, nitrofurantoin, α-and ß-naphthol were found to cause marked increases in the percentage of total glucose metabolized via the oxidative reactions of the hexose monophosphate shunt (14). These agents are thought to stimulate the HMP shunt by serving as an intermediary between NADPH and oxygen. They accept electrons from reduced NADP and transfer them to atmospheric oxygen, thereby regenerating the rate limiting factor NADP and stimulating CO_2 production from glucose. Marks has suggested that in vivo there may be some variation in the percentage of glucose metabolism by either one of the pathways, depending upon a variety of factors such as the concentrations of agents which can potentially cause the oxidation of NADPH.

The Pentose Phosphate Shunt

The pentose phosphate pathway provides ribose-5-phosphate, a precursor in the synthesis of various important metabolites in the erythrocyte, including pyridine nucleotides, ADP and ATP (15, 16). Ribose-5-phosphate is synthesized by either of two pathways, oxidation of glucose-6-phosphate, or reversal of the non-oxidative reactions catalyzed by transketolase and transaldolase (17-20).

The pentose phosphates can also serve as substrates in a sequence of reactions which lead to the formation of lactic acid and the phosphorylation of

ADP to ATP. This is mediated in part by the enzymes transketolase and transaldolase which generate fructose-6-phosphate and glyceraldehyde-3-phosphate. The subsequent conversion of these intermediates of glycolysis into lactic acid results in the concomitant formation of ATP from ADP at the level of phosphoglyceric kinase and pyruvic kinase. For every three molecules of pentose phosphate metabolized via this series of reactions, a net of 8 molecules of ATP and none of NADH are generated. The contribution of this system to the production of energy in freshly drawn erythrocytes is negligible. However, the role it plays in preserved cells is quite important since the pathway is not inactivated during several weeks of storage. It is this longevity which makes feasible the use of purine nucleosides for the production of energy long after utilization of glucose has declined to a negligible level.

Function of NADPH

The NADPH generated in the pentose phosphate pathway is not directly involved in the production of energy due to the absence of a cytochrome system for oxidation of the NADPH. Oxidation of NADPH with methylene blue or other electron-acceptor dyes does not represent true respiratory activity. Any energy liberated in the oxidation of glucose under these conditions is not utilized for the formation of high energy phosphate but is dissipated as heat.

NADPH serves as an essential cofactor in a number of important reactions responsible for maintaining the integrity of the erythrocyte; for example, the reduction of methemoglobin by NADPH-dependent methemoglobin reductase (21) and NADP linked glutathione reductase (22). NADPH plays a role in the re-

duction of glutathione which is necessary for the stability of certain sulfhydryl containing enzymes as well as in the maintenance of the native structure of hemoglobin (22-27).

NADPH appears also to function in several types of reactions involved in the metabolism of drugs. Brodie et al. (28) Migeon et al. (29) have shown its requirement for the transformation of estrone to 17B-estradiol in the reaction catalyzed by 17 estradiol dehydrogenase. The physiological importance of this enzyme is not yet clear.

2,3-Diphosphoglycerate (2,3 DPG) Metabolism

The human erythrocyte contains large amounts of 2,3 DPG (30), unlike other somatic cells and its own precursor cell, the reticulocyte, which have lower 2,3 DPG and higher α-glycerophosphate and ATP concentrations (11). The concentration of 2,3 DPG [400 μ moles/100 ml cells(31)] greatly exceeds that of any other organic phosphate within the cell and undergoes marked changes in concentration under various conditions. During storage the concentration of 2,3 DPG rapidly falls to one-fifth of the fresh cell level within two weeks, while the concentration of ATP drops 25 per cent or less during the same period of storage (32,33). Incubation of these cells with inosine or glucose and adenine results in the elevation of both 2,3 DPG and ATP.

Rapoport and Luebering (34-36) have investigated the reasons for the high level of 2,3-diphosphoglycerate in the erythrocyte. In their studies they found that when red blood cells are incubated in the presence of glycolytic inhibitors such as fluoride or iodoacetate a large portion of 2,3 DPG is degraded

to 3-phosphoglycerate which can be used in the production of energy, especially in stored cells where there is a failure in glycolysis (33, 34). Studies by Rapoport and Luebering have also shown that red cells have weak glycerate diphosphatase activity while muscle has strong diphosphatase activity. In this study and in subsequent work (35), the authors claim the existence of a diphosphoglycerate mutase which can convert 1,3-diphosphoglycerate to 2,3-diphosphoglycerate. This conversion involves a loss of potential energy because of the formation of a low-energy from a high-energy phosphate. However, in the fresh erythrocyte sufficient energy is produced from the utilization of glucose to permit the accumulation of 2,3 DPG despite the loss of potential energy.

Function of 2,3 DPG

Several functions have been associated with 2,3 DPG. Sutherland et al. (37) have shown that it serves as a cofactor in the glycerophosphate mutase reaction. Prankerd (38) suggested that it could have some raison d'etre in the economy of the red cell as a ready source of phosphate or as a store of organic phosphate.

Benesch and Benesch (39) and Chanutin and Curnish (40) have shown that 2,3 DPG and ATP (which is quantitatively less important) when present in physiological concentrations, greatly reduced the affinity of human hemoglobin for oxygen. This shift of the oxygen dissociation curve to the right is physiologically important since, although it may increase the pressure required for oxygenation, it will facilitate the unloading of oxygen from hemoglobin at relatively high tissue oxygen pressures. Further work by Lenfant et al. (41)

and Eaton et al.(42) indicate that during the early stages of acclimatization of humans to altitude there is an increase in 2,3 DPG concentration which is associated with a shift of the oxygen dissociation curve to the right.

2,3 DPG has also been found to be an allosteric inhibitor of the enzymes involved in several different reactions. Hershko et al. (43) claim that it is a potent inhibitor of PRPP synthetase at the physiological concentrations found in erythrocytes. Askari and Rao (44) have found that 2,3-DPG also has an inhibitory effect on AMP deaminase. Hexokinase inhibition by 2,3 DPG has been shown by Dische (45) and most recently Brewer (46). This inhibition is relieved by increasing concentrations of ATP and/or Mg^{2+}.

The role that 2,3 DPG plays as an inhibitor in the above reactions, as well as its important function in the regulation of oxygen transport by the human erythrocyte, suggest a possible feed back inhibition mechanism. By this mechanism the level of 2,3 DPG controls the rate of glycolysis and hence subsequent levels of 2,3 DPG and/or ATP which in turn regulates hemoglobin binding of oxygen.

Control of Glycolysis

The human erythrocyte is well suited as a model for the study of the control of glycolysis in living cells, since, having neither nuclei, mitochondria nor endoplasmic reticulum the possibility of compartmentalization of metabolites in this cell is negligible. Furthermore, the enzymes mediating glycogenesis, gluconeogenesis and glycogenolysis (58) are absent, while the alternate pathway of glucose utilization, the hexose monophosphate shunt, is relatively inoperative in the erythrocyte under normal conditions (6).

Investigations concerning the mechanism of glycolytic control have used a variety of experimental approaches. Chapman et al (59) using a reconstructed hemolysate system fortified with ATP, NAD, inorganic phosphate (Pi) and Mg^{2+}, have found that the pH optimum and the rate of lactate production are the same as those observed for the intact erythrocyte. Assay of the individual enzymes of the hemolysate and determination of turnover of substrate by these enzymes showed that hexokinase had the slowest rate of substrate turnover (10 μ moles substrate/hr/ml RBC) followed by aldolase (31 μ moles/hr/ml RBC), phosphofructokinase (82 μ moles substrate/hr/ml RBC), and enolase (95 μ moles substrate/hr/ml RBC).

Minakami and Yoshikawa (47, 48) working with intact red blood cells have calculated changes in free energy from the steady state concentrations of the intermediates and coenzymes in red cells under physiological conditions. The largest free energy changes occured at hexokinase $\Delta F=-8.0$, phosphofructokinase $\Delta F=-5.3$ and pyruvic kinase $\Delta F=-4.0$ enzyme catalyzed reactions.

The reactants of these enzymes are farthest from thermodynamic equilibrium. The authors conclude that these three steps may be the rate limiting steps in erythrocyte glycolysis and a change of reaction rate at any one of these steps could cause a change of the over-all glycolytic rate.

Other studies also using intact cells have looked for "cross over points" (49), using conditions which alter the flux of intermediates throughout the glycolytic pathway (50,51,52). The results of some of these studies will be presented later on.

Another experimental approach has been to study the effects of various glycolytic metabolites and cofactors on the activities of the individual enzymes. The results of such studies show a very intricate method of regulation of all enzymes and particularly the so called rate limiting enzymes of glycolysis. Table 1 shows the influence of various allosteric effectors on the three rate limiting enzymes of Minakami. The purpose of this table is simply to illustrate the complexity of the control mechanisms. From these recent findings several generalizations may be made concerning the mode of action of allosteric effectors. First, the influence of a modifier on an individual enzyme is not an absolute effect but is modified by other intracellular metabolites. An example of this is the release of inhibition of hexokinase and phosphofructokinase(caused by G-6-P and ATP)by inorganic phosphate. Secondly, allosteric modifiers may have different effects at different concentrations. A good example of this is found in the work of Haeckel et al. (57). Using pyruvic kinase isolated from yeast, this group found that low concentrations of ATP activated, while higher concentrations of ATP inhibited enzyme activity. Thirdly,

Table 1.

A SUMMARY OF THE ALLOSTERIC INFLUENCES ON THE "PHYSIOLOGICAL RATE-LIMITING ENZYMES (47)"

ENZYME	SOURCE	ALLOSTERIC PROPERTIES
Hexokinase	Ascites tumor cells	- susceptible to product inhibition by glucose-6-phosphate and ADP, both competitive with ATP (53). - inhibition by glucose-6-phosphate partly released by Pi but ADP inhibition not released by Pi (53)
	Human red blood cells	- also inhibited by 2,3 DPG relieved by ATP and/or Mg^{2+} (46)
Phosphofructokinase	Rabbit muscle	- susceptible to inhibition by ATP phosphoenolpyruvate, citrate (54) - reversal of ATP inhibition by ADP, AMP, Pi - inhibition by ATP - pH dependent - at pH 9.0 no inhibition but below pH 7.0 increased considerably (54)
Pyruvic kinase	Yeast	- ATP competitive inhibitor of phosphoenol-pyruvate (55)
	Human red blood cells	- in the presence of 1mM ATP and 0.15mM ADP; FDP (2mM), AMP (03mM), Pi (10mM) mild competitive inhibitors with phos-phoenolpyruvate (56)
	Yeast	- low ATP activates while high ATP inhibits; stimulated by FDP (57)

modifiers are ubiquitous and an individual modifier may be able to either decrease or increase the activity of a large group of enzymes, thereby controlling the rate of its own synthesis and subsequent intracellular concentration. The influence of ATP, ADP, AMP, Pi and 2,3 DPG on hexokinase, phosphofructokinase and pyruvic kinase, as well as a host of other enzymes serves to illustrate this point.

Several allosteric modifiers have been considered to have important functions in the control of glycolysis. There is good evidence that inorganic phosphate limits the rate of glycolysis in red blood cells. First, its concentration is normally very low 0.28 to 0.48 μ mole/ml cell (60) and therefore especially sensitive to small changes in concentration, produced either on esterification or hydrolysis of organic phosphate esters. Secondly, high inorganic phosphate concentration overcomes oubain inhibition of glycolysis (51). This suggests an important regulatory role for inorganic phosphate released by ATP'ase activity, especially in overcoming ATP inhibition of phosphofructokinase. While, a third point in evidence is the action of Pi in elevating glucose utilization and lactate production. This has been investigated in the red cell by several groups (62,52). Minakami et al. (52) have studied this effect using the "cross over"method of Chance and have attributed the effect of Pi to different factors at different pHs. At pH 7 they attribute the effect to an enhancement of hexokinase; at pH 8.0 to an increase in phosphofructokinase; and at pH 7.4 to enhancement of both hexokinase and phosphofructokinase.

Under conditions of an elevated glycolytic rate other factors have also been implicated as controlling agents. At high inorganic phosphate levels, Warm and Rose (56) suggest that as a result of a limiting NAD/NADH ratio, glyceraldehyde phosphate dehydrogenase is probably rate limiting. Saito and Minakami (61), also using conditions which stimulate glycolysis, found that when ATP is elevated by incubation with glucose and adenine there is a concomitant elevation of 2,3 DPG which they suggest is caused by ATP depression of phosphoglyceric kinase. Under these conditions the authors suggest that both phosphoglyceric kinase and aldolase may serve as additional rate limiting factors.

Nucleotide metabolism in erythrocytes

As glycolysis is presumably the only energy-generating system in the red cell there is a close relationship between glycolysis and ATP level in the cell. The concentration of nucleotides in erythrocytes is presented in Table 2A.

From this table it is apparent that ATP (1000 μmole/L red cells) forms the major portion of the total nucleotide content of the human erythrocyte. The concentration of ADP is 10-20% that of ATP while AMP is 1-2%. Small amounts of IMP have been reported by some investigators (64) while others were unable to detect its presence (63). The only other nucleotides present are guanosine triphosphate (GTP), GDP and GMP (63,64).

Of the pyridine nucleotides NAD, NADP, along with their reduced forms and uridine diphosphoglucose have been shown to be present in small amounts (65).

Biosynthesis of Adenine Nucleotides in Erythrocytes

Lowy et al. have shown that rabbit erythrocytes (67) and human erythrocytes (68) unlike other somatic cells cannot incorporate labeled precursors such as glycine and formate into purine nucleotides under conditions which allowed preformed purines to be incorporated. Lowy and Williams (68) subsequently found that the mature rabbit erythrocyte could synthesize ATP and GTP from labeled formate in the presence of 5 amino-1-ribosyl-4-imidazole carboxamide. This placed the failure of de novo synthesis earlier in the sequence before final ring synthesis.

The rabbit erythrocyte (68) has the ability to convert IMP (the first product of ring synthesis) to AMP and GMP (69). The human erythrocyte

Table 2A

CONCENTRATION OF SOME PHOSPHORYLATED COMPOUNDS IN HUMAN ERYTHROCYTES

Compound	Concentration μmoles per liter of red cells	Reference
ATP	900 - 1233	Bartlett (60)
ADP	190 - 245	"
AMP	10 - 20	"
GTP	62	Bishop et al. (63)
GDP	10	Mandel et al. (64)
IMP	30, 0	Mandel et al.(64), Bishop et al.(63)
UDPG	30	Mandel et al. (64)
NAD	60	Gross et al. (65)
NADH	50	" "
NADP	40	" "
NADPH	30	" "
Pi	280 - 480	Bartlett (60)
2,3 diphosphoglycerate	3600 - 5010	"

can not carry out this conversion but can however convert IMP to GMP (68).

The inability to convert IMP to AMP is thought to be due to an enzyme deficiency rather than to lack of GTP which is a required cofactor (68).

The ability of reticulocytes to synthesize purine nucleotides de novo was investigated by Lowy et al. (68). This group has demonstrated incorporation of formate or glycine into purine nucleotides by rabbit reticulocytes in vitro. Inasmuch as mature anucleate erythrocytes cannot completely synthesize purine nucleotides, and since these cells are derived from reticulocytes, it seems reasonable to assume that the ability has been lost during maturation.

Preformed purines such as adenine, guanine and hypoxanthine are readily incorporated into mature red cell nucleotides by both rabbit and human erythrocytes (71,68). However, due to the inability to convert IMP → AMP, the human erythrocyte can utilize only adenine for ATP synthesis while the rabbit red cell can utilize all purines.

In human erythrocytes in vivo, adenine-8-C^{14} is incorporated into AMP, ADP, AMP readily, but into NAD only after a number of days (72,66). Other researchers have found no incorporation into NAD (73).

Two important enzymes are involved in the incorporation of adenine into ATP:phosphoribosyl pyrophosphate synthetase and adenine phosphoribosyl transferase. Price and Handler (74) have shown that an acetone-powder preparation of human erythrocytes can bring about the synthesis of PRPP from R-5-P and ATP. The conversion of adenine or any other preformed purine or pyrimidine base into the corresponding nucleoside-5'-phosphate can then take place

in the presence of PRPP and the enzyme adenine phosphoribosyl transferase. This enzyme was first discovered by Kornberg et al (75) in yeast and liver and has since been shown to be present in the red blood cell (76).

The interconversion of AMP and ADP is brought about by adenylate kinase, the presence of which has been demonstrated in the human erythrocyte (77-80). The final conversion of ADP to ATP is carried out by substrate level phosphorylation involving phosphoglyceric kinase and pyruvic kinase of the Embden-Myerhof pathway.

Both glucose and nucleosides can serve as precursors for the ribose moiety of the nucleotides that are synthesized in the presence of adenine(78) (81). When purine nucleosides are used as precursors for the ribose moiety they are first deaminated and then acted upon by nucleoside phosphorylase to yield a purine base and R1P, which is then converted to R5P via a mutase.

Conversion of glucose to ribose phosphate (81,82)has been shown to occur mainly via the transketolase reaction between F6P and G3P yielding Xu5P and R5P. The Xu5P is then converted to Rib5P and eventually to R5P via the epimerase and isomerase enzymes of the HMP shunt.

The physiological significance of this "salvage"(83) pathway in the red blood cell is not known. A possible role in purine transport was suggested by the work of Laj tha and Vane who showed that when a hepatectomized rabbit was supplied with C^{14} formate little label was found in bone marrow adenine (84). Utilizing various controls they concluded that the liver was the main supplier of purine for bone marrow cells and perhaps for other tissues as well. The Henderson

and Le Page (85) work supports this view. From their experiments they conclude that purines might be distributed among mouse tissues by blood cells, adenine being taken up (perhaps in the red cell nucleotides) as the blood passes through the liver and later released in other tissues.

Breakdown of ATP

The breakdown of ATP was studied by Bishop (86) in blood stored at 4°C or incubated at 37°C and was found to proceed to hypoxanthine according to the following sequence: ATP → ADP → AMP → IMP → Inosine → hypoxanthine. In human erythrocytes the breakdown stops at hypoxanthine because there is no xanthine oxidase present. Jorgenson (87) observed that the sum of the above compounds was nearly constant in stored human blood.

Turnover of Nucleotides

Evidence for a metabolic turnover of the purine nucleotides of red blood cells was originally provided by Lowy et al. (69). Working with rabbit erythrocytes they reported that the purine moieties of ATP and GTP had been labeled in vivo by subcutaneous injection of [2-^{14}C] glycine, exhibited a considerably faster decline of radioactivity than the similarly labeled hemoglobin. Bishop (72) noted the renewal of human red cell nucleotides labeled in vivo by intravenous administration of [8-^{14}C] adenine. The interpretation of these results however is complicated by possible recycling of the isotope tracer. This problem was largely eliminated by Mager et al. (88, 89) by in vivo labeling red cell nucleotides with radioactive purines and reinjecting the tagged cells intravenously. The average biological half-times of the intracellular

nucleotides derived from exogenously supplied adenine, guanine and hypoxanthine in rabbit were estimated to be 25, 10 and 9 h respectively, while in man they were 8-9 days , 5-7 h and 1 h respectively. The disparity between the two species, the authors claim, is "inherent in the different extent of contribution of exogenous purines to their adenine pool, as determined by the capacity for converting hypoxanthine to adenine which is negligible in the human and substantial in the rabbit red cells."

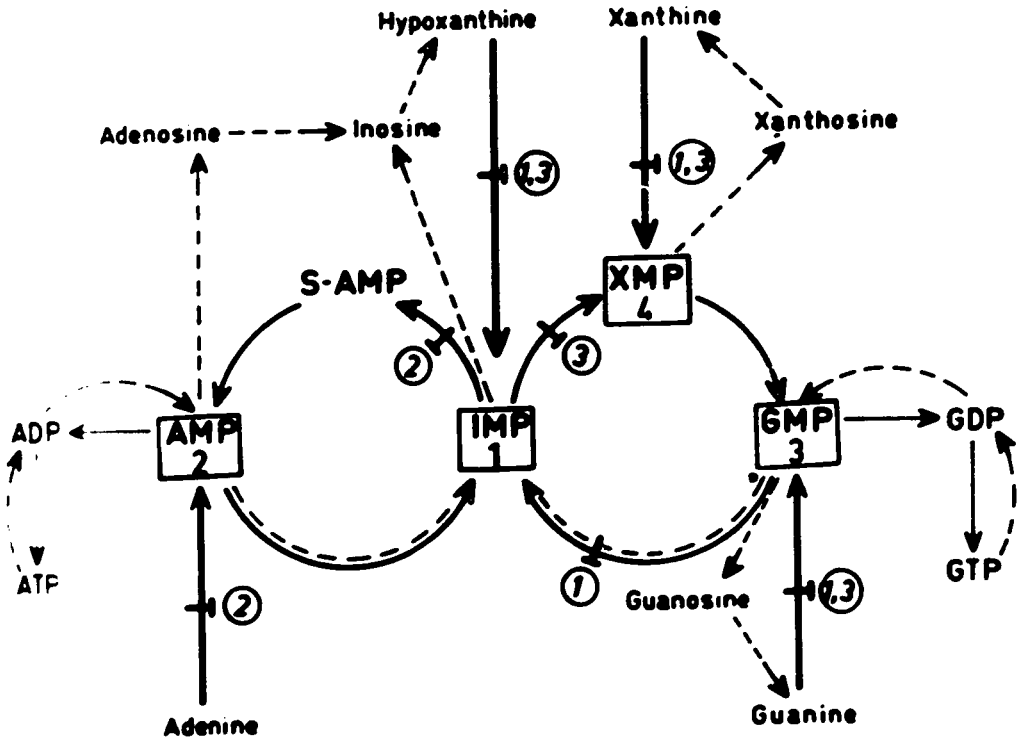
Pathways involved in the uptake and release of purine bases by erythrocytes

The series of reactions involved in the uptake and release of purine bases in erythrocytes is shown in Fig.1. The uptake of purine bases into purine nucleotides has been studied by Bishop et al. (72) and Lowy et al.(69). They found that [8-^{14}C] adenine and [8-^{14}C] guanine are incorporated mainly into the corresponding nucleotide triphosphates, while only a minor portion of the incorporated radioactivity appears in the form of the respective nucleoside diphosphate derivates of the red blood cell. Mager et al. (88-90) have shown that with hypoxanthine in contrast to adenine and guanine, there is no detectable incorporation into IDP or ITP, all the intracellular hypoxanthine being confined to IMP. In addition, a substantial part of the radioactive hypoxanthine taken up by the cells was incorporated into GTP in human red blood cells (89) and into both GTP and ATP in rabbit erythrocytes (88,90).

The release of the incorporated purine was investigated by Hershko et al.(90) who incubated rabbit erythrocytes, which had been labeled with [8-^{14}C] purine in a low phosphate containing medium. The radioactive material

Figure I

PATHWAYS OF PURINE NUCLEOTIDE METABOLISM AND THEIR ENZYMIC FEED-BACK CONTROL MECHANISMS.



Solid lines, biosynthetic pathways; dashed lines, catabolic pathways. The T signs cutting across the pathways denote feed-back inhibition and the numbers underneath refer to the inhibitory end-products identifiable by the corresponding numbers within the squares.

released into the suspending medium was analyzed and found to consist of hypoxanthine and xanthine when the cells were pre-labeled with adenine-8-^{14}C; hypoxanthine, xanthine and guanine when pre-labeled with guanine 8-^{14}C; and hypoxanthine, xanthine and xanthosine when pre-labeled with hypoxanthine 8-^{14}C. The predominance of hypoxanthine and xanthine among the purine compounds released indicates that conversion of AMP and GMP to IMP by AMP deaminase and GMP reductase is a necessary link in the process of purine release. The considerable release of xanthosine and xanthine observed in vitro is probably due to the piling up of XMP caused by inadequate supply of glutamine required in XMP conversion to GMP. In vivo, glutamine levels are high so purine catabolism may proceed entirely to IMP. IMP, unlike AMP and GMP, cannot be phosphorylated to the corresponding nucleotide triphosphate and is more vulnerable to the hydrolytic action of 5'-nucleotidase initiating the catabolic path leading to freely diffusible hypoxanthine.

Enzymes of adenine nucleotide biosynthesis

The reactions catalyzed by the enzymes involved in adenine nucleotide biosynthesis are presented in Table 2B.

Biosynthesis of phosphoribosyl pyrophosphate (PRPP)

In 1955 Kornberg et al. (75) and Remy et al. (91) demonstrated the conversion of ribose-5-phosphate and ATP to phosphoribosyl pyrophosphate (PRPP) and AMP in an enzyme preparation from pigeon livers. The enzyme catalyzing this reaction is called phosphoribosyl pyrophosphate synthetase and has been shown to require Mg^{2+} and Pi for activity (75, 90-93). Further investigation of the properties of the enzyme was carried out by Switzer (95). Kinetic studies done by this author indicate that the active substrate is the Mg-ATP complex and that the divalent cation also stimulates the system in some other way, possibly by activating the enzyme.

The PRPP synthetase reaction was demonstrated to be reversible. The equilibrium constant for the reaction in the direction of PRPP synthesis at pH 7.5 and 37° was determined to be 28.6 which corresponds to a standard free energy ΔF of -2.0 ± 0.5 K cal per mole for the reaction. The mechanism of phosphate transfer from ATP to ribose-5-phosphate and inorganic phosphate was studied by Khorana et al. (94), using a chemical procedure which brings about hydrolysis of phosphoribosyl pyrophosphate to ribose-1,2-diphosphate* and inorganic phosphate. These investigators showed that the degradation of PRPP derived from ribose-5-phosphate and ^{32}P-γ labeled ATP yielded quantitative amounts of radioactive inorganic phosphate and non radioactive ribose-1,2-diphosphate*. When the same experiment was done with ^{32}P-ß labeled ATP, chemical degradation of PRPP resulted in the formation of quantitative amounts of unlabeled ribose-5-

*Error, should read: 5-phosphorylribose-1,2,-cyclic phosphate

Table 2B

ENZYMES INVOLVED IN THE "SALVAGE PATHWAYS" OF PURINE NUCLEOTIDE BIOSYNTHESIS

TRIVIAL NAME	REACTION CATALYZED
Phosphoribosyl pyrophosphate synthetase	ATP + R5P $\rightleftarrows$ PRPP + AMP
Adenine phosphoribosyl transferase	Adenine + PRPP $\rightleftarrows$ AMP + P~P
Guanine -hypoxanthine phosphoribosyl transferase	Guanine + PRPP $\rightleftarrows$ GMP + P~P Hypoxanthine + PRPP $\rightleftarrows$ IMP + P~P
Adenosine kinase	Adenosine + ATP $\rightleftarrows$ AMP + ADP
Inorganic pyrophosphatase	P~P $\rightleftarrows$ 2Pi
Adenylic deaminase	AMP $\rightarrow$ IMP + NH_3
Nucleoside phosphorylase	Nucleoside $\rightleftarrows$ base + RIP
Adenosine deaminase	Adenosine $\rightleftarrows$ Inosine + NH_3
Adenylic kinase	AMP + ATP $\rightleftarrows$ 2ADP
Nucleoside diphosphokinase	$N_1TP + N_2DP \rightleftarrows N_1DP + N_2TP$ (N = purine or pyrimidine base)
Phosphoglyceric kinase	1,3 diphosphoglyceric + ADP $\rightleftarrows$ phospho-glyceric + ATP
Pyruvic kinase	Phosphoenolpyruvic + ADP $\rightleftarrows$ pyruvic + ATP

phosphate and radioactive inorganic phosphate. The authors have suggested that PRPP is synthesized as a result of transfer of an intact pyrophosphate group from ATP to ribose-5-phosphate in the presence of PRPP synthetase. Further studies on the mechanism of PRPP synthetase have been carried out by Switzer (92,95,96) using the enzyme obtained from Salmonella typhimurium. Incubation of enzyme with ^{32}P-terminally labeled ATP in the absence of ribose-5-phosphate resulted in the binding of radioactivity to the enzyme; however, incubation with uniformly labeled ^{14}C-ATP resulted in no binding of radioactivity to the enzyme. Therefore the adenosine moiety of ATP is not being bound to the enzyme. With ribose-5-phosphate (the pyrophosphate acceptor) the binding of radioactivity derived from ^{32}P-terminally labeled ATP to the enzyme is abolished. Switzer (92,95) suggested that an enzyme-pyrophosphate intermediate is formed in the PRPP synthetase reaction. The accumulation of this enzyme-pyrophosphate intermediate is prevented by the presence of the pyrophosphate acceptor, ribose-5-phosphate.

Studies with intact Ehrlich ascites tumor cells have shown that the rate of synthesis of PRPP from glucose is slower than the rate of glycolysis (97,98). Henderson and Khoo have demonstrated that the initial rate of PRPP synthesis is dependent on extracellular glucose up to 0.55 mM and independent thereafter. In the presence of adenine the concentration of PRPP within the cell is low compared to the level in its absence. In the presence of adenine, nucleotide synthesis continues at a constant rate indicating that PRPP synthesis must have also continued. In view of these findings Henderson and Khoo (97,98) suggested

that PRPP may inhibit its own synthesis by a feedback inhibition mechanism. Unfortunately, similar studies have not been carried out in erythrocytes. However, using a cell free reaction mixture, Hershko et al. (99) have not been able to show feedback inhibition by PRPP on PRPP synthetase. These researchers have found a potent inhibition of PRPP synthetase by ADP, GDP and 2,3 diphosphoglycerate at the concentrations of these metabolites normally found in the red cell. The inhibition could be alleviated only partly by increasing the concentration of Pi. Inhibition of the enzymatic activity of PRPP synthetase by ADP, GDP and 2,3 DPG is explained as part of a general mechanism for energy conservation, an hypothesis first proposed by Atkinson and Walton (100) and Atkinson and Fall (101). Having noted the inhibition of both the citrate cleavage enzyme of rat liver and the PRPP synthetase of Escherichia coli by ADP, they proposed that this inhibition by ADP is part of a general ATP conservation system that results in a decreased rate of ATP expenditure when the "energy charge" of the cell decreases slightly. The "energy charge" of the adenylate system is defined by these investigators as (ATP + 1/2 ADP) / (ATP + ADP + AMP) - that is, the ATP - ADP - AMP balance of the system.

Overgaard-Hansen (102), using extracts of L5178V lymphoma cells, and Tyrsted and Sartorelli (103) using Ehrlich ascites tumor cells, have shown the inhibition, by 3'-deoxyadenosine, of PRPP synthesis, from ATP and ribose-5-phosphate.

However, this effect is thought to be due to phosphorylation of 3'-deoxyadenosine to 3'-deoxyadenosine triphosphate which has been shown to

have a pronounced inhibitory effect on phosphoribosyl pyrophosphate synthesis (104).

Purine phosphoribosyltransferase

Kornberg et al.(75)and Remy et al.(91) have demonstrated the condensation of adenine and phosphoribosyl pyrophosphate to produce AMP and inorganic pyrophosphate in the presence of an enzyme preparation of pigeon liver. Subsequent investigations have established the existence of two phosphoribosyl transferases in red blood cells (105) one utilizing adenine as the prefered substrate, and the other acting on hypoxanthine and guanine. These phosphoribosyl transferases, along with the nucleoside kinases which catalyze the conversion of nucleosides to nucleotides, are known as "salvage" enzymes since they tend to conserve the purine ring (83).

The mechanism of the adenine phosphoribosyl transferase reaction has been kinetically investigated by Hori and Henderson (106), using the enzyme from Ehrlich ascites tumor cells. These investigators have concluded that the reaction mechanism was of the "ping-pong" type. More recent studies (107, 108)however, support the view that the reaction is ordered and involves the formation of a ternary complex of enzyme and both substrates. The ternary enzyme : Mg-PP-ribose-P: adenine complex is converted into an enzyme: Mg PPi: AMP complex. In the catalytic process the bond between the 1-carbon of PP-ribose-P and the oxygen attached to it is broken, the proton on the 9-nitrogen of adenine is removed and the bond between the 9-nitrogen of adenine and the 1-carbon of ribose-5-P is formed.

The rate of synthesis of AMP from adenine and PRPP is regulated by both Pi and nucleotide concentrations. Several groups have found (109, 110, that the products of the reaction, AMP and inorganic pyrophosphate, show a concentration-dependent inhibition of enzymatic activity. At 50 μ M AMP or inorganic pyrophosphate Holmsen and Rosenberg have observed a 40% inhibition in the rate of AMP synthesis, a finding confirmed by Murray working with Ehrlich ascites tumor cells. This group (110) has also shown that a combination of AMP (1.25 mM) plus ADP (1.25 mM) causes a greater inhibition than AMP alone. ATP may also have a regulatory effect; at low concentration (40 to 50 μ M) it stimulates adenine phosphoribosyl transferase while at concentrations greater than 250 μ M it inhibits. The stimulation was unaffected by PRPP but the inhibitory effect could be overcome by the addition of high concentrations of PRPP to the incubation medium. Similar results have been obtained by other groups using Bacillus subtilis (111)and other bacterial as well as mammalian cells (112). The mechanism of hypoxanthine-guanine phosphoribosyl transferase reaction has been studied by Henderson et al. (70) who found that the reaction is ordered and involves the formation of ternary enzyme-substrate complexes similar to those found in the adenine phosphoribosyl transferase reaction. Their results are consistent with the view that only one enzyme catalyzes the conversion of guanine and hypoxanthine to the corresponding nucleotides. These two substrates share a common binding site. Kelley et al. (113) have presented evidence that human erythrocyte hypoxanthine-guanine phosphoribosyl transferase will also catalyze the conversion of xanthine to

xanthylate but that this reaction proceeds at less than 1% of the rates of the other two reactions.

Loss of adenine or hypoxanthine-guanine phosphoribosyl transferase activity has been observed in several clinical reports. The complete loss of hypoxanthine guanine phosphoribosyl transferase activity in man (114) is associated with a severe neurological disease characterized by self-mutilation, mental retardation, spasticity and choreoathetosis (115). Partial losses (90 to 99%) of this enzyme are associated with certain types of gout and also with a mild neurological disease characterized by spinacerebular involvement (116). All these diseases are associated with excessive production of uric acid and accelerated purine synthesis. The association of excessive purine synthesis with a partial or complete HGPRT deficiency is evidence that normal function of this enzyme is required for regulation of purine biosynthesis, although the precise mechanism by which the enzyme defect gives rise to overproduction of uric acid has yet to be determined (116).

The presence of adenine phosphoribosyl-transferase deficiency has been reported by Kelley et al.(117). Despite apparent close functional similarity to HG-PRT ase, its partial deficiency (21-37% of normal) was not associated with any detectable abnormality of purine metabolism in the one case so far reported (118).

Adenosine Kinase

Adenosine kinase was first discovered in yeast and mammalian cells by Kornberg and Pricer (119). The presence of the enzyme has been shown in

rat kidney and liver (119), rabbit liver, Ehrlich ascites tumor cells (120), human tumor cells [H.Ep. No.2] (121) and salmon milt (122). While, indirect evidence for its existence in human erythrocytes has been provided by the work of Lowy et al. (123) and Bartlett et al. (124).

The properties of the enzyme have been studied more recently by two groups (121, 120) using enzyme preparations from different sources. Schnebli et al. (121) have purified the enzyme 175-fold from human tumor cells in culture [H.Ep.No. 2] and have shown it to be free from adenosine deaminase and adenosine monophosphate kinase activity. They found that the reaction requires a nucleoside triphosphate as a phosphate donor (most effective were ATP, GTP and ITP) and adenosine as the other substrate. Addition of a divalent cation was not required for the phosphorylation of adenosine. As Mg^{2+} was present in some of the buffers used during the isolation of the kinase, it is possible that the enzyme had already bound some Mg^{2+} during the isolation. Using a 50 mM phosphate buffer the pH optimum was found to be pH 6.2 - 6.8 and the Km for adenosine was 1.8×10^{-6} M. Linberg (121) et al., using rabbit liver as the source of enzyme, found the Km of adenosine to be 1.6×10^{-6} M.

The same research workers also found lower pH optima (pH 5.8 in phosphate buffer and pH 6.5 in citrate - phosphate) and a requirement for Mg^{2+}. It is this group's contention that the enzyme activity is inhibited if the molar ratio between ATP and Mg^{++} is not close to 1. The Kms found for adenosine by the two groups are in close agreement; however, the two groups differ in their opinion as to the role of Mg^{2+} and the pH optima for the enzyme.

It has been demonstrated (119, 120) that the enzyme phosphorylates several derivatives and analogues of adenosine to a greater extent than adenosine itself. While 6-mercaptopurine ribonucleoside, which is not itself phosphorylated, inhibits the phosphorylation of adenosine (120). Other nucleosides such as guanosine and inosine are also not phosphorylated but do not inhibit the phosphorylation of adenosine.

Inorganic Pyrophosphate

Inorganic pyrophosphate is produced in several metabolic reactions. Of special significance, to us, is its production in the formation of AMP by the enzyme phosphoribosyl transferase. The hydrolysis of pyrophosphate is an important reaction because pyrophosphate exerts inhibitory effects on adenine phosphoribosyl transferase. Therefore, its eventual breakdown will relieve this inhibition. Hydrolysis of pyrophosphate also produces orthophosphate, which is important in the regulation of the activities of many enzymes (56, 52, 99). Pyrophosphatase activity has been reported in erythrocytes (137, 138) this activity has been shown by Rapoport and Scheuch (126) to be linked with the oxidation reduction state of glutathione. Some properties of purified human erythrocyte inorganic pyrophosphatase have recently been investigated by Pynes and Younathan (125). These researchers found an optimal pH of 7.7, an apparent Michaelis constant of 9.7×10^{-6} M and optimal activity at a Mg^{2+}:PPi ratio of 1.

AMP deaminase

The presence of a high level of AMP deaminase activity in human

erythrocytes was first established by Conway and Cooke (127, 128) and the reaction has since been shown to be irreversible (136). The former authors suggested that the red cell adenylic acid deaminase is reversibly bound to some inhibiting substance. In view of its important role in controlling adenine nucleotide breakdown, studies on the control of adenylic deaminase activity have been carried out by various groups (136, 129, 44). In human erythrocytes, Askari and Rao (44) have found that both 2,3 diphosphoglycerate and inorganic phosphate inhibit adenylic deaminase activity, while ATP stimulates this activity. Recent investigations by Setlow and Lowenstein (129) with enzyme obtained from calf brain, indicate that the enzyme is stimulated by ATP and inhibited by GTP. Since GTP is also involved in IMP conversion to AMP in this tissue, the level of GTP plays an important role in regulating the size of the adenine nucleotide pool. In the red blood cell GTP and Pi are present in very small quantities, while 2,3 DPG is present in high concentrations. In view of the relatively large concentrations of 2,3 DPG in comparison with the other potential inhibitors, it is reasonable to assume that most of the inhibition of adenylate deaminase in the red cell is due to 2,3 DPG.

Nucleoside Phosphorylase

The enzymatic degradation of purine nucleosides has been known since 1911 (130). In 1945 Kalckar (131) made the observation that ribose-1-phosphate is one of the products of the purine nucleoside phosphorylase reaction. The natural purine bases, guanine, hypoxanthine and xanthine, as well as certain related structural analogues such as 8-azaguanine, serve as substrates for the reverse reaction. However, adenosine is inactive as a substrate (132).

The nucleoside substrate may contain a ribose or deoxyribose moiety; the products of nucleoside phosphorylase activity are then R-1-P and deoxy R-1-P respectively.

In addition to the above reaction, purine nucleoside phosphorylase from mammalian sources has been reported to catalyze purine base exchange or ribosyl transfer reactions in the absence of inorganic orthophosphate (133), or exchange between orthophosphate and ribose-1-phosphate in the absence of purine base (132,134).

The reaction mechanism of human erythrocyte nucleoside phosphorylase has been investigated by Kim et al. (135). Results of kinetic analysis were consistent with an "ordered Bi Bi reaction" mechanism, suggesting that the enzyme was multivalent with cooperative interactions between the active sites.

Adenosine Deaminase

Adenosine deaminase was demonstrated in human blood by Conway and Cooke (139) and later by Rubinstein and Denstedt (140), who found the pH optimum to be 7.0 in human red cell hemolysate. The deamination of adenosine by adenosine deaminase constitutes hydrolysis without the participation of cofactors. Further studies by Baer et al. (141), using a preparation from calf intestinal mucosa, showed that the enzyme exhibited maximal activity at pH 6.8. The Km for adenosine was 2.93×10^{-5} M. The enzyme was also capable of hydrolyzing various groups at the 6- position of purine ribonucleoside derivatives including 2-amino-N^6-methyl-adenosine, 6-chloro, 6-methoxy and 6-hydroxylamino groups.

Formation of dinucleotides from monucleotides

The conversion of AMP to ADP was first shown in 1943 by Colowick and Kaplan (142) who used a purified enzyme from rabbit skeletal muscle. The reversible conversion of ADP to AMP and ATP occurs according to the following scheme. 2 ADP $\rightleftharpoons$ AMP + ATP. The enzyme is known as myokinase or adenylate kinase (143). It is specific for adenine nucleotides (144) and requires Mg^{2+}. Kashket and Denstedt (145) have demonstrated the association of adenylate kinase activity with the red blood cell membrane. The kinetics of the reaction have been investigated by Colowick and Kaplan (142, 143) who showed that 60% of added ADP is converted to AMP and ATP in the presence of the enzyme obtained from rabbit muscle. Further investigations (143) revealed that this enzyme is activated by Mg^{2+}. The direction of the reaction depends on the ratio of Mg^{2+}/ADP. When this ratio is 0.5, the formation of AMP and ATP from ADP is favored, while formation of ADP is favored when the ratio is 1.0.

IMP phosphorylation

Evidence for the absence of ATP-dependent phosphorylation of IMP to ITP, similar to that mediating the conversion of AMP and GMP to the corresponding nucleoside diphosphates, has been obtained by Hershko et al. (90). They observed that the splitting of ATP induced by stroma-free hemolysates is markedly enhanced by the addition of GMP, but is not significantly affected by similar addition of IMP. Further evidence for the absence of IMP phosphorylation was obtained from erythrocytes; while hypoxanthine is incorporated only

into IMP (147, 148), adenine and guanine are incorporated mainly into their corresponding nucleoside triphosphates (88, 89).

In other studies Vanderheiden (149) found a large concentration of ITP (44-51 μg of phosphorus/g of hemoglobin) in erythrocytes obtained from two members of one family. These results led to detailed analysis of several samples of normal human erythrocytes which revealed the presence of small amounts of ITP in an average concentration of 1.8 μg of phosphorus/g of hemoglobin. Studies on in vitro synthesis of ITP from inosine-8-C^{14} in several blood samples showed variations in the ability of the individual samples to incorporate inosine into ITP. The ratio of radioactivity in ITP/IMP fell into three groups, 0.14 - 0.21, for the erythrocytes with high ITP concentrations; 0.01 - 0.1 for erythrocytes which can synthesize ITP; and which do not show increase in the concentration of this nucleotide on incubation with inosine. The authors explained the inability of other groups (147, 148) to detect ITP as being due to the small percentage of individuals possessing erythrocytes capable of synthesizing ITP. The mechanism of inosine incorporation into IMP could be accomplished either by guanine-hypoxanthine phosphoribosyl transferase (70) or by inosine phosphokinase. Direct evidence for the latter is lacking (150).

Conversion of dinucleotides to trinucleotides

The conversion of a dinucleotide to a trinucleotide may take place in the presence of nucleoside diphosphokinase (NDP) which was first described in 1953 (151, 152). The enzyme catalyzes the following type of reaction

$$N_1TP + N_2DP \rightleftharpoons N_1DP + N_2TP$$

When N = purine or pyrimidine base. Recently the enzyme from human erythrocytes has been purified 1400 - fold by Mourad and Parks Jr.(146). The enzyme is normally present in high concentrations in erythrocytes and is relatively non specific with regard to nucleotide substrates. ATP and GTP have Michaelis constants (Km) of $4 - 8 \times 10^{-4}$ and 1.5×10^{-4} respectively, while ADP has a Km of 4.0×10^{-5}. IDP and ITP were also shown to serve as substrates for the enzyme, while 5'-monophosphate nucleotides proved to be inhibitory. Results from kinetic studies are consistent with the hypothesis that the erythrocytic NDP kinase follows a "ping-pong" reaction mechanism involving a phosphorylated enzyme intermediate.

A second method for converting ADP to ATP involves substrate level phosphorylation which occurs in two reactions of the Embden- Meyerhof pathway. The first reaction involves the transfer of a phosphoryl group from the high energy phosphate ester 1,3-diphosphoglyceric acid to ADP resulting in the formation of 3-phosphoglyceric acid and ATP. The enzyme catalyzing this reversible reaction was first crystallized from yeast by Bucher (153) and requires Mg^{2+} for activity. The equilibrium of the reaction lies in the direction of production of 3-phosphoglyceric acid and ATP. Chapman et al. (59) have assayed the activities of the individual enzymes involved in glycolysis in human red blood cell hemolysates. They showed that phosphoglyceric kinase has the second highest velocity, exceeded only by triose phosphate isomerase. This finding supports the idea that a high activity of ADP - transphosphorylating

enzymes is in part responsible for the maintenance of a high ATP/ADP ratio in the red cell. The second reaction is catalyzed by pyruvic kinase and involves the conversion of phosphoenolpyruvic acid to pyruvic acid with a concomitant formation of ATP from ADP.

The equilibrium of this reaction lies in the direction of pyruvic acid and ATP formation. This enzyme is also Mg^{2+}-dependent and its activity is regulated by various allosteric effectors, as seen in Table 1. The reaction is stimulated by ADP and is inhibited by high concentrations of ATP. This enzyme plays an important role in adenine nucleotide regulation.

In vivo aging

A sample of normal human erythrocytes represents a population having a wide distribution of cell ages, from those newly formed to those at the end of their life span which in the case of human cells is about 120 days. Normally 0.8 percent of the erythrocytes are removed from the circulation daily (154) by a means as yet not clearly understood. In order to study the changes occurring in the red cell during the process of in vivo aging, it is desirable to separate the population of circulating red cells into groups of different ages. Various techniques have been used for this purpose, such as serial osmotic lysis (155) counter current distribution (156) and differential sedimentation by centrifugation (154, 157). Structural differences between young and old cells have been found by Leif and Vinograd (159) who have shown that old cells are more spherical. Marikovsky and Danon (158) have examined red blood cells of different ages using an electron microscope

and have noted the presence of filamentous protrusions on the surface of young cells, these were not seen on the oldest cells.

Chemical changes involving lowered concentrations of K^+ and Cl^- and lowered mean cell volume (159) as well as decreased Mg^{2+}, DPN (160, 162) and ATP concentrations (154, 163) have been reported in the oldest cell fraction. The ratio of ATP/ADP is 5 for young cells and declines for the older cell fraction. (160).

Enzyme activities in general were found to be higher in young cells and to decrease with cell age (160, 161, 155, 163). However, not all enzyme activity declined at the same rate. Brok et al. (163) have shown that the rate of hexokinase inactivation is more rapid than other enzymes, suggesting an important role for it and ATP (which was also shown to decline quickly) in determining the life span of normal human red blood cells.

Preservation of blood

The problem of preserving blood has been the subject of much investigation over the last sixty years. One of the first advances was made in 1915 by Weil (164), who introduced sodium citrate as an anticoagulant solution. In 1916 Rous and Turner (165) introduced the addition of glucose to the isotonic citrate medium, which greatly prolonged the period of preservation. Using this preservative medium Robertson (166) showed that blood could be usefully stored at 4°C for three to four weeks. Without additional glucose, the endogenous blood glucose would be completely depleted after about two weeks of storage.

The next major contribution to blood preservation technology was provided by Loutit (167) in 1943, who acidified the isotonic citrate-dextrose medium through the addition of citric acid. Acidification of the blood shifts the pH from 7.4 to about 7.0 and causes a further retardation of metabolic activity since the lower pH is further away from the pH optimum for glycolytic activity (59).

More recently an improved citrate-phosphate-dextrose preservative medium CPD has been introduced by Gibson et al. (168-170). There are three differences between CPD and ACD: 1) the initial pH of the blood collected in CPD solution is 7.2 compared to 7.0 with ACD; 2) citrate ion concentration in CPD solution is slightly less than that in the ACD; 3) in addition CPD contains phosphate (as its name would imply) in the form of dibasic sodium salt. The post-transfusion viability of erythrocytes stored for three to four weeks in this preservative has been claimed to be slightly better than that of a control stored in ACD.

It should be noted that the viability of the preserved erythrocytes is estimated by the proportion of the transfused red cells remaining in the recipient's circulation twenty-four hours after transfusion. When the per cent surviving in the circulation 24 hours after infusion is less than 70%, the preserved blood is considered unfit for clinical use. At 4°C the 70% survival value is reached in 3-5 days for cells preserved in citrate alone, 10-12 days for cells in citrate-dextrose and 21 days for cells in acidified citrate dextrose (ACD).

The preservation of erythrocytes results in numerous biochemical and structural changes, collectively referred to as the "storage lesion"(183). When blood is stored in acidified citrate dextrose (ACD) at 4°C the following changes take place: 1) a progressive impairment of glucose utilization as preservation proceeds (171); 2) a decrease in the concentration of organic phosphates with an accompanying increase in the inorganic phosphate concentration (171,33). The largest contributor to the increased pool concentration of inorganic phosphate is 2,3 diphosphoglycerate, the concentration of which is diminished to 20% of its initial fresh cell concentration within two weeks of preservation (50). The next largest contributor to the increased Pi pool size is ATP, the rate of its depletion is considerably slower and it reaches 20% of its initial fresh cell value only after six weeks of storage (50). 3) a loss of lipids commencing after the first or second day and reaching a maximum after about 12 days (172); 4) a decrease in intracellular K^+ and increase in intracellular Na^+ concentrations (171); 5) accompanying these chemical changes are various physical changes involving transformation of cell shape from biconcave to spherical with increase in volume and a decrease in diameter and surface area (171); a progressive increase in osmotic and mechanical fragility of the cells (171).

The reason for the decline in glucose utilization on prolonged storage has been the subject of much research. Bartlett and Barnet (33) have evidence to support the suggestion of an inactivation of phosphofructokinase. They studied the levels of organic phosphates in blood stored for 60 days in ACD

preservative medium and could not confirm the presence of F-1,6-di P even though G-6-P and F-6-P were found in relatively high concentrations. The work of Blanchaer et al. (173,177) supports this view. In their experiments this group has shown that in red cells phosphofructokinase loses 80% of its activity by the 25th day of storage.

Kashket et al. (174), on the other hand, claimed that the progressive impairment of the hexokinase enzyme is an important factor in the slowing down of glycolysis in preserved erythrocytes. They found the pH optimum for hexokinase to be 7.8. As preservation proceeds, lactic acid is accumulated through glycolytic activity. As a result, there is an increase in hydrogen ion concentration. Kashket et al. claim that it is this increase in hydrogen ion concentration which is responsible for the inhibition of hexokinase activity. Gabrio et al. (175) and Bishop (176) attribute the loss of glycolytic activity during storage to the gradual depletion of ATP. Bishop has been able to restore glucose utilization of four-week preserved erythrocytes to the fresh cell level by regenerating ATP concentration through preincubation of the erythrocytes with nucleosides.

The reason for the decline in organic phosphates and especially in 2,3 diphosphoglycerate and ATP, is not known. It is possible that the impairment of glycolysis is responsible for a decreased rate of ADP rephosphorylation, resulting in a decline in ATP concentration. Since ATP is needed for Na^+, K^+ (178,179,180) balance and so is directly involved in red cell shape, and osmotic and mechanical stability, as well as in hexokinase activity,

one would expect that a decline in ATP concentration is directly associated with most of the changes described in the "storage lesion".

An exception to this assumption may be lipid loss during storage. Reed et al.(181) and Rimon et al. (172) have investigated erythrocyte lipid changes during storage. Both groups have obtained similar results which indicate that starting from the first or second day of storage there is a steady loss of lipids (phospholipids and cholesterol) which levels off at approximately 25% of the total lipids after 12 days of storage and does not increase even after 42 days. This loss of lipid is not associated with the decline in ATP concentration nor is it associated with post transfusion viability. It seems therefore that the lipids lost during storage are not an essential part of the red blood cell membrane since their removal is not associated with decreased red cell viability.

The viability of preserved cells decreases with the duration of storage (171). Since the ATP content, more than any other factor, is associated with red cell function and stability, the efforts of investigators in this area have been directed toward improving the post-transfusion viability of red blood cells, mainly through increasing the ATP concentration of stored blood cells.

In 1954 Gabrio and Finch (175) made the first real breakthrough when they discovered that a regeneration of ATP concentration occurred when ACD-preserved blood was incubated with adenosine for 1 hour at 37°C. The regeneration of ATP content was accompanied by an improved post-transfusion viability when compared to a non incubated control (183). Further studies

reported that ATP regeneration is also associated with restoration of hexokinase activity, osmotic resistance, biconcave shape, intracellular Na^+, K^+ concentration and organic phosphate levels (185–190).

The addition of adenosine to the ACD preservative medium at the onset of storage was shown to improve the post-transfusion viability of the red blood cells (184). Further studies showed that other nucleosides such as inosine, deoxyadenosine, xanthine and guanosine had similar effects on viability (190).

The mechanism of the nucleoside action on the erythrocyte was attributed to the production of phosphorylated ribose as an energy substrate through the action of nucleoside phosphorylase (191). This enzyme was shown to act on inosine and guanosine (193) directly, but on adenosine only after its conversion to inosine by the erythrocyte enzyme adenosine deaminase (128). It is noteworthy that, in contrast to glucose-6-phosphate production from glucose, ribose-5-phosphate production from purine nucleosides proceeds without the expenditure of energy. The apparent superiority of adenosine as compared to inosine in both maintaining and regenerating the level of ATP in stored cells was explanined by Rubinstein et al. as being due to the NH_3 released on its deamination (194). But due to the hypotensive action of adenosine, its clinical use as a blood preservative is a potential hazard. Guanosine, xanthosine and inosine, on the other hand, in dosages equivalent to ten times the minimal hypotensive dosage of adenosine, do not cause an observable effect (5).

In 1959 Nakao et al. (185, 186) observed that while incubation of 8 week blood, preserved in ACD medium, with inosine for 3 hours at 37°C could not regenerate ATP, the addition of adenine together with inosine caused a marked increase in the ATP content in the same cells. Incorporation of adenine-8-C^{14} into adenine nucleotides was shown to occur. The regeneration of ATP in the eight week old blood incubated with inosine and adenine was postulated to occur via the "salvage pathway" which had been shown by Preiss and Handler (74) to be present in human red blood dried residue acetone extract. Inosine provides the ribose moiety of the newly synthesized nucleotide and also serves as a substrate for the generation of energy via the hexose monophosphate shunt and the Embden-Meyerof pathway. The failure of inosine alone to regenerate ATP is attributed to depletion of the adenine moiety .

The use of adenine as a additive to the ACD (195, 196) preservative medium in small amounts (0.75 ± 0.25 μ mole/ml ACD blood) without added nucleoside, maintained both higher ATP and total adenine nucleotide concentrations, resulting in satisfactory viability for at least five weeks. Supplementation of the ACD preservative medium with small amounts of adenine (0.75 ± 0.2 μ mole/ml ACD blood) and large amounts of inosine (10 μ moles / ml ACD blood) produced only a slight extension of viability (197). However, the elimination of inosine had the advantage of avoiding toxicity from uric acid overload in the blood of the recipient. The reported toxicity of adenine is low. No adverse clinical reactions, attributable to adenine,

were noted in a series of over 5,500 transfusions of blood stored up to 35 days in ACD-adenine preservative medium (198).

Modifications of the ACD-adenine solution have been made. Several groups (199,200) have shown that optimal pH for ATP maintenance in ACD-adenine solutions is 5.5 and not 5.0, as is the pH of the ACD medium. Supplementation of ACD adenine with Pi enhances the effect of adenine in generating and maintaining higher ATP levels (199). However, despite better ATP maintenance in the phosphate supplemented samples, no improvement of viability after 50 to 65 days of storage was found when compared to adenine supplementation alone (201). Unfortunately total adenine nucleotides were not determined, thus making a comparison of total adenine nucleotide concentration of cells preserved in this medium and the others already discussed impossible. While an association between high ATP concentration and post transfusion viability has been made with blood stored in ACD, ACD-adenine, and citrate phosphate dextrose CPD preservative media (202,187,203) ; whether this relationship is retained after 50 to 56 days of storage requires further elucidation.

Role of ATP in erythrocyte metabolism

The importance of ATP in the human red blood cell is well established, the release of energy from the hydrolysis of its terminal high energy phosphate bond is necessary for metabolic function and structural integrity (178,204,205,207). ATP expenditure is required for maintenance of cationic balance and electrical equilibrium via the sodium-potassium pump (178,179) as well as for mainten-

ance of extracellular volume, biconcave shape, and osmotic rigidity (204,205,207). High intracellular ATP concentrations are also necessary for maintenance of hexokinase (176) and phosphofructokinase activity (173,177) and subsequent glycolytic activity. In addition fatty acid esterification into phospholipids has been shown to be ATP dependent, with intracellular ATP diminution causing a decline in esterification (206). A wealth of evidence also indicates a direct correlation between intracellular ATP concentration and post-transfusion viability of red blood cells, as has been previously described (187,202,203).

Gross variations in ATP concentration has been associated with several different hereditary abnormalities (208-213). A low cellular ATP concentration has been associated with pyruvic kinase deficiency, a type of congenital nonspherocytic anemia (208,209). Pyruvic kinase deficient red blood cells are unable to maintain adenosine triphosphate levels and acquire a membrane lesion characterized by K^+ loss and crenation. The pyruvic kinase deficient reticulocyte, because of the presence of mitochondria, differs from the erythrocyte, in being able to maintain its concentration of ATP and is spared from destruction in the general circulation (213).

Elevated erythrocytic ATP levels have been found in three distinct enzymatic abnormalities (210,211,212). Two show distinct enzymatic abnormalities, in the first (210), twice normal pyruvic kinase activities and in the second (212), 2,3-diphosphoglycerate phosphatase deficiency, while in the third case (211), the primary biochemical abnormality has not been

elucidated. ATP levels in erythrocytes of these affected individuals varied from 128 to 198 percent of the normal level. Of particular interest, with regard to the importance of the elevated ATP, are the results obtained when post-transfusion viability was determined. After six weeks of storage in ACD solution under blood bank conditions, 74 percent of Cr^{51}-labeled autotransfused erythrocytes survived in the circulation 24 hours after transfusion, in contrast to 28 to 29 percent for similarly treated control erythrocytes (214).

In our laboratory Manohar (81,82) has shown that incubation of erythrocytes for 12 hours with glucose and adenine at 37°C causes a doubling of the level of nucleotide labile phosphorus. When [8-C^{14}] adenine incorporation into nucleotides was followed, most of the radioactivity was found to be incorporated into ATP; adenine incorporated into ADP was generally one-fifth that of ATP and incorporation into AMP, IMP and hypoxanthine (1-2%) was almost negligible. Adenine [8-C^{14}] incorporation into adenine nucleotides could be accounted for by the increment in nucleotide labile phosphorus produced following glucose and adenine incubation in fresh cells. Both adenine-8-C^{14} incorporation into nucleotides and elevation of nucleotide labile phosphorus increased linearly with time and reached a maximal at about 10-14 hours of incubation. The mechanism of ATP elevation in this system has been previously described (81,82).

The purpose of this research project was to study ATP elevation in human red blood cells. As previously described, Manohar reported the

doubling of the initial concentration of ATP in human erythrocytes by glucose and adenine incubation. Using this method for elevation of ATP concentration as a starting point, an investigation was undertaken in order to answer the following questions: First, can the level of ATP be elevated above twice the fresh cell value; second, what is limiting the synthesis of ATP under the experimental conditions studied and third what is the function of this "extra" ATP produced by the "salvage pathway" of synthesis?

EXPERIMENTAL

Materials

Adenine Grade A was obtained from Calbiochem Ltd., Los Angeles, California.

Adenosine and ATP were purchased from the Sigma Chemical Company, St. Louis.

Activated charcoal 'Norite S x 30 SG Extra' and Boehringer enzyme (test combination) kits for the determination of adenine nucleotides were obtained from Fisher Scientific Ltd., Montreal. The activated charcoal was found to be free of inorganic phosphorus and no UV-absorbing material could be eluted using 0.2N NH_4OH in 30% ethanol.

2,5-diphenoxyoxazole (PPO) and 1,4-bis-2-(5-phenoxazolyl) - benzene (POPOP) were obtained from Packard Instrument Company, Downer Grove, Illinois.

Phthalate esters methyl phthalate (s.g. 1.189) and di-n-butyl phthalate (s.g. 1.041) were obtained from Miles-Yeda Ltd., Rehovoth, Israel, through Brickman and Company, Montreal.

Adenine-8-^{14}C was purchased from Schwartz Bioresearch Corp., Orangeburg, N.J. Adenosine-8-^{14}C and hypoxanthine 8-^{14}C from the New England Nuclear Corp., Boston. All radioisotopes were found to be pure when checked by paper chromatography according to the method of Krebs and Hems (215).

ATP [γ^{32}P] was kindly supplied by Dr. Rhoda Blostein, Department of Hematology, Royal Victoria Hospital, Montreal.

Glucostat, glucose reagent and chromogen was obtained from Worthington Biochemical Corp., Freehold, N.J.

Reagents

Krebs-Ringer phosphate solution was prepared according to the method of Umbreit et al.(216) except for the omission of calcium chloride.

The scintillation counting fluid contained the following cocktail:

PPO (2,5-diphenyloxazole, scintillation grade)	12.0 g
POPOP (1,4-bis-2-(5-phenyloxazolyl benzene)	0.4 g
Toluene	4.0 liters

The nucleotide eluting solution for eluting adsorbed bases, nucleosides and nucleotides from activated charcoal consisted of the following:

Ammonium Hydroxide 28-29%	5.0 ml
Distilled water	200.0 ml
95% Ethyl alcohol	95.0 ml

Citrate buffers 0.45 M of pH 4.8 and pH 2.5 were used in paper electrophoresis and were prepared as follows:

Solution A	Crystalline Citric Acid	21.008 g
	1 N NaOH	200.0 ml
	Distilled water to make	1000 ml
Solution B	0.1 N HCl	

To make a citrate buffer of pH 4.8

9 parts of solution A

1 part of solution B

10 parts of distilled water

and a final adjustment of pH to 4.8

To make a buffer of pH 2.5

21.008 g of citrate was made to 2 L with distilled water and the final pH was adjusted to 2.5.

The chromatographic paper was used for separation of bases, nucleosides and nucleotides consisted of the following:

Isobutyric acid	300 ml
1M NH_4OH	180 ml
3.8% Ethylenediamine tetracetate (sodium salt)	4.8 ml

The chromatographic paper was Whatman 3 mm grade.

Methods

Preparation of Erythrocytes

Erythrocytes were obtained from normal donors and preserved at 4°C in acidified citrate dextrose solution. Samples were drawn under aseptic conditions from the preserved blood as required. In experiments where the freshly drawn blood was to be used immediately isotonic trisodium citrate (3.2%) served as the anticoagulant. Five ml of this solution was used for 20 ml of human venous blood. The sample was centrifuged in a 12 ml hard glass centrifuge tube in the 'International Clinical' centrifuge and the plasma along with the leucocytes* ("buffy coat") layer wereremoved by aspiration. The packed cells were resuspended in cold physiological sodium chloride (0.9%) and recentrifuged. The supernatant and any remaining leucocyte layer were removed. This procedure was repeated three or four times.

In experiments where it was desired to separate cells according to cell age (i.e. cell density) blood was collected into Erlenmeyer flasks and defibrinated with a glass rod. Two procedures were used, in one introduced by Bernstein (154) the defibrinated specimen was centrifuged in a siliconized glass centrifuge tube at 2000 x g for 15 minutes at 4°C. The supernatant serum and buffy coat was removed and the cells washed three times in an equal volume of Krebs-Ringer phosphate buffer pH 7.4. The erythrocytes were resuspended in Krebs-Ringer phosphate pH 7.4 and centrifuged in plastic tubes at 100 x g for 10 minutes,

* White cell contamination in all preparations never exceeded 50 W.BC/cu mm.

and then centrifuged for 45 minutes at 2000 x g . The supernatant was discarded and the top layer of cells representing 3 to 5 per cent of the total cell population and containing the youngest cells was removed by Pasteur pipette. The bottom layer of 3 to 5 per cent containing the oldest cells was collected by puncturing the bottom of the tube.

In the second procedure used by Danon and Marikovsky (157) a series of water immiscible phthalate esters ranging in specific gravity from 1.062 to 1.180 in increments of 0.004 was made using various proportions of stock solutions of methyl phthalate (s.g. 1.189) and di-n-butyl phthalate (s.g. 1.041). In a preliminary experiment it was necessary to find three phthalate solutions of the series which could best separate firstly, leucocytes from erythrocytes, secondly, 5% of the least dense cells (i.e. the youngest cells) and thirdly, 5% of the most dense cells (i.e. the oldest cells) from the total cell population. This was done using defibrinated blood in capillary hematocrit tubes centrifuged at 2000 x g for 15 minutes. From the resulting separation of cells using the complete series of phthalate solutions three tubes were noted which most closely fulfilled our criteria (as previously indicated). Three phthalate solutions in the series were then selected which were 0.004 s.g. higher than those used in the capillary tube test. As a rule these phthalate solutions had s.g. of 1.070, 1.078 and 1.106. For preparation of larger quantities of cell groups as required in our experiments, defibrinated blood was centrifuged in tubes (8 x 100 mm) with an appropriate amount of phthalate solution at 2000 x g for 15 minutes.

Two different runs were necessary the first to separate leucocytes from erythrocytes and the second to separate the least or most dense cells from the total population. Accordingly, following the first separation cells had to be washed and resuspended in an equal volume of Krebs-Ringer phosphate buffer, pH 7.4. Following the second centrifugation the fractions of oldest and youngest cells were collected and the remaining cells from the resulting separations were pooled. The three pools of cells were washed twice with Krebs-Ringer phosphate buffer and the cells were then incubated in an appropriate incubation medium.

Procedure for incubation of intact erythrocytes

To study the effect of adenosine and adenine and other substrates upon ATP level, one volume of packed erythrocytes was suspended in 4 volumes of Krebs-Ringer phosphate buffer, pH 7.4. containing the required substrates. During prolonged incubations* 0.6 mg each of penicillin and streptomycin per ml of incubation medium was added. The incubations were terminated by addition of an equal volume of cold 0.6 N perchloric acid. Following centrifugation at 2000 x g for 15 minutes at 4°C the supernatant also known as the protein free filtrate was divided into aliquots to be analyzed using the methods described in the section on 'Analytical Procedures'.

Assay of enzyme activity in human red blood cells hemolysates

Adenosine kinase (ATP: adenine-5-phosphate-transferase EC.2.7.1.20) activity was measured by a modification of the method of Schnebli et al.(120). Erythrocytes were hemolyzed by rapid freezing and thawing and then dialyzed for

* Refers to incubations exceeding 6 hours.

2 hours in 2 liters of a 0.001 M Tris HCl buffer, pH 7.4 at 4°C. About two mg of protein from the dialyzed hemolysate were incubated in 1.0 ml of a solution containing 2.5 mM* $MgCl_2$, 2.5 mM ATP, 50 mM phosphate buffer pH 7.0 and 22.4 mμ moles, adenosine 8-^{14}C for 20 minutes at 25°C. The reaction was terminated by heating in a boiling water bath for 1 minute. Nucleosides, nucleotides and bases were separated by paper ionophoresis or paper chromatography and incorporation of radioisotope determined as detailed in 'Analytic Procedures'.

Phosphoribosyl pyrophosphate synthetase (ATP: D-ribose-5-phosphate pyrophosphotransferase, EC.2.7.6.1) was assayed by the procedure of Hershko et al.(90) and adenine phosphoribosyl transferase (adenosine monophosphate: pyrophosphate phosphoribosyl transferase EC.2.4.2.7.) by the method of Kelley et al. (116).

Analytical Procedures

The hemoglobin content of the red cell suspension or the hemolysate was determined by the modified cyanmethemoglobin method of King (217) as further modified by Brownstone (218).

Nucleotide labile phosphorus from di and tri nucleotides was determined by the method of Crane and Lipmann (219). To ensure complete adsorption of nucleotides 20 mg of activated charcoal per ml of packed erythrocytes was used since preliminary experiments showed that this was more than sufficient.

The intracellular ATP, ADP and AMP values were measured enzymatically using Boehringer enzyme (test combination) kits according to the procedures

* mM or mM denotes millimolar

described by Adam (220).

Isolation and separation of bases, nucleosides and nucleotides was done according to the following method. Erythrocytes were hemolyzed and protein was precipitated by the addition of 0.6 N perchloric acid. The suspension was then centrifuged and the protein free filtrate removed. Activated charcoal was next added and the resulting suspension was throughly mixed with a glass rod. Adsorption of the purines was complete within ten minutes. This was confirmed by running a control and comparing the UV absorption of the supernatants after centrifugation of the charcoal. The supernatant was then discarded and the charcoal was washed twice more with distilled water. The nucleosides, nucleotides and bases were eluted from the charcoal with 5.0 ml of 0.2 N NH_4OH in 30 per cent ethanol by continuous mixing for about 10 minutes. The suspension was then centrifuged for 20 minutes at high speed. The supernatant containing eluted base derivatives was removed and lyophilyzed to dryness. The dried material was redissolved in 0.5 ml of distilled water for electrophoretic or chromatographic treatment.

Electrophoresis of the eluates was carried out with a Spinco apparatus of the Durrum Type, Model R, Series D, using 12 x 1.5 inch paper strips in a citrate buffer (0.045 M)pH 4.8 or pH 2.5 when a better separation of IMP from AMP was desired. Electrophoresis was usually run for four hours at a constant current of 50 ma. Under these conditions using a citrate buffer of pH 4.8 ATP, ADP, AMP, IMP separate from one another in distinct bands. Adenine

and adenosine also separated distinctly from one another and other nucleotides. However inosine and hypoxanthine ran close to adenosine. To separate these nucleosides paper chromatography proved more useful.

For chromatographic separation of bases, nucleosides and nucleotides a solvent mixture containing isobutyric acid NH_4OH (1.0 M) and EDTA (0.1 M) in a V/V ratio 100:60:1.6 (215) was used. Chromatograms were developed for about 15 hours in ascending fashion. AMP, ADP and ATP, adenine, hypoxanthine, inosine and adenosine were separated as distinct bands from each other. IMP and ATP however, moved closely together.

After separation the paper strips or chromatographic sheets were dried and the position of the bases nucleosides and nucleotides were revealed upon UV exposure from a mercury vapour lamp in a dark room as dark bands on the fluorescent paper background. The positions were marked with a soft lead pencil cut out and put in a separate scintillation counter vial. The method used was essentially that of Wang and Jones (221). The vials were filled with "phosphor" so that the paper specimens was completely covered. The radioactivity of each sample was then determined by placing the vials in the Packard Tri-Carb Liquid Scintillation spectrometer (Packard Model 754).

The concentration of nucleotides was estimated by elution from the paper with 0.01 N HCl. The optical densities of the eluates containing adenine nucleotides were measured at 257 mμ, and IMP at 249 mμ. The molar extinction coefficients were taken as 14.7×10^3 for ATP and ADP, 15.1×10^3 for AMP, and 11.7×10^3 for IMP (222).

Glucose was measured by the glucostat method (223, 224).

Osmotic fragility was determined using the Sanford method (225) and the Osmotic Fragilograph obtained from Kalmedic Co., N.Y.

RESULTS

PART I. - AN ATTEMPT TO FURTHER ELEVATE ATP CONCENTRATION ABOVE TWICE THE NORMAL RED CELL LEVEL

One of the original objectives of this research project was to elevate the initial level of erythrocyte ATP, to above twice the fresh cell level first achieved in our laboratory by S. V. Manohar (81,82). Optimal concentrations of glucose and adenine were found to be 21.0 mM and 1.5 mM respectively, for maximum nucleotide labile phosphorus elevation.

The influence of hydrogen ion and inorganic phosphate concentrations on nucleotide labile phosphate elevation had not been investigated. In view of the known stimulatory effect of high pH (52,59) and of inorganic phosphate (52,62) on both glycolytic rate and PRPP synthetase activity, it was reasonable to expect that hydrogen ion as well as inorganic phosphate concentrations would be important factors in ATP elevation following glucose and adenine incubation.

The effect of hydrogen ion concentration on nucleotide elevation by glucose and adenine incubation

In this series of experiments optimal concentrations of glucose and adenine were added to Krebs-Ringer solution containing 10 mM tris and 10 mM phosphate buffers adjusted to the required pH with NaOH or HCl. At the end of 12 hours incubation, nucleotide labile phosphorus was determined as described previously in Methods. The results presented in Table 3 indicate that maximal elevation of nucleotide labile phosphorus occurred over the broad range pH 6.5-8.5. However at all pHs studied the maximum level of nucleotide labile phosphorus did not exceed

Table 3.

THE EFFECT OF HYDROGEN ION CONCENTRATION ON GLUCOSE AND ADENINE ELEVATION OF NUCLEOTIDE LABILE PHOSPHORUS.

pH	Incubation period (hours)	Nucleotide labile phosphorus µg P/100 mg Hb	Increment in nucleotide labile phosphorus µg P/100 mg Hb
7.4	0	22	-
6.0	12	26	4
6.5	12	48	26
7.0	12	48	26
7.5	12	49	27
8.0	12	47	25
8.5	12	47	25

Conditions.

1.0 ml of erythrocytes were incubated with 4 ml of Krebs-Ringer solution containing 21.0 mM glucose and 1.6 mM adenine in a 0.01 M tris and 0.01 M phosphate buffer at the required pH . The results presented are typical of three such experiments.

twice that initially present in freshly drawn human erythrocytes. In subsequent experiments pH 7.4 was used since it is the most physiological pH and is in the optimal range for nucleotide labile phosphate elevation.

The effect of phosphate concentration on glucose and adenine elevation of nucleotide labile phosphorus and [8-^{14}C] adenine incorporation into adenine nucleotides

In this set of experiments a 12 mM tris HCl buffer pH 7.4 replaced the phosphate buffer of the Krebs-Ringer phosphate solution normally used. It can be noted from the results summarized in Table 4 that both elevation of nucleotide labile phosphorus and incorporation of adenine 8-^{14}C into ATP are phosphate dependent. In the absence of phosphate there is a negligible increase in nucleotide labile phosphorus and only a small incorporation of adenine into ATP. Maximal nucleotide labile phosphorus elevation and 8-^{14}C adenine incorporation into ATP occurs at 10 mM phosphate ion concentration. Increasing the level above 10 mM to as high as 50 mM had no further stimulatory effect. It should also be pointed out as Manohar has previously shown (81), that the observed values for adenine incorporation into nucleotides closely corresponds to that calculated from nucleotide labile phosphorus, on the assumption that the increment is due to newly synthesized ATP.

Having failed to further increase the final level of nucleotide labile phosphorus by either increasing pH, or inorganic phosphate concentration, we shifted the focus of the investigation from energy producing systems (i.e. ATP synthesis from ADP) to a study of factors effecting AMP synthesis from glucose

Table 4.

THE EFFECT OF PHOSPHATE CONCENTRATION ON GLUCOSE AND ADENINE ELEVATION OF NUCLEOTIDE LABILE PHOSPHORUS AND ADENINE - 8-^{14}C INCORPORATION INTO NUCLEOTIDES

Phosphate concentration	Incubation period	Nucleotide labile phosphorus	Adenine-8-C^{14} incorporation into adenosine triphosphate mμ moles/100 mg Hb	
mM	hours	μg P/100 mg Hb	observed	calculated
-	0	21		
0	12	23	33	32
5	12	35	182	224
10	12	41	275	320
20	12	40	281	304
30	12	42	279	336
50	12	40	268	304

Conditions.

1.0 ml human erythrocyte incubated with 1 μC adenine-8-C^{14}, 1.6 mM adenine 21.0 mM glucose at 37°C with varying concentrations of phosphate as indicated in Krebs-Ringer solution, 12 mM tris HCl buffer pH 7.4. The results presented are typical of three such experiments.

and adenine.

As has previously been described the synthesis of AMP from glucose and adenine involves glucose utilization to supply ribose-5-phosphate and other ribose moieties required in nucleotide synthesis. Catabolism of various purine nucleosides is also known to produce ribose-5-phosphate and PRPP (124) through an alternate pathway not involving the transketolase system. Thus, by the addition of nucleoside, the pool size of ribose phosphate derivatives may be increased. The effect of various nucleosides on stimulating nucleotide synthesis was therefore investigated.

Influence of purine nucleosides on the nucleotide labile phosphorus content of human erythrocytes in the presence and absence of glucose

The effect of three nucleosides, inosine, guanosine and adenosine at the fairly high concentrations of 32 mM, 12 mM, 32 mM respectively, were tested in incubation media containing 1.6 mM adenine. From the results presented in Table 5 it is evident that incubation of red cells with adenine but without glucose and/or nucleoside (i.e. the ribose phosphate precursors) resulted in a substantial drop in the level of nucleotide labile phosphorus from preincubation values. Incubation with inosine in the absence of glucose produced a doubling of the nucleotide labile phosphorus present prior to incubation. The elevation obtained was of the same degree as that seen following glucose and adenine incubation. This suggests that the catabolism of inosine can supply a sufficient amount of ribose phosphate for ATP synthesis. When the two ribose phosphate producing systems - the first involving the transketolase catalyzed reactions and the second nucleoside

Table 5.

INFLUENCE OF PURINE NUCLEOSIDES ON THE NUCLEOTIDE LABILE PHOSPHORUS CONTENT OF HUMAN ERYTHROCYTES IN THE PRESENCE AND ABSENCE OF GLUCOSE

Nucleoside	Nucleotide labile phosphorus µg P/100 mg Hb	
	+ glucose	- glucose
unincubated	24.3 ± 0.7 (10)	24.3 ± 0.7 (10)
none	48.1 ± 1.9 (10)	6.9 ± 1.2 (3)
inosine	48.2 ± 1.5 (3)	48.6 ± 1.8 (4)
guanosine	46.9 ± 1.6 (3)	-
adenosine	23.9 ± 1.1 (10)	24.6 ± 1.12 (4)

Conditions

Each incubation mixture contained 1.0 ml erythrocytes in 4.0 ml Krebs-Ringer phosphate solution, pH 7.4 containing 1.6 mM adenine and 21.0 mM glucose and was incubated for 12 hours at 37°C. Concentration of added nucleosides: adenosine, 32 mM; inosine, 32 mM; guanosine, 12 mM. The figures in brackets represent the number of experiments.

phosphorylase - were used simultaneously there was no additional increment in nucleotide labile phosphate, showing that ribose phosphate production is not limiting the syntheses of ATP from either glucose and adenine; or inosine and adenine. Of the other nucleosides tested the addition of guanosine produced comparable effects to those obtained with inosine incubation. However supplementation of the incubation media containing 1.6 mM adenine with 32 mM adenosine in the presence or in the absence of glucose, failed to elevate the fresh cell value of nucleotide labile phosphorus. This observation was of particular interest and was further investigated. The results of this study are presented in the second part of this section.

Although inosine cannot elevate the level of nucleotide labile phosphorus above twice that of fresh cells, it was thought that it might have an effect on the rate of nucleotide synthesis. This possibility was therefore investigated.

Influence of inosine on the rate of nucleotide labile phosphorus synthesis from glucose and adenine

The results in Table 6 indicate that addition of inosine to the incubation media caused no significant change in the rate of nucleotide labile phosphorus synthesis from glucose and adenine. Throughout the incubation period the rate of nucleotide labile phosphorus synthesis is linear with maximum synthesis occurring at about 12 hours; prolonged incubation up to 14 hours had no additional effect.

During glucose and adenine incubation there is an accumulation of various metabolites, including lactic acid and hydrogen ion. The possibility that these metabolites may limit the synthesis of nucleotides, especially during

Table 6.

INFLUENCE OF INOSINE ON THE RATE OF NUCLEOTIDE LABILE PHOSPHORUS FORMATION FROM GLUCOSE AND ADENINE

Period of Incubation	Nucleotide labile phosphorus μg/ 100 mg Hb	
	without inosine	with inosine
0	23	23
3	28	28
6	33	32
9	39	40
12	45	46
14	46	46

Conditions. 1.0 ml of packed human red blood cells were incubated with 4.0 ml Krebs-Ringer phosphate buffer pH 7.4 containing 1.6 mM adenine and 21.0 mM adenine at 37°C. The results presented are typical of three such experiments. Maximum nucleotide labile phosphorus formation is reached from 10 to 14 hours with the rate of synthesis being linear with time.

prolonged incubation, was investigated by renewing the incubation medium periodically.

The results of this series of experiments are presented in Table 7. In these experiments red blood cells were incubated for 12 hours with glucose and adenine, following which the cells were removed, washed three times in 0.9% saline, and then reincubated for an additional twelve hours with glucose, glucose and adenine, or glucose, adenine and inosine in Krebs-Ringer phosphate buffer pH 7.4. As the results indicate, replacement with any of the three incubation media did not significantly alter the final level of nucleotide labile phosphorus nor adenine incorporation into ATP. It may also be noted as previously demonstrated that the increment in nucleotide labile phosphorus can be almost entirely accounted for by new ATP synthesis. Furthermore, it is evident that once a maximal nucleotide labile phosphorus concentration or adenine incorporation into nucleotide has been reached, the maintenance of this maximal level does not require the presence of adenine, provided glucose is available as a substrate for glycolysis.

PART II. - INHIBITION OF NUCLEOTIDE SYNTHESIS IN HUMAN ERYTHROCYTES BY HIGH CONCENTRATIONS OF ADENOSINE

The relationship between the inhibition of nucleotide labile phosphate synthesis in the presence of adenosine and the incorporation of adenine-8-^{14}C into cellular nucleotides was investigated.

Adenosine inhibition of adenine incorporation into adenine nucleotides and related compounds

Table 7.

INFLUENCE OF CHANGE OF MEDIUM ON THE NUCLEOTIDE LABILE PHOSPHORUS CONTENT AND ADENINE INCORPORATION INTO ADENINE NUCLEOTIDES IN RED CELLS INCUBATED FOR 24 HOURS.

Incubation medium	Period of incubation	Nucleotide labile phosphorus	Adenine-8-C^{14} incorporation into nucleotides	
	hours	µg P/100 mg Hb	mµmole/100 mg Hb	
			observed	calculated
Glucose + adenine	0	22.9	-	-
Glucose + adenine	12	44.0	316	344
Glucose + adenine replaced with G + A	24	44.9	335	359
Glucose + adenine replaced with G	24	44.9	354	359
Glucose + adenine replaced with G+A+I	24	45.8	326	313
Glucose + adenine	24	47.1	376	395

Conditions. 1.0 ml of erythrocytes were incubated with 4 ml of Krebs-Ringer phosphate buffer pH 7.4 containing 1.5 m$\underline{M}$ adenine, 21.0 m$\underline{M}$ glucose and 1 µc adenine-8-C^{14}. After 12 hours cells were centrifuged and the incubation medium removed and replaced with the required incubation solution. Inosine concentration 32 m$\underline{M}$. The results presented are typical of three such experiments.

G - denotes glucose

A - denotes adenine

I - denotes Inosine

Table 8.

INCORPORATION OF ADENINE-8-^{14}C INTO THE ADENINE NUCLEOTIDES AND RELATED COMPOUNDS*

	Adenine-8-^{14}C	Adenine-8-^{14}C +adenosine
ATP	218.7	12.8
ADP	67.2	6.6
AMP	3.8	2.5
IMP	11.0	5.0
Adenosine, Inosine, Hypoxanthine	2.5	2.3

*mμmoles adenine-8-^{14}C incorporated/100mg Hb.
Note: Each incubation mixture contained 1.0ml erythrocytes in 4.0 ml Krebs-Ringer phosphate solution. The mixture contained 21.0 mM glucose, 1.5 mM adenine, 2000,000 c.p.m. and 16 mM adenosine where required. Incubation time, 6h; temperature, 37°C; gas phase air.

The results in Table 8 indicate that the incorporation of adenine-8-^{14}C into ATP and ADP is almost completely inhibited by the presence of adenosine. Incorporation into AMP, IMP, adenosine, inosine and hypoxanthine is relatively small both in the controls and in the presence of adenosine.

Both inosine and guanosine have been found to be capable of replacing glucose as a source of ribose phosphate for the synthesis of nucleotides from adenine. If the reason for adenosine inhibition of nucleotide synthesis is an inhibition of glucose production of ribose phosphate, supplementation of the incubation medium with either inosine or guanosine would relieve the inhibition.

The effect of inosine and guanosine addition in overcoming adenosine inhibition of nucleotide synthesis from glucose and adenine

The results presented in Table 9 show that supplementation of the incubation medium with inosine or guanosine could not alleviate adenosine inhibition, indicating that the inhibition is probably not in the production of ribose phosphate.

Since both adenine and adenosine are similar in structure, a competitive inhibition was considered. This possibility was investigated by increasing adenine concentration in an attempt to overcome the inhibition by adenosine. The results of this study are presented in Table 10. In these experiments adenine concentrations ranging from 1.6 mM up to ten times that normally used were tested, but it was found to be impossible to relieve the inhibition of nucleotide labile phosphorus synthesis by adenosine.

Erythrocytes have been shown to possess a potent adenosine deaminase

Table 9.

THE EFFECT OF INOSINE AND GUANOSINE ADDITION IN OVERCOMING ADENOSINE INHIBITION OF GLUCOSE AND ADENINE ELEVATION OF NUCLEOTIDE LABILE PHOSPHORUS.

Nucleoside	Incubation period	Nucleotide labile phosphorus
		µg P/100 mg Hb
none	0	22
none	12	46
adenosine	12	23
adenosine + inosine	12	22
adenosine + guanosine	12	23

Conditions.

0.5 ml of packed erythrocytes were incubated at 37°C in 4.0 ml Krebs-Ringer phosphate buffer pH 7.4 containing 1.6 mM adenine and 21.0 mM glucose; 16 mM of each nucleoside added as indicated. The experiment presented is typical of 2 such experiments.

Table 10.

THE EFFECT OF INCREASING CONCENTRATIONS OF ADENINE IN OVERCOMING THE INHIBITION BY ADENOSINE OF THE ELEVATION OF NUCLEOTIDE LABILE PHOSPHORUS PRODUCED BY GLUCOSE AND ADENINE INCUBATION OF RED BLOOD CELLS

Hours of incubation	Adenine concentration (mM)	Adenosine concentration (mM)	Nucleotide labile phosphorus µg P /100 mg Hb)
0	0	0	22.2 ± 1.1
12	1.6	0	45.8 ± 3.1
12	3.2	16	23.7 ± 1.2
12	4.8	16	23.3 ± 1.1
12	8.0	16	23.6 ± 1.7
12	16.0	16	22.7 ± 0.7

Conditions.

0.5 ml of red blood cells were incubated for 12 hours in 4 ml Krebs-Ringer phosphate pH 7.4 containing 21.0 mM glucose and varying concentrations of adenine and adenosine as can be seen from the Table. Temperature: 37°C ; gas phase, air.

(139). The study of adenosine as an inhibitor is therefore complicated by the fact that it is deaminated with formation of ammonia (140) and inosine, resulting in an increase in pH (140). The possibility that these catabolic metabolites of adenosine breakdown might inhibit the synthesis of nucleotide labile phosphorus was then investigated. The results of these experiments are tabulated in Table 11. They indicate that neither ammonium ions alone nor ammonium ions in the presence of inosine have any inhibitory effect on nucleotide labile phosphorus synthesis. In addition, the results of a series of incubations as shown in Table 3 indicated that over pH range 6.5-8.5 there is no change in the level of nucleotide labile phosphate synthesized after 12 hours of incubation with glucose and adenine.

The possibility of reversing adenosine inhibition was then studied. In these experiments erythrocytes were incubated in the presence of adenosine with glucose and adenine for periods of 2, 6 and 12 hours. At the end of the 2 or 6 hour incubation period the cells were washed and reincubated with glucose and adenine but without adenosine. The synthesis of nucleotide labile phosphorus for these two samples and those incubated for the full 12 hours with adenosine is presented in Table 12. As in previous experiments, after 12 hours of incubation, the level of nucleotide labile phosphorus was doubled in the absence of adenosine but there was no increase when adenosine was present for the entire incubation period. Removal of adenosine after 2 hours, followed by reincubation in the absence of the inhibitor for an additional 10 hours, produced a level of nucleotide labile phosphorus similar to that seen when adenosine was absent for the entire

Table 11.

INFLUENCE OF ADENOSINE, AND NH_4Cl, AND NH_4Cl + INOSINE ON THE LABILE PHOSPHATE CONTENT OF HUMAN ERYTHROCYTES

Additions to cell suspensions	Incubation time(h)	Labile phosphate (µg P/100 mg Hb)
Adenine	0	-24.5
Adenine	12	47.9
Adenine + adenosine	12	30.3
Adenine + NH_4Cl	12	50.0
Adenine + NH_4Cl + inosine	12	50.1

Note: Each incubation mixture contained 1.0 ml erythrocytes in 4.0 ml Krebs-Ringer phosphate solution, pH 7.4, containing 21.0 mM glucose. 1.5 mM adenine, 16mM NH_4Cl, 16mM adenosine, and 16mM inosine were added to the incubation mixture when required.

Table 12.

EFFECT OF THE REMOVAL OF ADENOSINE ON THE INHIBITION OF NUCLEOTIDE LABILE PHOSPHATE SYNTHESIS.

Hours of incubation		Nucleotide labile phosphate (µg P/100 mg Hb)	
with adenosine	After removal of adenosine	Expt. 1	Expt. 2
0	0	22.5	18.7
0	1 2	44.9	36.5
1 2	0	21.5	18.7
2	10	42.1	39.3
6	6	32.8	29.5

Note: Incubation mixture contained 1.0 ml erythrocyte in 4.0 ml Krebs-Ringer phosphate solution, pH 7.4 containing 1.5 mM adenine, 21.0 mM glucose, and 32mM adenosine where required. The cells were washed once with saline for the removal of the adenosine. Temperature, 37°C; gas phase, air.

12 hour incubation period. When cells were incubated with adenosine for 6 hours prior to the removal of the inhibitor, synthesis of nucleotide labile phosphorus occurred during the remaining 6 hours, although apparently there was insufficient time for the level to reach that of the control.

Influence of varying concentrations of adenosine on the level of nucleotide labile phosphorus following glucose and adenine incubation

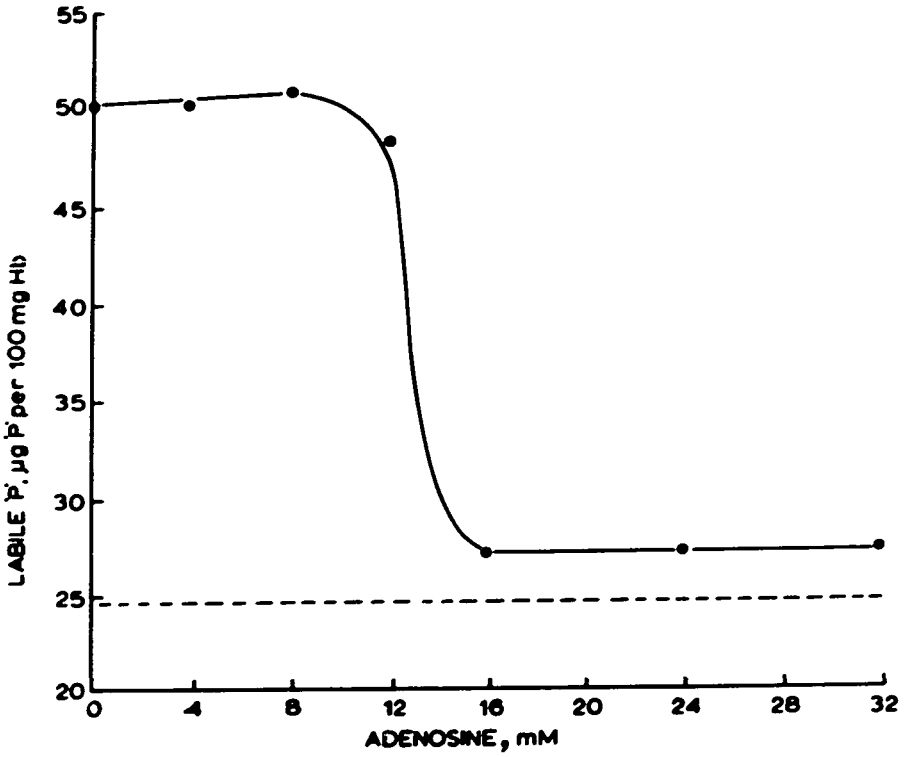
To determine the concentration of adenosine required for inhibition of nucleotide labile phosphorus elevation, red blood cells were incubated with a variety of adenosine concentrations ranging from 0-32 mM. In the experiments cited in Fig. 2 a concentration of 12 mM adenosine did not inhibit the synthesis of nucleotide labile phosphorus. However with 16 mM adenosine the inhibition is virtually complete. In the series of experiments of this type it was found that the lowest concentration of adenosine producing a measurable inhibition of nucleotide labile phosphorus synthesis varied between 12 and 16 mM.

The relationship between the levels of adenosine remaining in the incubation medium and the incorporation of adenine-8-^{14}C into nucleotides of incubated erythrocytes

The relationship between the deamination of adenosine and the inhibition of adenine incorporation into nucleotides was studied at three concentrations of adenosine-8-^{14}C. In parallel experiments the incorporation of adenine-8-^{14}C into nucleotides and adenosine-8-^{14}C remaining in the incubation medium was determined under identical conditions. The results are plotted in Fig. 3. After 3 hours of incubation 90% of the adenosine had been removed when the initial

Figure 2

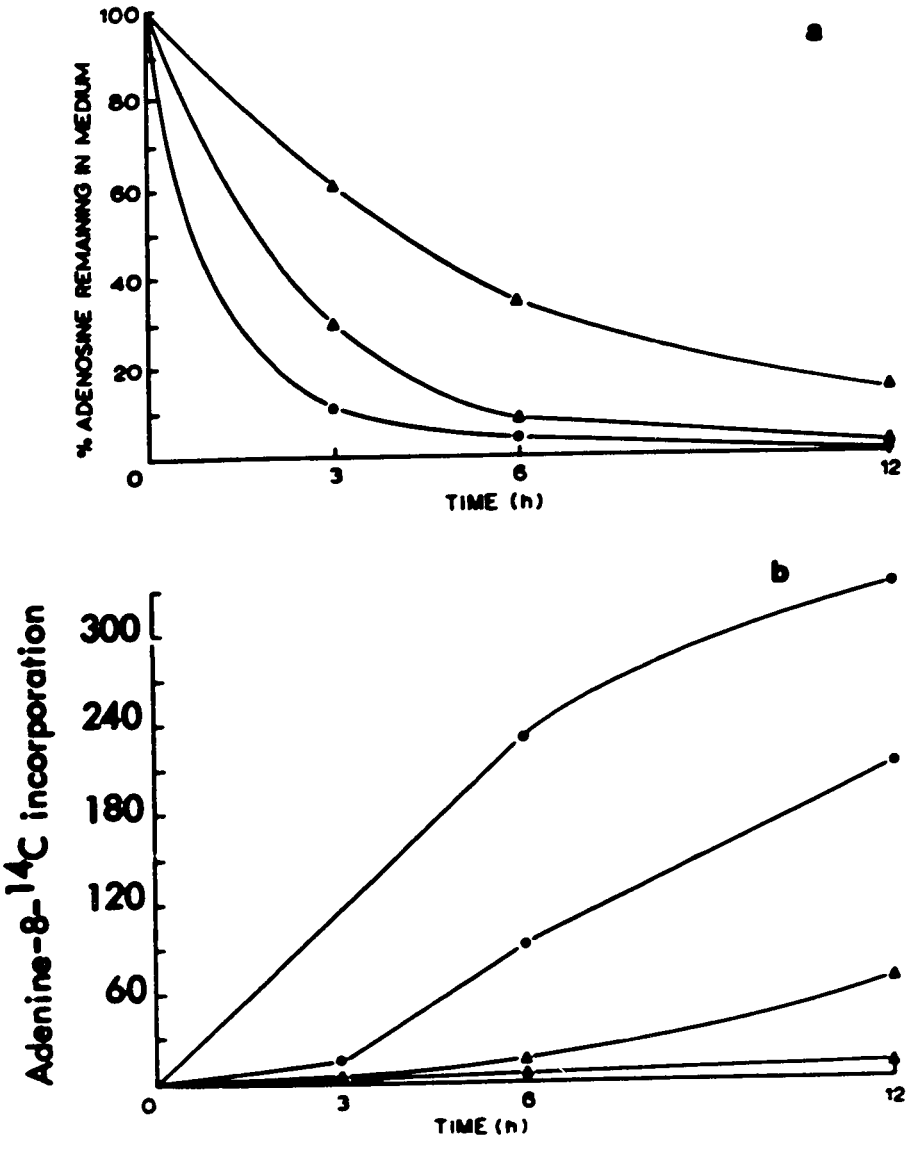
THE INFLUENCE OF VARYING CONCENTRATIONS OF ADENOSINE ON THE LEVEL OF NUCLEOTIDE LABILE PHOSPHORUS IN THE HUMAN ERYTHROCYTE FOLLOWING INCUBATION WITH ADENINE AND GLUCOSE.



The broken line represents the level of labile phosphate in the erythrocytes prior to incubation. Incubation conditions as outlined in table 12.

Figure 3

THE RELATIONSHIP BETWEEN THE LEVELS OF ADENOSINE REMAINING IN THE MEDIUM AND THE INCORPORATION OF ADENINE-8-^{14}C INTO NUCLEOTIDES OF THE INCUBATED ERYTHROCYTES.



* Units - mμ moles/100 mg Hb

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Figure 3 Legend

(a) % of adenosine-8-^{14}C remaining in the incubation medium; (b) incorporation of adenine-8-^{14}C into erythrocyte nucleotides. (●), control (no adenosine present); (Δ), 32 mM adenosine present at the start of the incubation; (▲), 16mM adenosine present at the start of the incubation; (o), 12 mM adenosine present at the start of the incubation. Incubation conditions as described in Table 12. Each flask contained 1μc adenine-8-^{14}C or adenosine-8-^{14}C as required. Each point represents 1ml of the incubation mixture.

level of the nucleoside was 12 mM (Fig. 3); during this time there was considerable inhibition of adenine-8-^{14}C incorporation into nucleotides (Fig. 3b). As the adenosine disappeared from the incubation medium the rate of adenine-8-^{14}C incorporation into nucleotides markedly increased. A similar phenomena was found to occur when 16 mM adenosine was the initial concentration, however with this higher concentration of nucleoside a greater delay in adenine-8-^{14}C incorporation into nucleotides was observed. In the presence of 32 mM adenosine, 12 hours of incubation was insufficient to bring about complete deamination of the nucleoside and no incorporation of adenine-8-^{14}C into nucleotides was observed. The increment of nucleotide labile phosphate in these experiments, not shown here, followed the pattern of adenine-8-^{14}C incorporation into nucleotides.

In view of the inhibitory effects of AMP on adenine phosphoribosyl transferase (110,111) and the inhibitory effects of ADP on PRPP synthetase (90), the possibility that the apparent adenosine inhibition was due to alteration of the levels of these nucleotides was investigated.

The effect of adenosine incubation on nucleotide concentration and [8-^{14}C] adenine incorporation into the individual nucleotides

For these experiments 32 mM adenosine was used along with 1.6 mM adenine. Nucleotide concentrations were determined after electrophoretic separation of the nucleotides as described previously in Materials and Methods. The nucleotides were eluted from the paper with 0.01 N HCl and optical densities of the eluates were measured. The results shown in Table 13 indicate

Table 13.

THE EFFECT OF ADENOSINE INCUBATION ON INTRACELLULAR NUCLEOTIDE CONCENTRATIONS †

	Control**	+ Adenosine	Unincubated	Control	+ Adenosine
ATP	324	30	310	650	295
ADP	60	12	75	115	120
AMP	10	2	32	40	41
IMP	8	3	40	40	290
Nucleotide labile phosphorus*	43	22	21	43	22

* Last horizontal row of figures represents μg P/100 mg Hb.

† Concentration of nucleotides expressed in μmoles/100 mg Hb.

** Incubated with glucose and adenine.

Conditions as previously described.

that adenosine did not significantly alter the concentrations of ADP and AMP when compared to a control incubated with glucose and adenine alone. These results demonstrate again the inhibition of adenine-8-^{14}C incorporation into ATP by adenosine with a concomitant failure to elevate the original level of nucleotide labile phosphorus. The large increase in IMP concentration suggests that adenosine is deaminated and then undergoes phosphorolysis prior to conversion of the purine into IMP. The possibility that IMP is responsible for the inhibition so far attributed to adenosine was investigated.

A comparison of the rate of adenine-8-^{14}C incorporation into nucleotides with the rate of IMP synthesis in the presence of adenosine

In this experiment an intermediary level of adenosine (14 mM) which produces partial inhibition of both nucleotide labile phosphorus synthesis and adenine incorporation into nucleotides was chosen. The results seen in Table 14 indicate that the largest accumulation of IMP occurs between the 6th and 12th hour of incubation. This is at the time when the inhibition of adenine incorporation into nucleotides, as well as, inhibition of nucleotide labile phosphorus synthesis is being relieved. Furthermore IMP accumulation is also associated with inosine incubation (124). In view of these findings it is unlikely that IMP is an inhibitor of ATP synthesis. Thus, the evidence points to adenosine itself and not nucleotide derivative as being responsible for the inhibition of adenine-8-^{14}C incorporation into nucleotides and concomitant ATP synthesis.

There are several steps in the synthesis of nucleotides at which adenosine

Table 14.

A COMPARISON OF THE RATE OF ADENINE-8-C^{14} INCORPORATION INTO NUCLEOTIDES AND THE RATE OF IMP SYNTHESIS IN THE PRESENCE OF ADENOSINE

Incubation period	Adenosine-8-C^{14} incorporation into IMP	Adenine-8-C^{14} incorporation into adenine nucleotides
	mμ moles / 100 mg Hb	mμ moles/100 mg Hb
3	26	7
6	75	15
9	120	60
12	150	130

Conditions.

1.0 ml packed erythrocytes incubated with 4 ml Krebs-Ringer phosphate solution containing 21.0 m$\underline{M}$ glucose 1.6 m$\underline{M}$ adenine and 14 m$\underline{M}$ adenosine, 1μC of either adenosine-8-C^{14} or adenine-8-C^{14}. Parallel experiments were run. The results are representive of three such experiments. Temperature: 37°C.

may produce its inhibitory action. In view of the structural similarity of adenine and adenosine, one of the most likely sites for adenosine action is the adenine phosphoribosyl transferase reaction. Studies to locate the site of adenosine inhibition required the use of red blood cell hemolysates instead of intact cells which have been used in all the experiments so far discussed. A preliminary investigation of the adenine phosphoribosyl transferase activity in human red blood cell hemolysates found it to be very low, thus making prolonged incubations necessary.* This in turn resulted in deamination of the adenosine; depleting available substrate. The poor activity of the hemolysate prepared by Manohar's procedure lead to the search for an alternate source of the enzyme. Fortunately a partial purified enzyme preparation of Ehrlich ascites tumor cells free of adenosine deaminase activity was kindly donated by J. F. Henderson.

The effect of adenosine on a partially purified adenine phosphoribosyl transferase activity from Ehrlich ascites tumor cells

Adenine phosphoribosyl transferase activity was assayed according to the method of Henderson and Khoo (97). From the results in Table 15 it can be seen that adenine incorporation into AMP is linear up to 24 minutes of incubation. The addition of a large concentration of adenosine did not alter the rate of adenine incorporation into AMP.

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* Manohar unpublished results

Table 15.

EFFECT OF ADENOSINE ON ADENINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY OF A PARTIAL PURIFIED ENZYME PREPARATION FROM EHRLICH ASCITES TUMOR CELLS

Period of incubation	AMP formed mμ moles	
Time minutes	Without adenosine	With adenosine
0	0	0
6	5.27	-
12	8.47	9.41
24	15.09	14.54

Conditions.

Each incubation flask consisted of 2 m$\underline{M}$ $MgSO_4$, 0.4 $\underline{M}$ Tris HCl, pH 7.4, 1 μc of 0.0455 m$\underline{M}$ adenine-8-C^{14}, 5.0 m$\underline{M}$ PRPP, 50 μg of enzyme and 16.12 m$\underline{M}$ adenosine when required. To the total volume of 0.4 ml incubation medium 0.1 ml of 4 N formic acid was added at the required time to terminate the reaction.

Whether adenosine exerted an inhibition on both nucleotide labile phosphorus and adenine incorporation into nucleotides in Ehrlich ascites tumor cells, similar to that observed in red blood cells, was investigated. In these experiments 0.2 ml of packed Ehrlich ascites tumor cells were incubated in 9 ml of Krebs-Ringer phosphate buffer pH 7.4 containing the usual concentrations of glucose and adenine. From the results presented in Table 16 it can be noted that in the presence of glucose and adenine the level of nucleotide labile phosphorus is elevated 1.7 fold. This is in agreement with others (227) and is close to the two fold increase found in red blood cells (82). The increment in nucleotide labile phosphorus also closely corresponds to the adenine incorporation into nucleotides. Incubation in the presence of (5 mM) adenosine inhibited nucleotide labile phosphorus synthesis, while incubation with 32 mM adenosine caused a substantial drop in nucleotide labile phosphorus concentration. It may also be noted that while the Ehrlich ascites tumor cell can maintain its initial level of nucleotide labile phosphorus in the absence of added glucose; the red blood cell cannot and a decline in nucleotide labile phosphorus concentration is observed (Table 5).

The effect of adenosine on adenine phosphoribosyl transferase activity of human red blood cell hemolysates

The preliminary problems in assaying adenine phosphoribosyl transferase activity in human hemolysate were largely overcome by using the assay procedure of Kelley et al. (116). This technique was designed for human hemolysates and gave higher enzyme activities than those obtained previously by Manohar.

Table 16.

EFFECT OF ADENOSINE ON ADENINE-8-C^{14} INCORPORATION INTO ADENINE NUCLEOTIDES AND NUCLEOTIDE LABILE PHOSPHORUS IN EHRLICH ASCITES CARCINOMA CELLS

Incubation medium	Adenine-8-C^{14} incorporation into adenine nucleotides	Nucleotide labile phosphorus
	μ moles/cc packed cells	μ mole P/ml packed cells
Unincubated	0	2.94 ± 0.06
None	0	2.97 ± 0.09
Glucose	0	3.09 ± 0.03
Glucose + adenine	1.117 ± 0.0	5.05 ± 0.07
Glucose + adenine + 5 mM adenosine	0.153 ± 0.017	2.31 ± 0. 4
Glucose + adenine + 32 mM adenosine	-	1.10 ± 0.1

Conditions.

Each incubation mixture contained 0.2 ml packed cells in 9 ml of incubation medium containing 21.0 mM glucose, 1.6 mM adenine, 1 μc adenine-8-C^{14} and the required amount of adenosine. All incubations were carried out at 37°C in air for a period of 30 minutes. The values presented for nucleotide labile phosphorus represents the average of three experiments while the values for adenine-8-C^{14} incorporation represents the average of two experiments.

From the results presented in Table 17 it can be seen that adenosine had no effect on the activity of adenine phosphoribosyl transferase of human red blood cell hemolysates. This finding is comparable to the results found for Ehrlich ascites tumor cells and suggests that adenine phosphoribosyl trransferase activity is not affected by adenosine.

The most likely site of adenosine inhibition is the formation of phosphoribosyl pyrophosphate from ATP and ribose-5-phosphate. Of particular interest is the finding by Bloch and Nichol (228) that decoyinine, [9-ß-D-(5,6-psicofuranoseenyl)-6-aminopurine] an adenine nucleoside, inhibits the synthesis of phosphoribosyl pyrophosphate in cell-free extracts of S. Faecalis. The effect of decoyinine on nucleotide labile phosphorus synthesis was then investigated. In these experiments 32 mM decoyinine was used. The results presented in Table 18 indicate that decoyinine does not inhibit nucleotide labile phosphorus synthesis as does adenosine. The reason for this lack of inhibition of nucleotide synthesis is not known. Two possibilities may be considered. First of all, decoyinine cannot cross the plasma membrane and secondly, decoyinine does not inhibit PRPP synthetase of human red blood cells. Direct experiments on the effect of adenosine on PRPP synthetase of human hemolysate have been attempted by the present author. These experiments are complicated by the fact that assay of PRPP synthetase activity involves conversion of [8-^{14}C] hypoxanthine to IMP. Since catabolism of adenosine also results in hypoxanthine production an isotope dilution of the [8-^{14}C] hypoxanthine makes interpretation of data obtained from these experiments

Table 17.

EFFECT OF ADENOSINE ON ADENINE PHOSPHORIBOSYL TRANSFERASE ACTIVITY OF HUMAN RED BLOOD CELL HEMOLYSATE

	Adenine-8-C^{14} incorporation into adenine nucleotides (μ moles/g Hb)
Control	10.5
+ adenosine	10.8

Conditions.

Incubation mixtures contained 55 mM Tris buffer, pH 7.4 5 mM $MgCl_2$, 1 mM 5-phosphoribosyl-1-pyrophosphate (PRPP) 0.6 mM adenine, 1 μC adenine-8-C^{14} and 6.0 mM adenosine where required along with 0.3 to 0.6 mg of protein from dialyzed erythrocyte hemolysate in a final volume of 1.0 ml. Time: 20 minutes. Temperature: 37°C.

Table 18.

THE EFFECT OF DECOYININE ON GLUCOSE AND ADENINE ELEVATION OF NUCLEOTIDE LABILE PHOSPHORUS

Nucleoside additive	Incubation period	Nucleotide labile phosphorus
		µg P/100 mg Hb
none	0	21
none	12	44
adenosine	12	23
decoyinine	12	42

Conditions.

1.0 ml of packed erythrocytes were incubated with 4.0 ml Krebs-Ringer phosphate buffer, pH 7.4 containing 1.6 mM adenine, 21 mM glucose concentration of added nucleoside 32 mM adenosine 32 mM decoyinine. Temperature: 37°C The experiment is typical of three such experiments.

extremely difficult. Elucidation of this problem requires further experimentation and the development of other assay procedures. At this time however it was thought more profitable to carry on with other aspects of this research project.

PART III. - ELEVATION OF NUCLEOTIDE LEVELS IN PHYSIOLOGICALLY AGED AND IN PRESERVED RED BLOOD CELLS

Elevation of adenine nucleotide levels in human erythrocytes of different ages

A sample of blood represents a population of red blood cells varying in age from one to 125 days. This set of experiments was designed to establish whether the addition of adenine produces a build up of high energy nucleotides equally in erythrocytes of all ages.

Influence of glucose and adenine incubation on elevation of adenine nucleotides in different fractions of human erythrocytes separated according to the method of Danon and Marikovsky

In this series of experiments red blood cells were separated into youngest and oldest cell fractions using the method of Danon and Marikovsky (157), then incubated for 12 hours in the presence of glucose and adenine. The technique used separates 5% of the least dense and youngest cells, designated in Table 19 as upper and 5% of the most dense and oldest cells, designated as lower. The fraction designated as "remainder" refers to 95% of the remainder of the upper and the 95% of the remainder of the lower which have been pooled. From the results in Table 19 it can be noted that the level of nucleotide labile phosphate initially present in the oldest fraction of cells is lower than that found in the

Table 19.

THE EFFECT OF GLUCOSE AND ADENINE ON ELEVATION OF NUCLEOTIDE LABILE PHOSPHORUS AND ADENINE INCORPORATION INTO ADENINE NUCLEOTIDES IN DIFFERENT FRACTIONS OF HUMAN ERYTHROCYTES (USING THE DANON METHOD (157))

Fraction of cell population	% of total population	N.L.P.* µgP/100 mg Hb		Adenine-8-^{14}C incorporation into A.N.† mµmoles/100 mg Hb	
		Before incubation	After Incubation	Expected	Observed
Unseparated	100	19.7	39.1	296.8	316.0
Youngest	5.8	20.6	35.4	238.7	242.0
Oldest	5.1	12.2	21.1	143.6	126.0
Remainder	100	18.4	32.2	238.7	237.0

Conditions.

1.0 ml of human erythrocytes from each fraction was incubated with Krebs-Ringer phosphate solution pH 7.4 containing 1.6 mM adenine, 1 µc adenine-8-^{14}C and 21.0 mM glucose. Incubated for 10 hours at 37°C. The results are typical of four such experiments.

* Denotes nucleotide labile phosphorus.

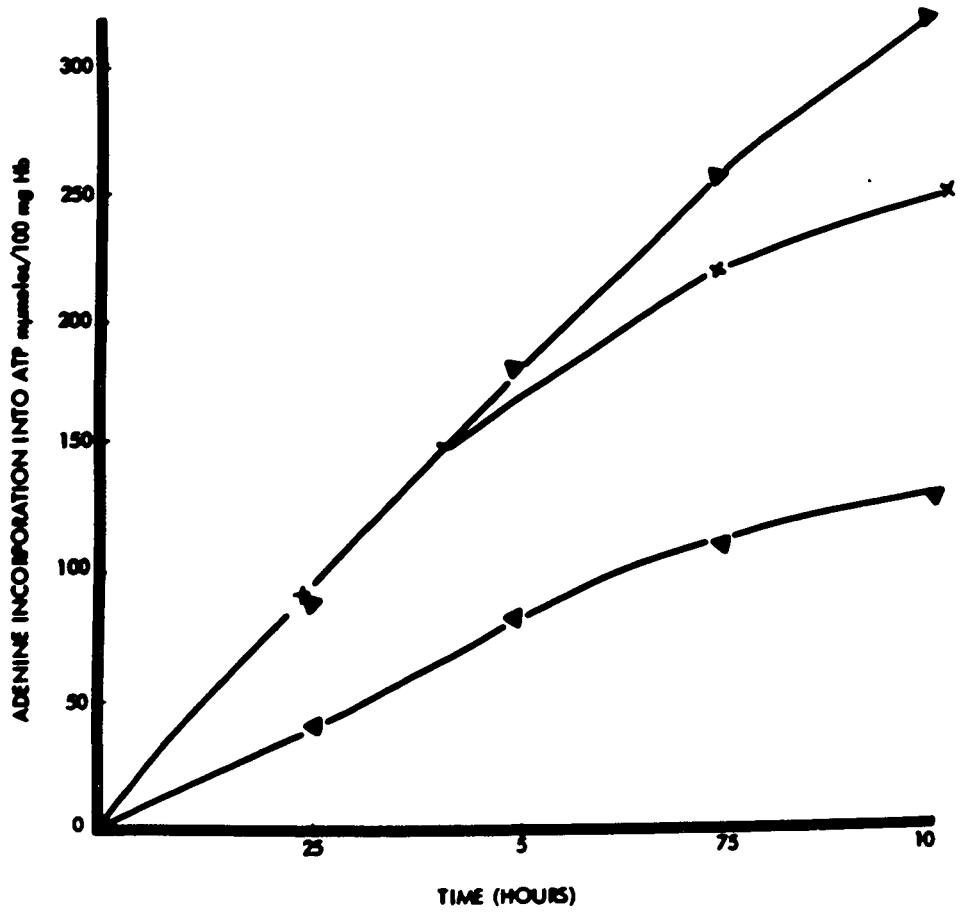
† Denotes adenine nucleotides.

complete, upper or remainder fractions. Inspection of the values for nucleotide labile phosphorus and adenine-8-^{14}C incorporation into nucleotides of the fractionated cells reveals lower levels of both after 12 hours of incubation with glucose and adenine in the oldest 5% of the cell population. While the youngest 5% cell fraction when compared with the remaining fraction showed no major difference in both nucleotide labile phosphorus and adenine-8-^{14}C incorporation into nucleotides. It can also be pointed out that these values are lower in cells fractionated than those unseparated. When the rate of adenine incorporation into nucleotides in the various fractions was investigated (Fig. 4) it was found that although the amount of nucleotide synthesized is less in the older cells the pattern of incorporation is similar in both young and old cells, reaching a maximal after 10 hours of incubation. Again it can be seen that adenine incorporation in the fraction not separated by phthalate treatment is higher than the other cell fractions. This difference is even more apparent after 7.5 to 10 hours of incubation. The reason for the higher values for both nucleotide labile phosphorus and adenine-8-^{14}C incorporation into nucleotides (Table 19, Fig. 4) in the fraction not subjected to the separation technique may be attributable to the possible deleterious effects of phthalate ester treatment and centrifugation required in this separation procedure. These effects are intensified during long incubation, C.F. Fig. 4. In view of these detrimental effects it was decided to change the separation procedure.

Elevation of adenine nucleotides in different fractions of human erythrocytes

Figure 4

ADENINE-8-^{14}C INCORPORATION INTO DIFFERENT FRACTIONS OF RED BLOOD CELLS SEPARATED BY A MIXTURE OF METHYL AND n-BUTYL PHTHALATE ESTERS.



(▲) control complete cell population , no phthalate ester used.
(x) youngest fraction 5.8% of total cell population.
(▼) oldest fraction 5.1% of total cell population.

using the separation procedure of Berstein

In this series of experiments red blood cells were fractionated according to density following centrifugation using the procedure of Berstein (154). Adenine nucleotide concentrations were determined enzymatically. The results are presented in Table 20 and show essentially the same general pattern as seen using the phthalate ester separation procedure in that first of all, there was no difference in the ATP level between the youngest cell fraction and the "remainder" and secondly, there is a lower ATP concentration in oldest cells as compared to the general population of erythrocytes. The results of individual nucleotide analysis also revealed that incubation with glucose and adenine produced a two fold increase in the levels of both ATP and ADP in all cell populations while the level of AMP was not effected.

In view of the association between ATP concentration and post transfusion viability of preserved red blood cells (187, 202, 203, 206) an elevation of intracellular ATP concentration might have some practical application in preservation. It was therefore of interest to study two factors: firstly, the feasibility of replacing the Krebs-Ringer phosphate buffer incubation medium normally used with serum and secondly, the ability of red blood cells, preserved under standard conditions, to synthesize ATP upon glucose and adenine incubation.

The effect of replacing Krebs-Ringer phosphate incubation solution with serum on the ability to synthesize nucleotide labile phosphorus and incorporate adenine-8-^{14}C into nucleotides in fresh red blood cells

Table 20.

ELEVATION OF ADENINE NUCLEOTIDES IN DIFFERENT FRACTIONS OF HUMAN ERYTHROCYTES BY GLUCOSE AND ADENINE (USING THE METHOD OF BERSTEIN (154))

Fraction of cell population	% of total population	Adenine nucleotides (μmoles/g Hb)					
		ATP		ADP		AMP	
		before*	after*	before	after	before	after
Youngest	5	3.1	5.8	0.6	1.1	0.2	0.3
Oldest	5	1.7	3.2	0.5	1.0	0.2	0.2
Remainder	90	2.9	5.7	0.6	1.1	0.2	0.2

Incubation conditions: as in Table 19

* Before and after 12 hrs incubations

In this series of experiments blood was defibrinated using a rod covered with multi-processes. The serum was separated by centrifugation and incubated at 37°C with 2 μmoles of adenine, 5 mg of glucose per ml of serum until all substrates were completely dissolved. The serum was then incubated with erythrocytes. An inspection of the data presented in Table 21 reveals that incubation of erythrocytes in serum supplemented with glucose and adenine caused only a slight adenine-8-^{14}C incorporation into nucleotides and no increase in nucleotide labile phosphorus, while a control sample incubated in Krebs-Ringer phosphate showed the normal doubling of nucleotide labile phosphorus with adenine-8-^{14}C incorporation into nucleotides corresponding to the increment in nucleotide labile phosphorus. However, supplemention of the serum with 12 mM Na_2HPO_4 resulted in an increase of both nucleotide labile phosphorus concentration and adenine incorporation into nucleotides. These values were slightly less than the corresponding amounts found upon incubation in Krebs-Ringer phosphate buffer solution. The reason for this may be due to the fact that red blood cells tend to sediment more quickly in serum which would result in a possible decreased availability of required substrates.

The effect of glucose and adenine on elevation of nucleotide labile phosphorus in fresh red blood cells and in cells stored for 4 and 6 weeks in ACD* medium

To evaluate the ability of glucose and adenine to elevate the levels of nucleotide labile phosphorus standard blood donations were stored for 4 and 6 weeks at 4°C. Samples of the blood were withdrawn initially and after 4 and

* ABBOTT Formula A

Table 21.

THE EFFECT OF REPLACING THE KREBS RINGER PHOSPHATE INCUBATION SOLUTION WITH SERUM, ON THE ABILITY OF RED BLOOD CELLS TO INCREASE THEIR NUCLEOTIDE LABILE PHOSPHORUS CONTENT

Incubation medium	Period of incubation (hours)	Nucleotide labile phosphorus (µg/100mg Hb)	Adenine 8-^{14}C incorporation into adenine nucleotides (mµmoles/100 mg Hb)
None	0	25	0
Krebs Ringer phosphate	12	49	370 (384)*
Serum	12	22	40
Serum + phosphate	12	42	246 (224)

Conditions.

1.0 ml of erythrocytes was incubated with either 4 ml Krebs Ringer phosphate or 4 ml of serum, or 4 ml of serum and phosphate. All incubation solutions contain glucose and adenine and were adjusted to pH 7.4. Concentration of additives: glucose 21mM, adenine 1.5mM, 1 µc adenine 8-^{14}C, 0.012 M $NaHPO_4$. The result presented is typical of four such experiments.

* The values in brackets represent those expected from the increment in nucleotide labile phosphorus.

6 weeks preservation and then incubated for 12 hours at 37°C with either glucose or glucose and adenine. The results of nucleotide labile phosphorus analysis are shown in Table 22. As the preservation period progressed the level of nucleotide labile phosphorus present in the red blood cells declined. Although incubation with glucose alone was able to maintain the preincubation level of nucleotide labile phosphate in fresh blood cells during the 12 hour incubation, it could not do so with 6 week preserved erythrocytes. Following 12 hours of incubation with glucose and adenine the level of nucleotide labile phosphate was twice the preincubation level in both 4 and 6 week preserved blood. The ratio between incubated and unincubated red blood cells was maintained throughout the storage period, but due to the fall of nucleotide labile phosphate during preservation the actual level in the preserved cells after incubation for 12 hrs. was less than that found for fresh red blood cells.

The reason for the decline in the ability of preserved cells to synthesize nucleotides may be due to several factors including enzyme deterioration, causing a limitation in both the rate of glycolysis and/or the availability of the necessary intermediates necessary for de novo purine nucleotide synthesis. To partially ameliorate both of these deficiencies the use of nucleosides was employed.

The effect of adenosine supplementation upon nucleotide synthesis after glucose and adenine incubation for 12 hours in preserved red blood cells

Table 22.

THE EFFECT OF GLUCOSE AND ADENINE UPON ELEVATING THE LEVEL OF NUCLEOTIDE LABILE PHOSPHORUS CONCENTRATION IN RED BLOOD CELLS STORED FOR 0, 4, 6 WEEKS IN ACD

Period of storage weeks	Incubation medium	Nucleotide labile phosphorus µg/100 mg Hb	
		Before incubation	After incubation
0	glucose	22	21
	glucose + adenine		39
4	glucose	8	5
	glucose + adenine		16
6	glucose	4	2
	glucose + adenine		8

Conditions.

1.0 ml of erythrocytes was incubated with 4 ml of Krebs-Ringer phosphate buffer pH 7.4 containing 1.6 mM adenine and 21.0 mM glucose for 12 hours at 37°C.

Table 23.

THE EFFECT OF ADENOSINE SUPPLEMENTATION UPON THE LEVEL OF NUCLEOTIDE LABILE PHOSPHORUS AND ADENINE-8-^{14}C INCORPORATION INTO ADENINE NUCLEOTIDES OF 6 WEEK RED BLOOD CELLS STORED IN ACD

Incubation medium	Nucleotide labile phosphorus	Adenine-8-^{14}C incorporation into nucleotides	
	µg P/100 mg Hb	mµmoles/100 mg Hb	
		Observed	Calculated
Unincubated	5.1		
Glucose + adenine	9.4	62	59
Glucose + adenine + 8 mM Adenosine	42.1	151	576

Conditions.

1.0 ml of erythrocytes was incubated with 4 ml of Krebs-Ringer phosphate solution pH 7.4 containing the required additives, for 12 hours at 37°C. Concentration of additives glucose 21.0 mM, adenine 1.5 mM, 1 µc adenine-8-^{14}C. The result presented is typical of 6 such experiments.

In view of the inhibitory effects of adenosine at concentrations above 12 mM, as discussed earlier, for this series of experiments it was desirable to use an 8 mM concentration of adenosine which has been shown to have no inhibitory effect. From the results of the experiment cited in Table 23, it is seen that supplementation of glucose and adenine with 8 mM adenosine resulted in a dramatic elevation of nucleotide labile phosphorus in 6 week preserved cells. Adenine-8-^{14}C incorporation into nucleotides was also increased considerably. However, only a small proportion of the increment in nucleotide labile phosphorus could be accounted for by the observed adenine incorporation into nucleotides. This suggested that another pathway for nucleotide synthesis may be involved in the elevation of nucleotide level.

To further study this possibility it was necessary to evaluate the role of both adenine and adenosine as purine precursors in the synthesis of nucleotides in fresh and preserved red blood cells. In order to elucidate the role of adenine and adenosine as precursors of adenine nucleotides, blood was stored in ACD preservative medium for 6 weeks at 4°C. Samples were withdrawn periodically, divided into aliquots, washed and incubated for 12 hours at 37°C with adenosine; glucose; adenine and glucose; or glucose, adenine and adenosine. The effect of these incubations on the level of nucleotide labile phosphorus is shown in Fig. 5. As has previously been shown, during storage the level of nucleotide labile phosphorus fell rapidly reaching one half of its original value after 4 weeks and one quarter after six weeks of preservation. Incubation with glucose alone

Figure 5

NUCLEOTIDE LABILE PHOSPHORUS LEVELS FOLLOWING TWELVE HOURS INCUBATION OF PRESERVED ERYTHROCYTES WITH GLUCOSE, ADENINE AND ADENOSINE.

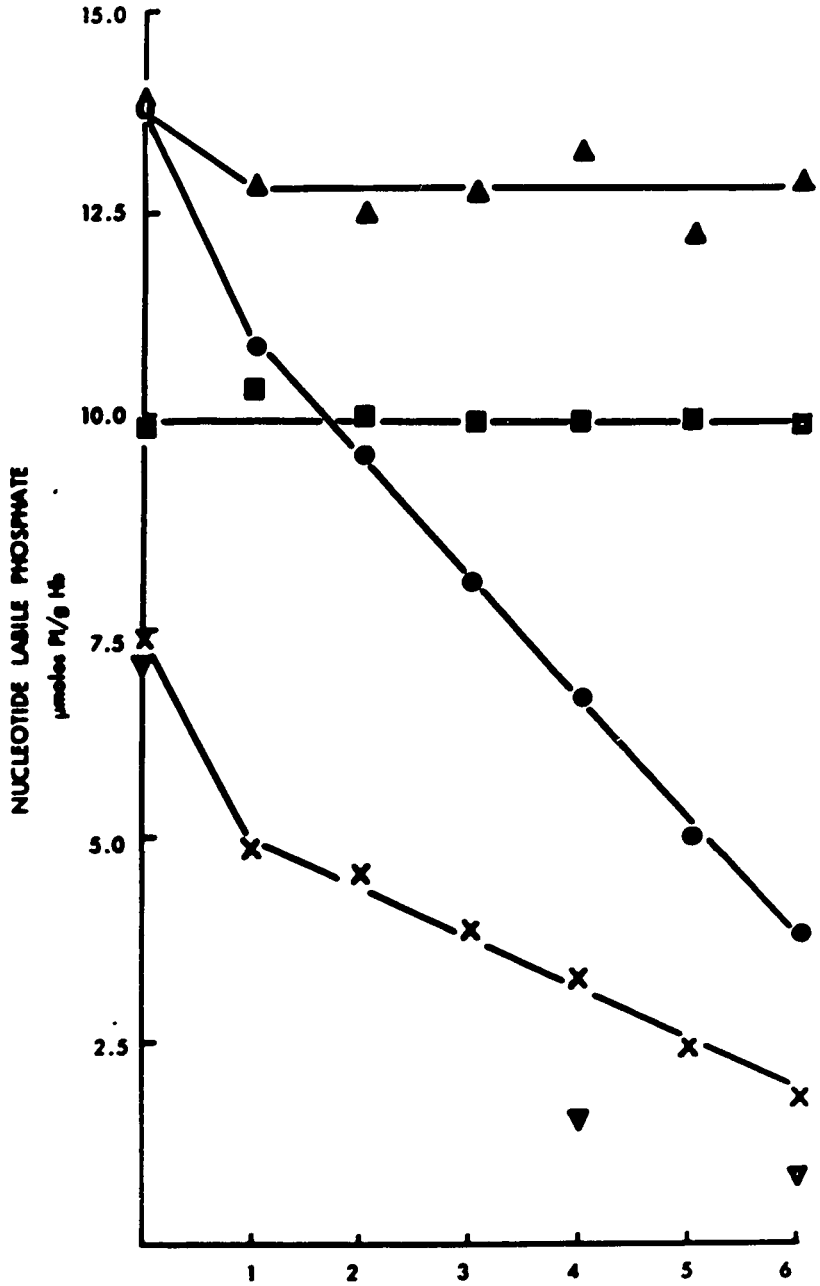


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Figure 5 Legend

x : before incubation, ● : glucose and adenine

■ : adenosine, ▲ : glucose, adenine, and adenosine

▼ : glucose.

Incubation conditions: 1.0 ml packed erythrocytes, 1.6mM adenine, 8 mM adenosine, 21.0 mM glucose and 0.1 M phosphate buffer, pH 7.4, made up to 5.0 mls with Krebs-Ringer solution. Temp. 37°.

Figure 6

INCORPORATION OF ADENINE-8-^{14}C, AND /OR ADENOSINE-8-^{14}C INTO ADENINE NUCLEOTIDES FOLLOWING TWELVE HOURS INCUBATION OF PRESERVED ERYTHROCYTES.

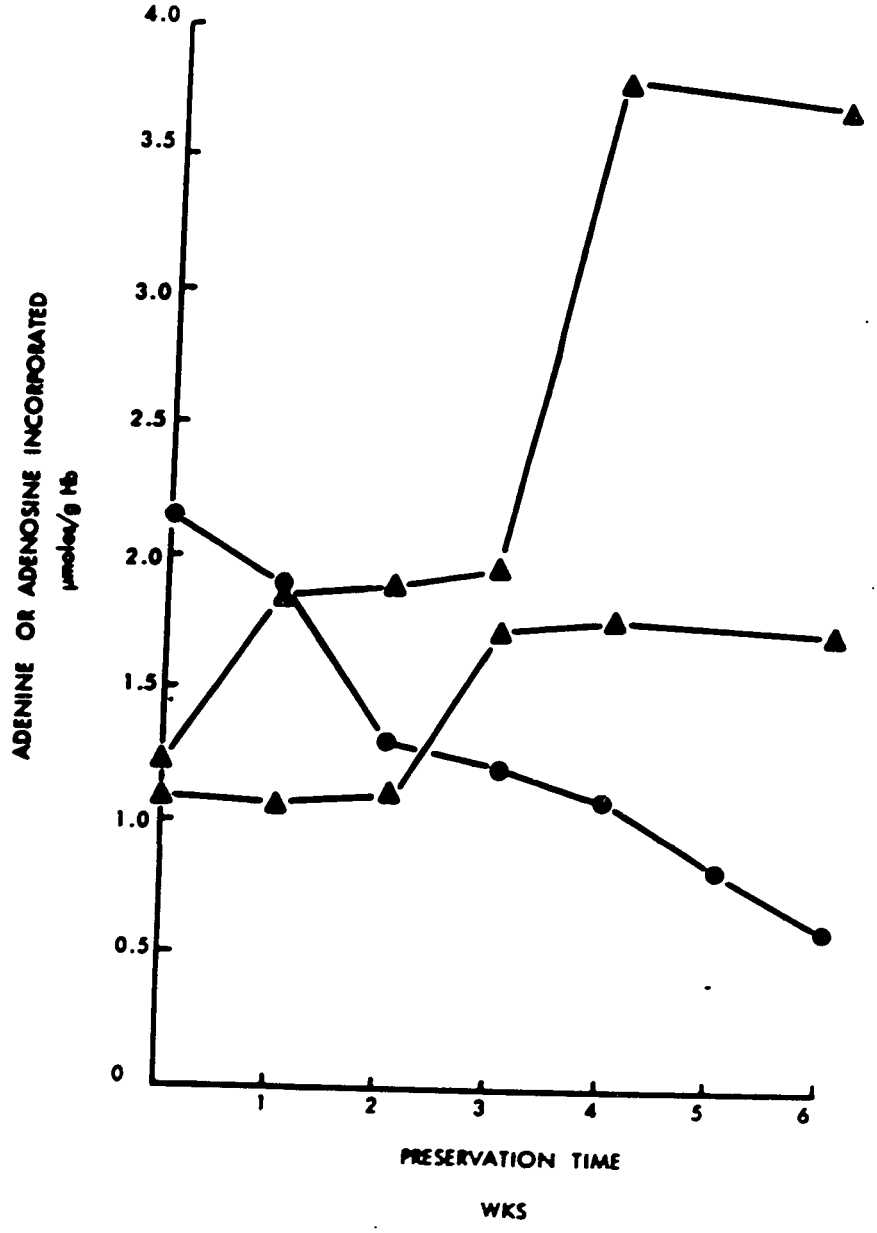

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Figure 6 Legend

● : [8-^{14}C] adenine incorporation during incubation with glucose and adenine, ▲ : [8-^{14}C] adenosine incorporation and ▲ [8-^{14}C] adenine incorporation both during incubation with glucose, adenine and adenosine. Incubation conditions: as in Fig. 5 , containing 1μc [8-^{14}C] adenosine or 1μc [8-^{14}C] adenine.

could not maintain the preincubation level of nucleotide labile phosphorus with 4 or 6 week preserved erythrocytes,although it could with fresh blood cells. Following incubation with glucose and adenine the level of nucleotide labile phosphorus was elevated to twice preincubation values. However, due to the low concentration of nucleotide labile phosphorus in preserved cells the actual amount of nucleotide labile phosphorus synthesized decreased. Incubation with adenosine alone also elevated the level of nucleotide labile phosphorus in fresh cells and this level was maintained by erythrocytes withdrawn throughout the storage period. Thus a large increment in nucleotide labile phosphorus was noted in incubated 6 week preserved blood. When glucose adenine and adenosine were all present the level of nucleotide labile phosphate was raised to approximately double that of fresh cells following incubation even with blood cells which had been preserved for 6 weeks.

The incorporation of [8-^{14}C] adenine or [8-^{14}C] adenine and [8-^{14}C] adenosine during incubation of erythrocytes stored for various time intervals was also studied. The results, using the same blood samples which provided the data for Fig. 5, are shown in Fig. 6. When glucose and adenine are present in the incubation medium there is a decreased adenine-8-^{14}C incorporation into nucleotides as preservation proceeds, this corresponds to the decrease in nucleotide labile phosphorus (as presented in Fig. 6). When fresh erythrocytes were incubated with adenosine and adenine, both purines were incorporated in approximately equal quantity and together they corresponded to the adenine incorporation obtained when adenine was the sole purine precursor present. As

the preservation period progressed, most of the purine for the adenine nucleotides, which is required to elevate the falling ATP level to twice that of the fresh erythrocytes, was supplied by adenosine which was incorporated in increasing amounts. Thus, the large increment of nucleotide labile phosphorus observed upon glucose, adenine, adenosine incubation could be accounted for by two different pathways of nucleotide synthesis; one pathway incorporating adenine while the other incorporating adenosine. The mechanism of adenosine incorporation into adenine nucleotides is unclear. Its incorporation may involve direct phosphorylation of adenosine via an adenosine kinase. The presence of this enzyme in human erythrocytes has been suggested by the work of Lowy et al. (123) and Bartlett (124) but as yet there is no direct evidence for its existence. However, evidence for its existence in human red blood cells has been obtained by the present author and will be presented in the next section of the results.

The reason for the decreased nucleotide synthesis from adenine, as preservation proceeds either when it is the sole precursor or in the presence of adenosine may be due to the inhibitory effects associated with the build up of AMP or ADP; this possibility was investigated.

Adenine nucleotide concentrations in fresh and 6 week preserved erythrocytes prior to and following 12 hour incubation at 37°

Washed erythrocytes were incubated with glucose and adenine; adenosine or glucose, adenine and adenosine. The results of this study are presented in

Table 24.

ADENINE NUCLEOTIDE LEVELS IN FRESH AND PRESERVED ERYTHROCYTES AFTER 12 HRS. INCUBATION AT 37°

Preservation (weeks)	Incubation	Adenine nucleotides (μmoles/g Hb)		
		ATP	ADP	AMP
0	unincubated	2.9	0.6	0.2
0	glucose + adenine	5.7	1.0	0.2
0	adenosine	4.8	1.1	0.2
6	unincubated	0.9	0.3	0.3
6	glucose + adenine	1.9	0.4	0.3
6	glucose, adenine + adenosine	5.3	0.8	0.2

Incubation conditions: as in Figs. 5 and 6.

Table 24. A comparison of adenine nucleotide values from fresh cells to those of six week preserved cells indicated a decreased level of ATP and ADP while AMP remained fairly stable. However, due to the more rapid decline of ATP, the ratio of ATP/ADP is higher in the 6 week preserved cells. It will be noted that in fresh red blood cells the initial levels of ATP and ADP were virtually doubled by incubation with glucose and adenine; or adenosine, while the level of AMP remained unchanged. In 6 week preserved red blood cells, even though the concentration of ATP had fallen to 30% of that in fresh cells, there still was a doubling of this level following glucose and adenine incubation. Incubation with glucose, adenine and adenosine raised the level of ATP in the preserved cells to almost twice that seen with unincubated fresh cells. The degree to which adenine and glucose raised the level of ADP in preserved cells was variable but it was consistently doubled following incubation with adenosine. The level of AMP remained unchanged under all incubation conditions. These data are also in agreement with those obtained by measuring nucleotide labile phosphorus concentrations and confirms that these values are a valid index of the energy potential of the cell. The fact that no ADP or AMP accumulates in 6 week preserved red blood cells suggests that these nucleotides are probably not involved in the decreased effectiveness of adenine. However, the change in ATP/ADP ratio may be of some importance in controlling nucleotide synthesis. Furthermore, the elevation of ADP in both fresh and preserved cells under conditions which facilitate maximal nucleotide labile phosphate elevation or ATP synthesis may also have some significance in understanding the mechanism

regulating intracellular ATP concentration. Following several weeks of storage, the glucose utilization by erythrocytes is markedly diminished (171). This raised the possibility that the failure of the preserved erythrocytes to synthesize significant amounts of nucleotides from adenine during incubation might be due to the decreased glycolytic rate. In view of the observation that inorganic phosphate increases glucose utilization (52,62) and stimulates the incorporation of adenine into erythrocyte nucleotides, the effect of elevating inorganic phosphate concentration above the 12 mM used in the incubations cited above was investigated.

The effect of inorganic phosphate levels on nucleotide labile phosphate synthesis in preserved cells

In the experiment cited in Table 25, 12 mM or 40 mM inorganic phosphorus was added to the usual incubation medium consisting of glucose and adenine; adenosine; or glucose, adenine and adenosine. The results indicate that increasing the inorganic phosphate concentration to 40 mM had no effect on the synthesis of nucleotide labile phosphorus following incubation of either fresh cells or preserved cells with adenosine. The higher concentrations of inorganic phosphate increased the nucleotide labile phosphorus formation only when preserved erythrocytes were incubated with adenine and glucose. This suggests that the decrease in glycolytic rate observed on storage plays a role in decreasing the ability of adenine to synthesize nucleotides.

The relative ineffectiveness of adenine to be incorporated into nucleotides

Table 25.

EFFECT OF INORGANIC PHOSPHATE LEVELS IN THE MEDIUM ON NUCLEOTIDE LABILE PHOSPHATE FOLLOWING 12 HRS. INCUBATION

Preservation period (weeks)	Incubation	Nucleotide labile phosphate (µmoles P/g Hb) before incubation	after incubation	
			12 mM P:	40 mM P:
0*	glucose + adenine	6.72	13.1	13.4
0	adenosine	6.72	11.2	10.9
6*	glucose + adenine	1.34	2.6	4.8
6	adenosine	1.34	11.2	10.5
6	glucose + adenine + adenosine	1.34	10.3	9.6

* Different blood donations were used for the fresh and preserved specimens.

compared to adenosine may be due to a decrease in the activity of either phosphoribosyl pyrophosphate synthetase or adenine phosphoribosyl transferase. These two enzymes along with adenosine kinase were assayed in human hemolysates of fresh, three and six week preserved erythrocytes. Phosphoribosyl pyrophosphate synthetase was assayed by the method of Hershko et al. (90); adenine phosphoribosyl transferase was assayed by the method of Kelley et al. (116); while adenosine kinase was assayed by a modification of the method of Schnebli et al. (120). Preliminary experiments (not shown here) were necessary to establish optimal conditions for each enzyme. The results tabulated in Table 26 indicate that there is no significant change in the activity of any of these enzymes during preservation at 4°C even up to six weeks of storage.

The decrease in glucose utilization during preservation raises the possibility of a decreased supply of ribose moiety required in phosphoribosyl pyrophosphate synthesis. Since, inosine can be readily utilized by preserved red blood cells for the production of ribose phosphate (124), the effect of inosine on the synthesis of adenine nucleotides from adenine was investigated. An inspection of the data presented in Table 27 indicates that for six week preserved red blood cells glucose and adenine incubation doubled the pre-incubation value of nucleotide labile phosphorus. However, during storage there is a sharp decline in nucleotide labile phosphorus, thus the actual increase in nucleotide labile phosphorus was quite small. However the addition of inosine, either in the presence or absence of glucose, raised the preincubation value of nucleotide labile phosphorus to much higher levels to approximately twice that of fresh

Table 26.

EFFECT OF PRESERVATION ON THE ACTIVITIES OF ERYTHROCYTE ENZYMES CONCERNED WITH ADENINE NUCLEOTIDE SYNTHESIS

Enzyme	units (μmoles/g Hb)	Preservation periods (wks)		
		0	3	6
Phosphoribosyl pyrophosphate synthetase	[8-^{14}C] hypoxanthine incorporation into IMP	18.1	18.1	15.1
Adenine phosphoribosyl transferase	[8-^{14}C] adenine incorporation into nucleotides	10.5	11.0	10.8
Adenosine kinase	[8-^{14}C] adenosine incorporation into nucleotides	1.7	1.6	1.7

Table 27.

EFFECT OF INOSINE ON THE SYNTHESIS OF ADENINE NUCLEOTIDES FROM ADENINE BY ERYTHROCYTES

	Storage periods (wks)			
	0	6	0	6
	nucleotide labile phosphate (μmoles P/g Hb)		[8-^{14}C] adenine incorporation (μmoles nucleotide/g Hb)	
unincubated	7.4	2.6	-	-
glucose + adenine	13.8	4.8	2.8	0.8
glucose + adenine + inosine	14.1	12.2	2.7	4.3
inosine + adenine	14.4	12.5	2.8	4.4

Incubation conditions: as in Figs. 1 and 2. 8 mM inosine was used where necessary.

unincubated cells. Therefore it can be surmised that the effect of inosine on nucleotide labile phosphorus elevation and [8-^{14}C] adenine incorporation into nucleotides of fresh cells resembles the pattern seen with glucose, but only inosine has a significant effect in 6 week preserved red blood cells. Thus, it appears that when inosine is available as a source of ribose phosphate, phosphoribosyl pyrophosphate synthetase plus adenine phosphoribosyl transferase can function as well in preserved as in fresh erythrocytes.

As the preservation period progressed and the requirement for adenine nucleotides increased the incorporation of adenosine becomes predominant (c.f. Fig. 6), suggesting that this system may be more active or more stable during the 12 hour incubation than the system incorporating adenine. However, as the enzymes themselves are not decreased in activity during storage the rate at which adenine and adenosine are incorporated into adenine nucleotides may be different. This possibility was next investigated.

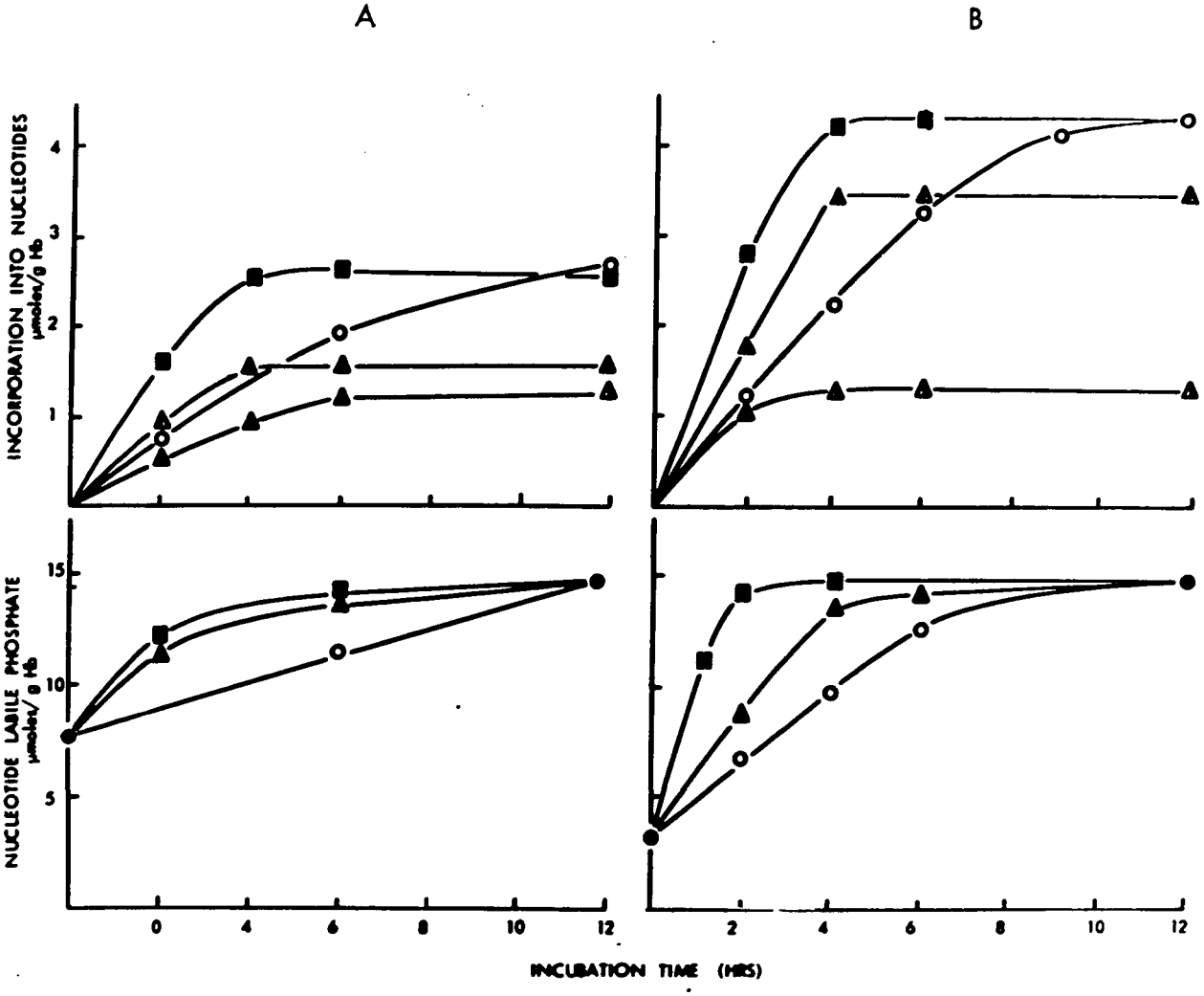
Time course for purine precursors incorporation into adenine nucleotides in fresh and 4 week preserved red blood cells

Fresh and four week preserved red blood cells were incubated with adenosine; glucose, adenine and adenosine or adenine and inosine at 37°C. At various time periods samples were removed and analyzed for nucleotide labile phosphorus concentration and purine precursor incorporation into adenine nucleotides. When adenine and adenosine both served as purine precursors in the same incubation medium, parallel incorporation experiments were performed.

The results of these experiments are plotted in the graphs of Fig. 7. Incubation with adenosine alone required four hours to enable either the fresh or the four week preserved erythrocyte to reach a level of nucleotide labile phosphorus twice that of fresh cells. When [8-^{14}C] adenosine incorporation into adenine nucleotides is followed a maximum purine incorporation is also found after 4 hours of incubation for both the fresh and four week preserved red blood cells. On the other hand when adenine was the sole purine precursor, a maximum for both nucleotide labile phosphorus elevation and [8-^{14}C] adenine incorporation into adenine nucleotides required about 10 hours of incubation, for both fresh and four week preserved red blood cells. When both purine precursors were present a maximum incorporation of adenosine and adenine was reached after about 4-6 hours in fresh and 4 week preserved red blood cells. At this time the incorporation into nucleotide due to adenine plus the incorporation due to adenosine was equivalent to the maximum incorporation achieved when either adenine or adenosine served as the sole purine precursor. This maximum level corresponded to twice the fresh cell value. It seems that once this maximum value was reached by any pathway no further incorporation of either purine precursor is possible. It can also be noted that incorporation of purine precursor into adenine nucleotides was greater when either adenine or adenosine served as the sole purine precursor as opposed to when they were both present. This indicates a constraining influence on nucleotide synthesis on either pathway when both precursors are present together.

Figure 7

TIME COURSE ADENINE-8-^{14}C AND ADENOSINE-8-^{14C} INCORPORATION INTO NUCLEOTIDES, AND THE SYNTHESIS OF NUCLEOTIDE LABILE PHOSPHATE FOLLOWING INCUBATION OF FRESH AND 4 WEEK PRESERVED ERYTHROCYTES.



A Fresh
B 4-week preserved

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Figure 7 Legend

For each type of cells, the upper panel represents incorporation into adenine nucleotide, the lower panel represents levels of nucleotides labile phosphate. ■ : incubation with adenosine-8-^{14}C; o: incubation with adenine-8-^{14}C glucose and inosine (8mM); ▲: incubation with adenine, adenosine, and glucose; ◭ ; adenosine-8-^{14}C incorporation. ◭; adenine-8-^{14}C incorporation. Incubation conditions: as in Figs. 5 and 6.

PART IV. - EVIDENCE FOR THE EXISTENCE OF AN ADENOSINE KINASE IN HUMAN ERYTHROCYTES

The finding that incubation of fresh human blood cells with 8 mM adenosine resulted in a doubling of nucleotide labile phosphorus was quite unexpected and led us to investigate the role of inosine in fresh red cells, to see if it has a similar effect.

The effect of inosine or adenosine on nucleotide elevation in fresh red blood cells

Incubation contained either 8 mM adenosine or inosine. From the results in Table 28 it can be seen that inosine unlike adenosine has no effect on elevating nucleotide labile phosphorus. The elevation of nucleotide labile phosphorus could have occurred from an adenine contamination of adenosine. However chromatographic analysis of adenosine using an isobutyric solvent system (as previously described in Methods) showed no detectable adenine contamination. Furthermore, incorporation of [8-^{14}C] adenosine into adenine nucleotides (Table 28) could account for the increment in nucleotide labile phosphorus. Analysis of [8-^{14}C] adenosine by the same chromatographic system as used above, verified the radiochemical purity as specified by the manufacturer. The incorporation of adenosine into adenine nucleotides is most probably brought about by an adenosine kinase involved in the following reaction:

$$\text{adenosine} + \text{ATP} \rightleftharpoons \text{AMP} + \text{ADP}$$

While the smaller incorporation into IMP is probably brought about by the hypoxanthine-guanine phosphoribosyl transferase reaction. In man IMP cannot

Table 28.

THE EFFECT OF INOSINE OR ADENOSINE ON NUCLEOTIDE ELEVATION IN FRESH RED BLOOD CELLS

Nucleoside	Nucleotide labile phosphorus	8-^{14}C Adenosine incorporation mμ moles/100 mg Hb		
		Adenine nucleotides expected	observed	IMP
unincubated	22			
adenosine	43	336	328	69
inosine	22			

Conditions.

Each incubation mixture contained 1.0 ml of packed red blood cells and 4 ml of Krebs-Ringer phosphate buffer pH 7.4 concentration of nucleosides 8 mM. Incubation time: 12 hours. Temperature: 37°C. The results presented are typical of three such experiments. 1 μC adenosine-8-C^{14} was added along with adenosine.

be converted to AMP.

The presence of adenosine kinase in human red blood cells has been suggested (123, 124). Further investigations were carried out in order to demonstrate the presence of this enzyme in human erythrocytes. The presence of an adenosine kinase in red blood cells would clarify the mechanism of the adenosine incorporation into adenine nucleotides.

Assay of adenosine kinase activity in red blood cell hemolysates

A dialyzed human red blood cell hemolysate was incubated as detailed in the Method section. The results of a time course for adenosine-8-^{14}C incorporation into adenine nucleotides is seen in Fig. 8. From this graph it can be seen that adenosine is incorporated into adenine nucleotides. The rate of adenosine incorporation is linear up to approximately 24 minutes of incubation.

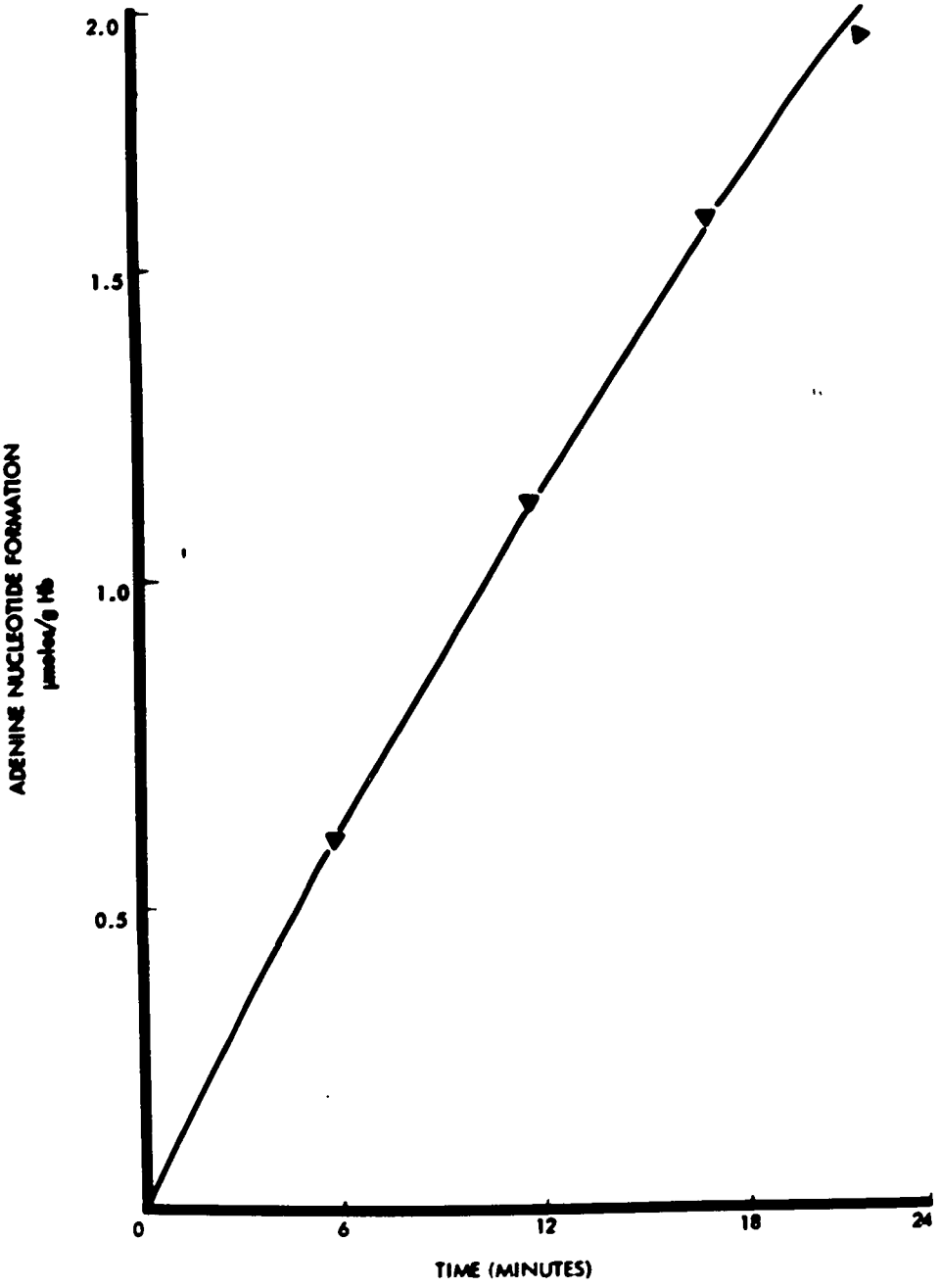
The possibility that adenosine was not being incorporated directly but was being broken down to adenine which was then incorporated or deaminated and subsequently incorporated as inosine or hypoxanthine was next investigated.

The effect of non labeled hypoxanthine, inosine or adenine on adenosine-8-^{14}C incorporation into adenine nucleotides in human red blood cell hemolysates

In these experiments 20.0 and 200 μM of hypoxanthine, inosine or adenine were tested. The results are presented in Table 29 and indicate no decrease in incorporation of adenosine-8-^{14}C into adenine nucleotides in the presence of either concentration of non labeled purine when compared to a control incubated under identical conditions without addition of non labeled purine. The results

Figure 8

ASSAY OF ADENOSINE KINASE ACTIVITY IN RED BLOOD CELL HEMOLYSATES.



Conditions as detailed in methods

Table 29.

EFFECT OF COLD HYPOXANTHINE, INOSINE AND ADENINE ON ADENOSINE-8-C^{14} INCORPORATION INTO ADENINE NUCLEOTIDES IN HUMAN RED BLOOD CELL HEMOLYSATE

Purine additive	Adenosine-8-C^{14} incorporation into adenine nucleotides (c.p.m.)
none	20,310
20.0 mμ moles hypoxanthine	18,520
200 mμ moles hypoxanthine	19,400
20.0 mμ moles inosine	20,430
200 mμ moles inosine	21,560
20.0 mμ moles adenine	20,020
200 mμ moles adenine	20,350

Conditions. 1.0 ml of incubation suspension containing 11.25 mμ moles adenosine-8-C^{14} specific activity 17.8 mc / m mole, 2.5 mM ATP, 2 mg protein, 2.5 mM MgCl in 50 mM potassium phosphate buffer pH 7.0 Incubated at 25°C for 20 minutes. The experiment was terminated by boiling in a 100°C water bath for 1 minute.

show that the incorporation into adenine nucleotides is not due to incorporation of adenine, inosine or hypoxanthine.

Several synthetic nucleosides are known to inhibit adenosine kinase activity from mammalian tumor cell cultures [Hp. No.2], the most prominent being 6-methylmercaptoribonucleoside. The effect of this nucleoside derivative on human red blood cell adenosine incorporation into adenine nucleotides was investigated. In this experiment 100 μM of 6-methylmercaptoribonucleoside was used under incubation conditions as previously described for 10 and 20 minutes incubation periods. The results presented in Table 30 indicate that adenosine incorporation into nucleotides of red blood cell hemolysates is also inhibited by 6-methylmercaptoribonucleoside, providing further evidence that the incorporation of adenosine is due to the presence of an adenosine kinase.

More direct evidence which rules out the possibility of adenosine, ATP exchange involves the demonstration of incorporation of the terminal phosphate of ATP into AMP; this was next studied.

Incorporation of ^{32}P from [γ-^{32}P] ATP into AMP by red blood cell hemolysates

Adenosine and [γ-^{32}P] ATP were incubated with a dialyzed hemolysate under the conditions used for the adenosine kinase assay. Following incubation, the nucleotides were adsorbed on activated charcoal, hydrolyzed according to the procedure of Crane and Lipmann (219), eluted with 0.2 N NH_4OH in 30% ethanol, concentrated by lyophilization, and separated by paper ionophoresis. The AMP band was then cut out and counted directly on the paper. The large incorporation of ^{32}P from terminally labelled ATP into AMP (Table 31) confirms

Table 30.

INHIBITION OF ADENOSINE KINASE ACTIVITY IN HUMAN RED BLOOD CELL HEMOLYSATES BY 6-METHYLMERCAPTO RIBONUCLEOSIDE

Inhibitor 6 methylmercapto ribonucleoside	Incubation time minutes	Adenosine kinase activity µg AMP/g Hb
-	10	1.0
+	10	0.4
-	20	1.7
+	20	0.6

Conditions.

2.5 mM ATP 2.5 mM $MgCl_2$ 22.4 mµ moles adenosine-8-C^{14}

in 50 mM phosphate buffer pH 7.4, 1 mg hemoglobin.

6 methylmercapto ribonucleoside, 100 µM.

Table 31.

INCORPORATION OF[P^{32}] FROM [γ-P^{32}] ATP INTO AMP BY RED BLOOD CELLS HEMOLYSATE.

	[P^{32}] AMP (c.p.m./mg Hb)
Without adenosine	200
With adenosine	284,000

Conditions.

Hemolysate containing 3 mg hemoglobin, 1.05 μM γ-P^{32} ATP, specific activity: 1470 c.p.m./μμmoles, 1.05 μM adenosine, 50 mM phosphate buffer, pH 7.0, in a total volume of 1.0 ml. Incubation time: 20 minutes. Temperature: 25°C. The reaction was terminated by boiling in a water bath for 1 minute.

the presence of adenosine kinase in human erythrocytes.

Several additional studies on the adenosine kinase activity of human erythrocyte hemolysate were carried out.

The effects of varying the concentration of ATP and Mg^{2+} on adenosine kinase activity of human red blood cell hemolysates

The effect of $MgCl_2$ on adenosine kinase activity was studied at two concentrations of ATP, 0.5 mM and 2.5 mM (Fig. 9b). Using 0.5 mM ATP, the optimal Mg^{2+} concentration is 0.5 mM while in the presence of 2.5 mM ATP the Mg^{2+} concentration becomes less critical with an optimum around 2.5 mM. The effects of varying the concentration of ATP are shown in Fig. 9a. The optimum concentration of ATP using 0.5 mM Mg^{2+}, is about 0.5 mM, both of these levels being within the physiological range (46). Higher concentrations of ATP are inhibitory. When 2.5 mM Mg^{2+} is used the optimal ATP concentration is also elevated, but the activity is fairly constant over a wide nucleotide concentration. Thus, it can be seen that the greatest activity is obtained when the ratio of ATP to Mg^{2+} is 1:1.

Effect of varying adenosine concentrations on adenosine kinase activity of human red blood cell hemolysates

In this experiment 2.5 mM ATP and 2.5 mM $MgCl_2$ were incubated with varying concentrations of adenosine. From the results presented in Fig. 10 it can be seen that increasing adenosine concentrations up to 4.5×10^{-5} M caused an increase in adenosine kinase activity. A maximum of activity was found at 4.5×10^{-5} M, while higher concentrations proved to be inhibitory.

Figure 9

EFFECT OF ATP AND $MgCl_2$ ON ADENOSINE KINASE ACTIVITY IN HEMOLYSATES.

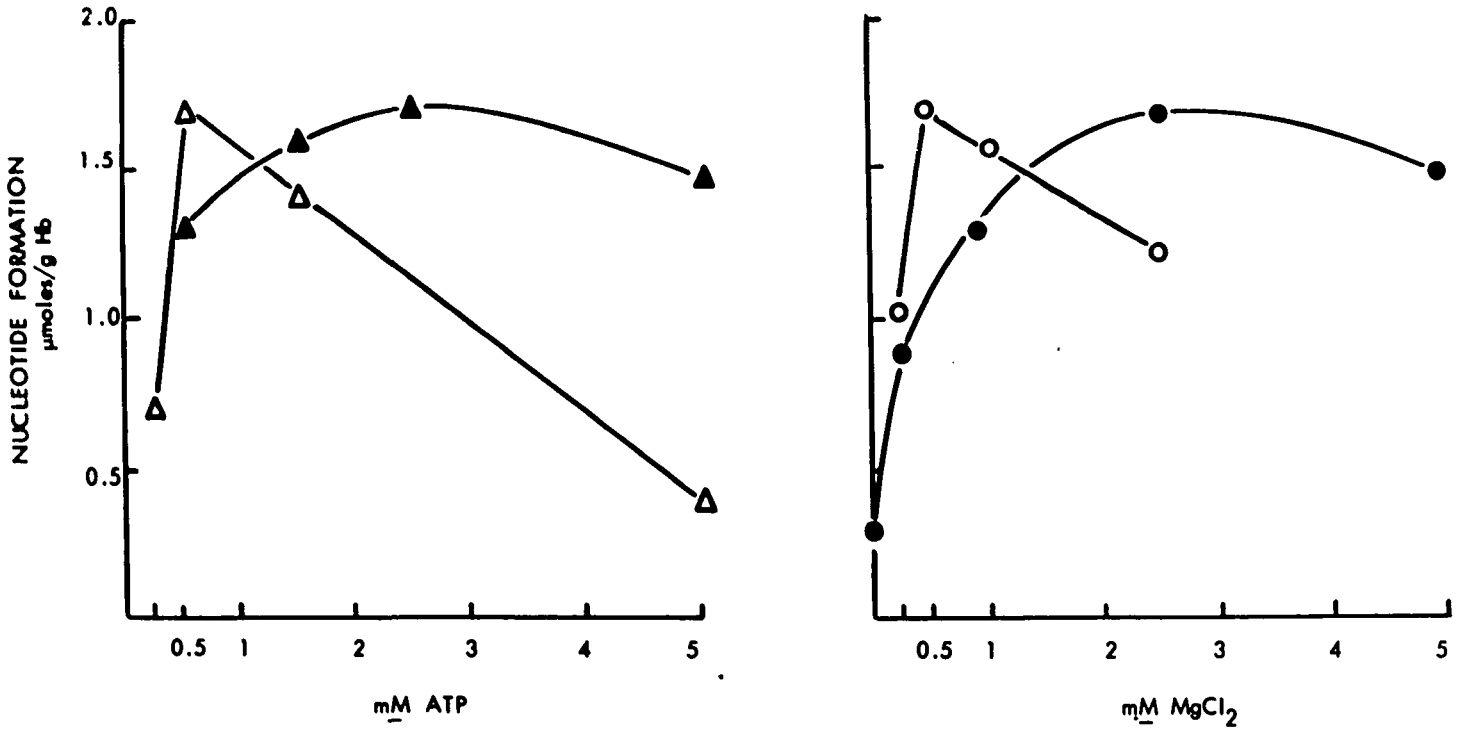


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Figure 9 Legend

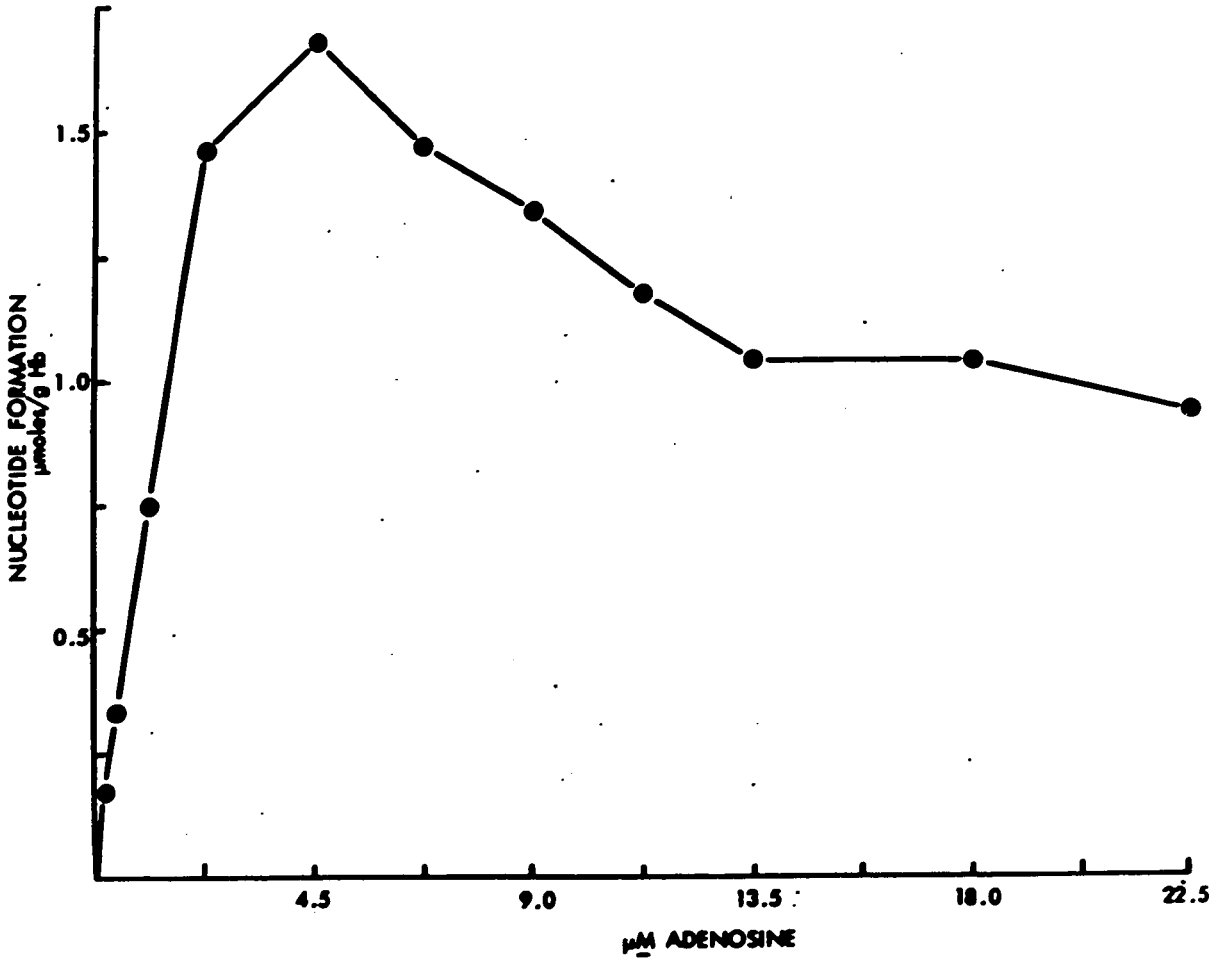
A: effect of ATP with; △ : 0.5 mM $MgCl_2$; ▲ : 2.5 m $MgCl_2$;

B: effect of $MgCl_2$ with: o: 0.5 mM ATP; ●: 2.5 mM ATP.

Assay conditions: as indicated in section on methods, using [8-^{14}C] adenosine with specific activity of 17.9 mc/m moles.

Figure 10

EFFECT OF ADENOSINE ON ADENOSINE KINASE ACTIVITY IN HEMOLYSATES.



Incubation conditions: as indicated in section on methods
The concentrations of adenosine shown are 1/10 that used.

Due to the presence of an active adenosine deaminase in human erythrocytes (139,140) the amount of the nucleotide available for phosphorylation by the kinase may be limited. Thus the fate of varying concentrations of adenosine in the hemolysates used to study adenosine kinase was investigated. The results of these experiments are seen in Fig. 11. At low nucleoside concentrations most of the adenosine was incorporated into the adenine nucleotides. At higher concentrations deamination becomes the predominant feature, while the absolute amount being incorporated into adenine nucleotides remains unchanged. It has previously been shown that with higher concentrations than those shown in Fig. 11, there is inhibition of nucleotide labile phosphorus formation by intact erythrocytes.

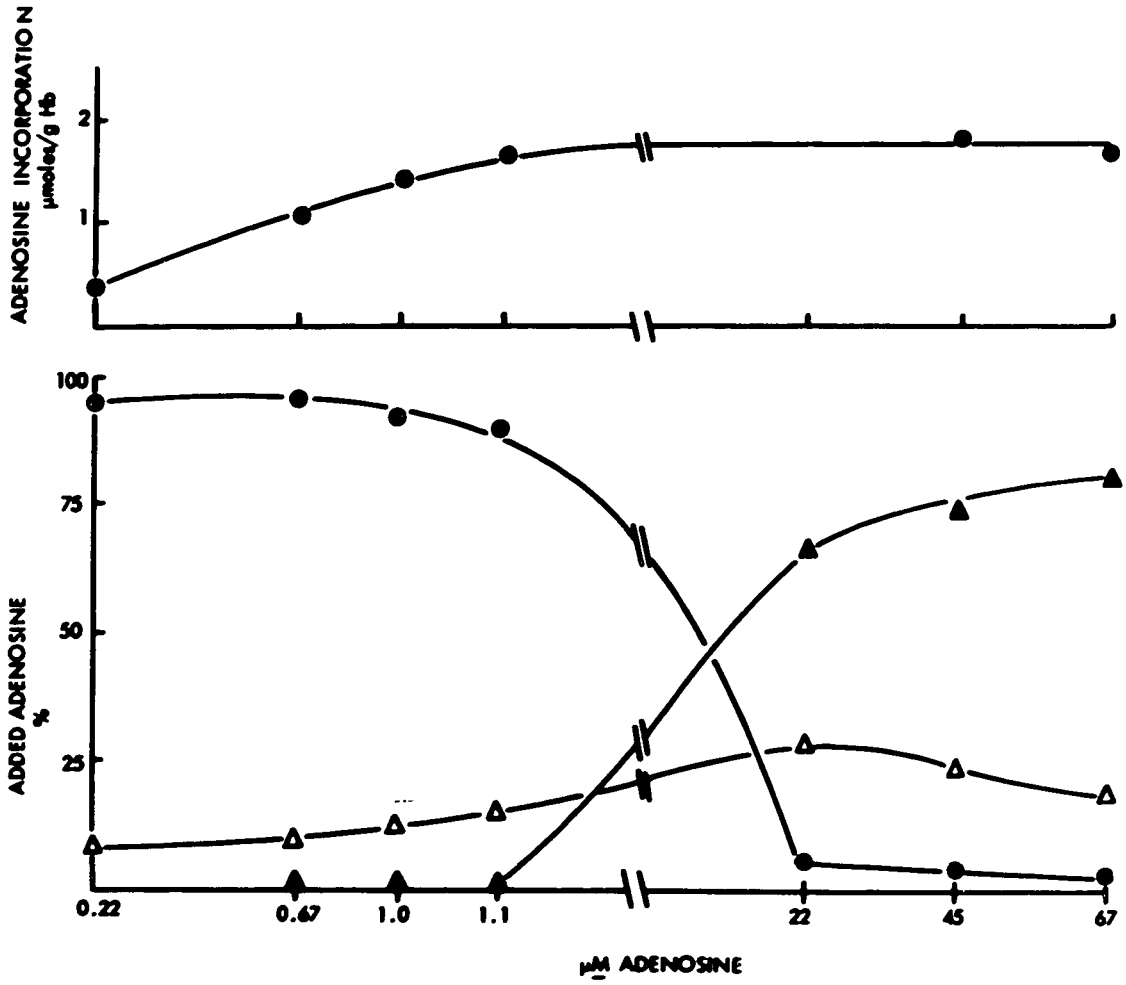
As previously demonstrated during storage there is no significant change in the activities of the three enzymes directly involved in AMP synthesis. There is however a progressive decrease in intracellular ATP concentration. In view of the importance of ATP as a substrate for the synthesis of AMP from either adenosine or adenine via adenosine kinase and phosphoribosyl pyrophosphate synthetase respectively, the relative affinities of the various enzymes for ATP involved in AMP synthesis may have an important role in determining the dominant pathway for purine nucleotide synthesis when both purine precursors are present.

Km of adenosine kinase for ATP

The Michaelis constant of adenosine kinase for ATP was obtained using a standard Lineweaver-Burke plot of reciprocal substrate concentrations vs

Figure II

FATE OF ADENOSINE IN HEMOLYSATE INCUBATED UNDER CONDITIONS USED FOR THE ADENOSINE KINASE ASSAYS.



Legend ●: adenine nucleotides; △ : inosine and hypoxanthine; ▲ : adenosine.

Upper graph represents adenosine incorporation into adenine nucleotides.
Lower graph represents % of adenosine in adenine nucleotides or inosine and hypoxanthine or in adenosine.
Incubation conditions: as indicated in section on Methods.

reciprocal initial velocities (Fig. 12). The substrate concentrations of ATP ranged from 0.25 mM - 5.0 mM. The Km for ATP was shown to be 4.4×10^{-4} M as determined from the regression line of the Lineweaver-Burke plot. The significance of these findings will be examined later on in the Discussion.

PART V. - FUNCTION OF THE ELEVATED ERYTHROCYTE ATP

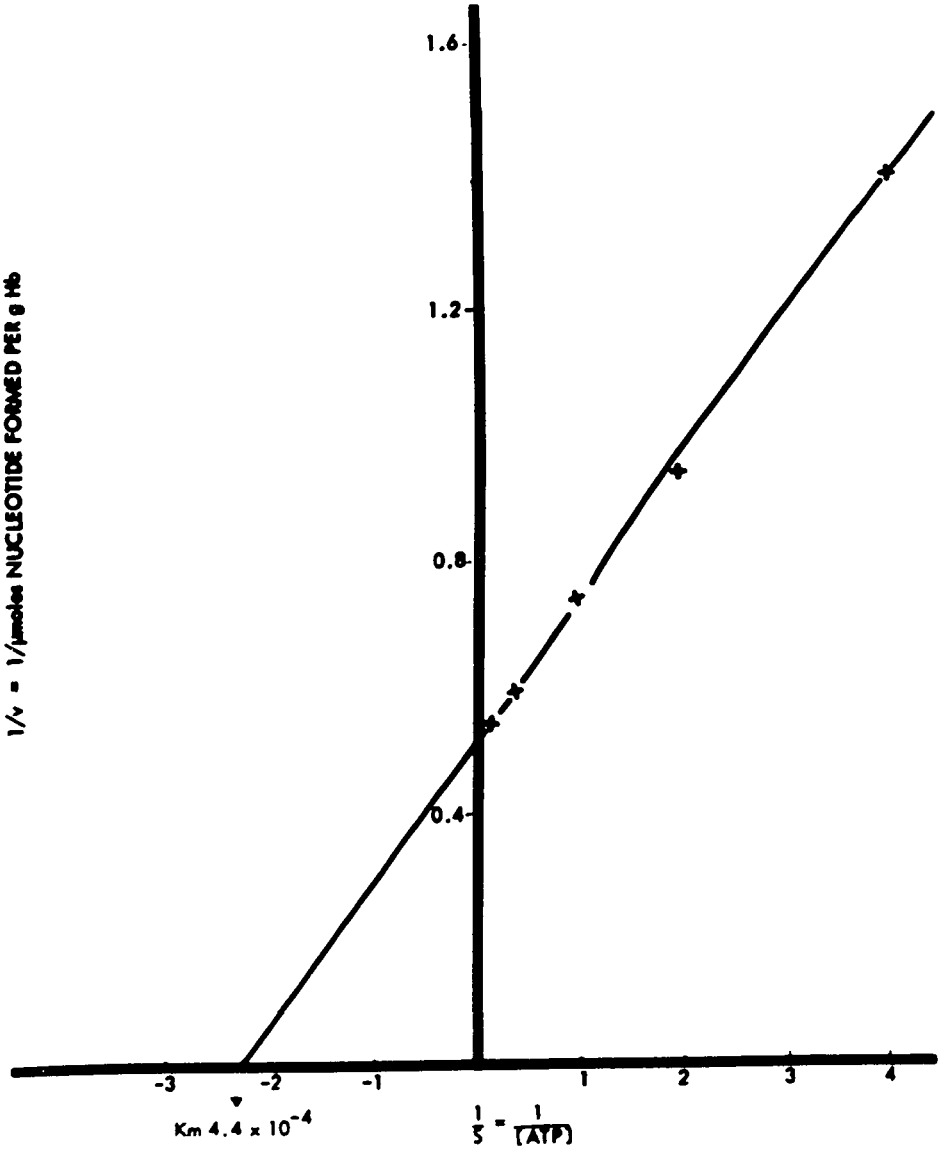
The association of ATP with Na^+, K^+ transport, glucose utilization, osmotic fragility and post transfusion viability is well known. However, most studies in this area have involved the study of normal intracellular ATP concentrations and subnormal concentrations usually brought about through preservation. In our studies it has been possible to double the fresh cell value of ATP. In view of the importance of ATP in many metabolic functions it was of interest to study the effect of the elevated ATP levels on various metabolic capacities.

Influence of increased nucleotide labile phosphorus on glucose utilization and osmotic fragility

In this series of experiments, freshly drawn erythrocytes were divided into two aliquots; one was incubated with glucose, while the other with glucose and adenine for 12 hours at 37°C. Glucose utilization was determined using the glucostat method (223, 224) by measuring the difference in glucose concentration prior to and following incubation. Osmotic fragility was measured using Sanford's method (225) and further verified by the use of an Osmotic Fragilograph, though the data for the latter are not shown. The results presented in Table 32, indicate

Figure 12

Km VALUE OF ADENOSINE KINASE FOR ATP.



Conditions as outlined in methods.

Table 32.

INFLUENCE OF INCREASED NUCLEOTIDE LABILE PHOSPHORUS ON GLUCOSE UTILIZATION AND OSMOTIC FRAGILITY

Incubation medium	Nucleotide labile phosphorus	Glucose utilization	Onset of hemolysis	Completion of hemolysis
	µg P/100 mg Hb	µ moles / hour	% NaCl solution required	
Glucose	24 (23)	1.83	0.42	0.34
Glucose + Adenine	45 (23)	1.83	0.42	0.34

Conditions.

1.0 ml of erythrocytes were incubated in 4.0 ml of Krebs-Ringer phosphate, pH 7.4 containing either 21.0 mM glucose or 1.6 mM adenine for 12 hours at 37°C. Glucose was measured using the glucostat method and osmotic fragility was measured using Sanford's method. The results presented are typical of three such experiments. The numbers in brackets represents the preincubation fresh cell value of nucleotide labile phosphorus.

that red blood cells incubated with glucose and adenine attained twice the fresh cell concentration of nucleotide labile phosphorus while glucose incubated cells only maintained their fresh cell levels. Following 12 hours of incubation the red blood cells were washed in 0.9% sodium chloride and glucose utilization, as well as osmotic fragility were determined. As can be noted there was no detectable difference in either glucose utilization or osmotic fragility in cells containing normal and elevated nucleotide labile phosphorus concentrations. The significance of these and other findings of this investigation will be examined in the Discussion which follows.

DISCUSSION

PART I. - AN ATTEMPT TO ELEVATE ERYTHROCYTE ATP CONCENTRATION ABOVE TWICE THE FRESH CELL VALUE

This study involves the investigation of factors controlling the elevation of ATP in human red blood cells. In the first part of the work an attempt was made to further elevate the level of nucleotide labile phosphorus above twice that obtained with glucose and adenine incubation of fresh human erythrocytes as has been previously demonstrated by Manohar (82). Three different experimental approaches were used to further elevate adenine nucleotide concentration of human erythrocytes. The first involved increasing the rate of glycolysis through elevation of inorganic phosphate or hydrogen ion concentration. Increasing the pH from 7.4 to 8.0 (52) and/or increasing the inorganic phosphate concentration from 10 mM to 30 mM (62) has been shown to increase the rate of glycolysis and therefore presumably ADP phosphorylation to ATP. Increasing the glycolytic rate by elevation of inorganic phosphate concentration or pH caused no further increase in nucleotide labile phosphorus synthesis. However inorganic phosphate and hydrogen ion concentrations are not the only rate limiting factors in glycolysis. The possibility that the rate of glycolysis is in fact limiting the elevation of adenine nucleotides to twice the fresh cell value requires further elucidation. Studies involving the change of flux of glycolytic intermediates under conditions of maximal nucleotide synthesis are being presently undertaken in this laboratory. The results of these experiments may help determine whether the glycolytic rate is indeed rate limiting and if so, at what step.

A second experimental approach was to study the possible limitation in available ribose phosphate which is required as a precursor for AMP formation via adenine phosphoribosyl transferase (185). Both ribose-5-phosphate and phosphoribosyl pyrophosphate pools can be increased by the addition of inosine (124). Inosine is broken down by nucleoside phosphorylase (131,135) yielding ribose phosphate and thereby bypassing the necessity for glucose utilization. Glucose is required in reactions catalyzed by hexokinase and transketolase, and is the usual method employed to synthesize pentose phosphate required for nucleotide synthesis. Evidence that inosine can supply ribose phosphate and phosphoribosyl pyrophosphate is demonstrated by the approximate doubling of nucleotide labile phosphorus in fresh cells incubated in the absence of glucose. Both glucose and inosine alone could supply sufficient ribose moiety to elevate nucleotide levels two fold in fresh cells. In the presence of both ribose phosphate precursors no additional increment in nucleotide labile phosphorus concentration occurred. This indicates that ribose phosphate production is not the rate limiting step in the formation of adenine nucleotides in fresh cells. The possibility that the rate of phosphoribosyl pyrophosphate formation may be limiting, as is the case for Ehrlich ascites tumor cells (97,98), is unlikely as an increase in ribose diphosphate, which is believed to be a breakdown product of a pre-existing pool of phosphoribosyl pyrophosphate, has been shown to accumulate during the metabolism of inosine (124). Furthermore, the accumulation of large quantities (1.6 μmoles P/ml erythrocytes) of IMP upon inosine incubation suggest

that PRPP must be readily available.

A third attempt to further elevate adenine nucleotide concentration above twice the fresh cell value involved changing the incubation medium with an extension of the incubation period. The ineffectiveness of this approach in increasing further the formation of nucleotides suggests that accumulation of a readily diffusible metabolite is not involved in limiting nucleotide labile phosphorus formation to twice that observed for fresh cells.

A discussion of the factors which may limit the synthesis of ATP to a maximal level which corresponds to twice that of fresh cells will be dealt with later on in PART III of this section.

PART II. - INHIBITION OF ADENINE NUCLEOTIDE SYNTHESIS IN HUMAN ERYTHROCYTES BY HIGH CONCENTRATIONS OF ADENOSINE

The observation that adenosine inhibits the synthesis of nucleotide labile phosphorus was surprising, as this compound is fairly rapidly deaminated by human red blood cells (139). The possibility that the observed inhibition may be due to the products of adenosine deamination or subsequent metabolism(resulting in an increased pH) have been ruled out. Adenosine itself must be present to exert the inhibitory effect, and if it is removed from the incubation medium after 2 to 6 hours, synthesis of nucleotide labile phosphorus in excess of the amount found in the fresh cell can then be achieved. In addition, as the adenosine is removed, primarily by deamination, the inhibition is relieved. Concentrations of adenosine which are inhibitory throughout the entire incubation are probably greater than that required to saturate erythrocyte adenosine deaminase during

this period. This accounts for the change from a slight inhibitory effect to almost complete inhibition over a relatively narrow range (12 mM to 16 mM) of adenosine concentrations.

Several possible explanations for the inhibition of adenine incorporation into adenine nucleotides by adenosine have also been ruled out. The results indicate that there is little likelihood that the added adenosine dilutes the adenine label (adenine-8-^{14}C). Thus, while a decrease in adenine-8-^{14}C incorporation into ATP might have been expected if the adenosine were merely diluting the labeled purine, no decrease in nucleotide labile phosphate or ATP concentration was observed.

Atkinson and Fall (101) and Hersko et al. (99) have reported ADP inhibition of phosphoribosyl pyrophosphate synthetase of E. coli and human red blood cells respectively. However the presence of inhibitory concentrations of adenosine does not alter the level of ADP achieved after 12 hours of incubation (Table 13). Therefore the possibility that adenosine is exerting its effect through ADP can likewise be eliminated. It has also been reported that adenine phosphoribosyl transferase is inhibited by AMP in Ehrlich ascites tumor cells (110,230). The presence of adenosine does not alter the AMP level found in these cells after 12 hours of incubation. The largest increase in nucleotide concentration with adenosine supplementation is in IMP, which cannot be converted to AMP in the human erythrocyte (68). It is unlikely that IMP is inhibitory since it was observed that the largest accumulation of this nucleotide occurs between the 6th and 12th hour, at a time when the inhibition of adenine incorporation and

ATP synthesis is being relieved by the action of adenosine deaminase. Furthermore IMP accumulation has been shown by Bartlett (124) to occur in the presence of inosine. The finding of an elevated IMP concentration following adenosine or inosine incubation suggests that adenosine is deaminated and then undergoes phosphorolysis prior to its conversion of IMP via the guanine-hypoxanthine phosphoribosyl transferase enzyme of human erythrocyte (70). The evidence strongly suggests that it is adenosine itself, and not a nucleotide derivative, which inhibits the incorporation of adenine-8-^{14}C into adenine nucleotides and the synthesis of ATP. There are several steps in the synthesis of nucleotides at which adenosine may produce its inhibitory action. As there is no accumulation of ADP or AMP seen with adenosine incubations, it is unlikely the adenosine is effecting adenylate kinase or substrate level phosphorylation of ADP to ATP. The two enzymes directly involved in the synthesis of AMP namely phosphoribosyl pyrophosphate synthetase and adenine phosphoribosyl transferase are the most likely sites for the inhibitory action of adenosine to take place. However our assay of adenine phosphoribosyl transferase activity of both a partially purified enzyme preparation from Ehrlich ascites tumor cells and human red blood cell hemolysate showed no inhibition of activity in the presence of adenosine. More recently, Blair et al. (231) have reported no adenosine inhibition of adenine phosphoribosyl transferase from bacterial or from Ehrlich ascites cells, thus supporting our finding.

The remaining possible sites for adenosine action are firstly, the entry of adenine into the cell; secondly, the synthesis of ribose phosphate and thirdly,

the synthesis of phosphoribosyl pyrophosphate.

It is the author's contention that phosphoribosyl pyrophosphate synthesis is the most likely site of adenosine action. Indirect evidence for this view comes from two sources. First of all, the finding of Block and Nichol (228) that PRPP synthetase of S. Faecalis is inhibited by decoyinine, which like adenosine is an adenine nucleoside; and secondly, the observation by Bartlett and Bucolo (124) that incubation of human red blood cells with adenosine produced no phosphoribosyl pyrophosphate accumulation, while incubation with inosine, under identical conditions, resulted in increased concentrations of PRPP. As has been previously mentioned in the Results, our experiments with PRPP synthetase from human red blood cells have been complicated by the fact that there was isotopic dilution of hypoxanthine-8-^{14}C used in the assay of PRPP synthetase. To determine whether PRPP synthetase is the site of adenosine action requires development of a different assay technique for PRPP synthetase.

PART III. - ELEVATION OF NUCLEOTIDE LEVELS IN PHYSIOLOGICALLY AGED AND IN PRESERVED RED BLOOD CELLS

In the present study it was found that the level of ATP was lower in the oldest 5% of the cells when fractionated by either the method of Berstein (154) or Danon and Marikovsky (157). This is in agreement with the findings of Brok et al. (163) and Berstein (154) but in contrast to those of Shojania et al. (232) who hc ɔ found no difference in ATP concentration in relatively young and old erythrocyte populations. The apparent discrepancy between the observations of Sho jania et al. and our own, may be due to the percentage of the oldest cells

involved in the determination. The old cell fraction of Shojania et al. represented one third to one tenth of the total red blood cell population, while the fraction separated by this author contained only one twentieth of the total population representing a much older population of cells.

Shojania et al. also expressed their values for ATP in terms of 100 ml of red blood cells while this author preferred to express the values on the basis of hemoglobin. Throughout the lifespan of the red blood cell the absolute amount of hemoglobin present in the cell remains constant (69), however, since the erythrocytes lose water and electrolytes (155) during their life span the actual cell volume is decreased. Therefore, the expression of values per cell volume would result in higher values than those expressed per weight of hemoglobin. A third possible reason for the discrepancy may be that Shojania et al. have studied rabbit while the author used human red blood cells.

We were unable to demonstrate a significantly elevated level of ATP in the 5% of the youngest cells. This is in agreement with the finding of Shastri et al. (206) and in disagreement with the findings of Brok et al. (163) and Berstein (154). This discrepancy may be due to variation in the number of reticulocytes trapped in the top 5% of the cell column. It has been shown that reticulocytes contain 2-3 times as much ATP as normal cells (11, 154). Berstein (154) reports about 10% reticulocytes in his young cell population whereas in our experiments they have been removed by a prior centrifugation. The possibility that a cell fraction of still younger cells might show an elevated ATP concentration cannot be ruled out. However, the amount of cells required for complete adenine

nucleotide analysis make it extremely difficult to obtain a sufficient amount of blood to supply a very young (i.e. only 1-2% of the total cell population) cell fraction. Two different procedures were employed to separate cells according to age and both gave similar results. However, some decrease in enzyme activity seems to occur using the phthalate ester method of Danon and Marikovsky. Evidence for this is seen by a decrease in adenine-8-^{14}C incorporation into nucleotides especially after 9 and 12 hours of incubation. No such problem was encountered when the method of Berstein was used. Both the young and old cell fractions were found to have the abi lity to double the initial concentration of ATP and ADP on incubation with glucose and adenine for 12 hours at 37°C. Initially the oldest cell population had a much lower ATP concentration, therefore, the actual amount of nucleotide synthesized is less in these cells. An investigation of the ability of preserved red blood cells stored in acidified citrate dextrose medium (ACD) to elevate nucleotide labile phosphorus following glucose and adenine incubation gave similar results to those obtained from physiologically aged human red blood cells.

The incorporation of adenine into nucleotides in the presence of glucose, appeared to be a function of the intracellular level of ATP prior to incubation in both physiologically aged and ACD preserved human red blood cells. When adenosine was used as the purine precursor for the elevation of adenine nucleotide concentration, the rate of its incorporation was not found to depend on the initial level of red cell adenine nucleotide but rather on the ultimate level of ATP synthesized by the cell. ATP is required in the synthesis of adenine nucleotides

from both purine precursors; in the case of adenine, it is used for PRPP synthesis while with adenosine; it is utilized for direct AMP synthesis. Hershko et al. (99) working with red blood cell hemolysate have found the Km value of PRPP synthetase for ATP to be 5×10^{-5} M, our work with adenosine kinase of human red cell hemolysate shows a Km value of 4.4×10^{-4} M for ATP. Whether the affinity of PRPP synthetase is actually greater than that exhibited by adenosine kinase cannot be assessed as the activity of PRPP synthetase observed in intact red cells was found to be 200-fold lower than the corresponding rate of PRPP synthesis in cell-free preparations. Further investigation is required to determine which enzyme has a greater affinity for ATP. However, in view of other findings to be discussed later, it seems unlikely that the reason for the relative ineffectiveness of the adenine incorporating system as compared to that of adenosine is a greater ATP affinity in the latter system. The reason for the apparent ineffectiveness of adenine in the presence of glucose to elevate nucleotide labile phosphorus in preserved cells to twice than seen in fresh cells was further investigated. That this is not due to inactivation of the enzymes directly involved in AMP formation was seen upon assay of the enzymes involved. No significant change in either phosphoribosyl pyrophosphate synthetase or adenine phosphoribosyl transferase could be detected in blood stored in acidified citrate dextrose medium for periods of up to six weeks. The most likely reason for the apparent adenine ineffectiveness in the presence of glucose in preserved blood cells is the inability of glucose to supply the ribose phosphate moiety, under conditions which result in a decrease in the intracellular ATP level (171, 174, 176). Thus, the addition

of inosine to an incubation medium containing adenine supplies the ribose phosphate moiety required for the elevation of the nucleotide labile phosphorus level to twice that of fresh cells. In addition, inosine also supplies glycolytic intermediates to stimulate the decreased rate of glycolysis (observed upon storage) and thereby promotes phosphorylation of ADP to ATP.

PART IV. - EVIDENCE FOR THE EXISTENCE OF AN ADENOSINE KINASE IN HUMAN ERYTHROCYTES

The large increase in nucleotide labile phosphorus level following incubation with 8 mM adenosine was quite surprising. The increase in nucleotide labile phosphorus could be accounted for by adenosine-8-^{14}C incorporation into adenine nucleotides. This suggested the presence of an adenosine kinase which would directly incorporate the entire adenosine moiety into adenine nucleotides according to the following reaction scheme: Adenosine + ATP → AMP + ADP. The presence of this enzyme has been found in yeast, liver, kidney (119), salmon milt (122), Ehrlich ascites tumor cells (120) and human tumor cells (H.Ep. No.2) in culture (121). Its presence in red blood cells has also been suggested from the work of Bartlett and Bucolo (124) and Lowy *et al.* (123). Lowy *et al.* have found incorporation of adenosine-8-^{14}C into adenine nucleotides despite a previous report to the contrary (68). Bartlett and Bucolo (124) have found an increase in ATP concentration following incubation with adenosine of four week preserved cells. Bartlett and Bucolo attributed their finding to either an adenosine kinase or to adenosine breakdown to adenine with subsequent adenine incorporation

into adenine nucleotides.

Our results provide direct evidence for the presence of an adenosine kinase in human red blood cells. An assay of adenosine kinase activity was developed for the human red blood cell using a modification of the procedure of Schnebli et al. (120) for human tumor cells of type H. Ep. No.2. The possibility that adenosine is being broken down to adenine which is then incorporated into AMP via the adenine phosphoribosyl transferase enzyme was investigated. Addition of unlabeled adenine to the enzyme assay incubation medium failed to dilute the radioactive adenosine-8-^{14}C incorporation into adenine nucleotides, thus ruling out this possibility. Similarly addition of unlabeled hypoxanthine or inosine had no effect. This strongly suggests that adenosine-8-^{14}C incorporation is due to adenosine incorporation and not due to the incorporation of one of its metabolic breakdown products. The possibility that the adenosine incorporation could be accounted for by an exchange of either adenine or adenosine with the corresponding moiety of an adenine nucleotide was also investigated. The demonstration that the terminal phosphate of ATP labeled with ^{32}P can be converted to the phosphate ester of AMP along with the results of isotope dilution experiments rules out the possibility of an exchange of the purine moiety and provides direct evidence that adenosine is phosphorylated in human erythrocytes. Erythrocyte adenosine kinase was shown to have similar properties to those reported for the enzyme purified from rabbit liver (121). Both enzyme preparations have optimal activity when the ATP:Mg^{2+} ratio is 1. When using physiological concentrations of Mg^{++} (0.5 mM)(46) the optimal ATP concentration was found also to be 0.5 mM. Higher concentrations of ATP are

inhibitory. The optimum concentration for adenosine was found to be 4.5×10^{-5} M, higher concentrations of adenosine proved inhibitory although inspection of the data of Lindberg et al. (121) does not reveal a similar inhibition with the rat liver enzyme. The inhibition of adenosine kinase by high concentrations of adenosine could explain the failure to elevate nucleotide labile phosphorus following incubation of erythrocytes with high concentrations of adenosine. The Km of human erythrocyte adenosine kinase for adenosine is 2.3×10^{-5} M compared to 1.6×10^{-6} M for the rabbit liver enzyme (120). The reason for the higher Km of our preparation can be explained by the fact that our work was done with red cell hemolysate while the enzyme from rabbit liver represents a preparation of much greater purity. Due to the presence of an active adenosine deaminase in human erythrocytes (139) the amount of the nucleoside available for phosphorylation may be limited. The enzyme was also shown to be inhibited by 6-methylmercaptopurine ribonucleoside as is the case for the enzyme from human tumor cells [H. Ep. No. 2] in culture (121) and to have a similar Km for ATP to that of the rabbit liver enzyme (120).

The physiological role of this enzyme is unknown, however, it has been suggested that it may serve to remove any adenosine which may accumulate and thus eliminate the hypotensive effects associated with this nucleoside (5). The results of our experiments support this suggestion since at low adenosine concentrations in red blood cell hemolysates, adenosine is preferentially phosphorylated, however at high concentrations, the deamination becomes the predominant pathway.

The inhibition of nucleotide synthesis from adenosine by high concentrations of adenosine may be due to two factors. First, the inhibition of adenosine kinase by high adenosine concentrations and secondly, an elevation of pH above the optimum required for the phosphorylation of adenosine resulting from deamination of large amounts of adenosine. The pH optimum for human tumor cells of type H. Ep. No. 2 is 6.2 to 6.8 (121) while for the rabbit liver enzyme it is pH 5.8 in phosphate buffer and pH 6.5 in citrate phosphate buffer (120).

PART V. - POSSIBLE FACTORS CONTROLLING SYNTHESIS OF ELEVATED ATP CONCENTRATIONS IN HUMAN ERYTHROCYTES

The fortuitous discovery that low concentrations of adenosine could double the fresh cell level of nucleotide labile phosphorus proved to be a valuable tool for studying the mechanism controlling ATP synthesis. This system, along with the adenine incorporating system, provides two different pathways to elevate nucleotide labile phosphorus in red blood cells. The role of both adenine and adenosine was studied when they were both present in the incubation medium, as well as under conditions where they served as the sole precursor in both fresh red blood cells and in cells preserved for periods up to six weeks. Incubation with high levels of inorganic phosphate (above the 12 mM normally used) had no effect on adenosine incorporation in fresh or 6 week preserved erythrocytes. However, high concentrations of inorganic phosphate in the incubation medium increased adenine incorporation into nucleotides of cells preserved for six weeks, probably by increasing glycolysis (52,62). Inorganic phosphate is also required

by phosphoribosyl pyrophosphate synthetase (43). Hershko et al. (43) have suggested that phosphoribosyl pyrophosphate synthetase is phosphate dependent, the level of ribose phosphate becoming rate-limiting only when the phosphate requirement has been satisfied. However, even at a concentration of 10 mM phosphate, inosine produces optimal adenine incorporation and nucleotide labile phosphate levels. Thus, it appears that the prime role of the phosphate in preserved cells is to stimulate the lagging glycolytic rate, probably at the hexokinase and/or phosphofructokinase steps (52). These are by-passed by the use of inosine or adenosine. Mager et al. noted that human erythrocytes require 15 mM phosphate (89) and rabbit 30 mM phosphate (90) for optimal adenine incorporation. Similar results with rabbit erythrocytes have been obtained by two workers in our laboratory.[1,2] The reasons for the difference between the two species of erythrocytes is not clear. When adenine and adenosine are both present in the incubation medium containing fresh cells, incorporation of each is diminished so that the total incorporation is equal to that seen with adenine when it is the sole purine precursor. As the period of preservation proceeds a larger portion of the nucleotide required to elevate the falling nucleotide concentration to twice that of the fresh cell level is supplied by adenosine, while the adenine incorporation only slightly increases. The interpretation of these results is consistent with the finding that the rate of

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[1] E. Warrendorf unpublished results.

[2] F. Novosad unpublished results.

nucleotide synthesis, under optimal conditions, is controlled by the ultimate level of ATP being achieved. Further evidence for this view comes from time course experiments in which the rates of incorporation of the two purine precursors, both together and separately, were followed during a twelve hour incubation period. When adenine was the sole purine precursor and inosine the source of ribose phosphate a maximal adenine incorporation occurred after 12 hours in both the fresh or four week preserved erythrocytes. On the other hand, when adenosine was the only purine precursor present the same degree of nucleotide incorporation and nucleotide labile phosphate formation was achieved within four hours. However, when both purine precursors were present together, adenosine incorporation was more rapid than that of adenine with no further incorporation of either occurring once the maximal doubling of nucleotide labile phosphorus had been achieved at about four to six hours in both fresh and four week preserved erythrocytes. At this time the level of nucleotide labile phosphorus corresponded to that seen with adenosine or adenine alone after 4 or 12 hours of incubation respectively. It seems likely that once a maximum of ATP or nucleotide labile phosphate concentration (which corresponds to twice the fresh cell value) has been reached by either pathway no additional incorporation of either adenine or adenosine can occur. In other words the system is controlled by the final level of ATP which is attained. The reason for the larger incorporation of adenosine when both precursors are present is due to the more rapid rate of adenosine incorporation.

The mechanism by which the rate of synthesis is regulated or amount of ATP is limited to twice that of fresh cells is not known. It is not a direct effect of the purine base or nucleoside since it has been previously shown that 8 mM concentrations of adenosine do not effect adenine incorporation into nucleotides while adenine or the products of adenosine catabolism have been shown to have no effect on adenosine kinase. Two possibilities may be considered. First of all, ATP itself may limit further nucleotide synthesis. In this regard, our investigations have shown that elevated levels of ATP inhibit erythrocyte adenosine kinase, especially at the Mg^{2+} concentrations found in erythrocytes. Brewer (46) claims that less than 15% of the Mg^{2+} normally present is unbound thus giving free Mg^{2+} an important regulatory role by making ATP inhibition even more pronounced. In Erhlich ascites cells high concentrations of ATP also inhibit adenine phosphoribosyl transferase (110). The increase in ATP has also been shown to be accompanied by a parallel increase in ADP which inhibits the synthesis of phosphoribosyl pyrophosphate (99) and the adenine phosphoribosyl transferase of B.subtilis (111) (if this enzyme can be considered analogous to the erythrocyte enzyme). In addition 2,3 diphosphoglycerate which is known to accumulate in red blood cells during incubation with glucose and adenine or with inosine (61) also inhibits phosphoribosyl pyrophosphate synthetase (99). The provision of energy for nucleotide synthesis represents a second mechanism which may control the rate of adenosine and adenine incorporation into nucleotides and especially in limiting the total ATP accumulation to twice that of fresh cells.

In the presence of high levels of ATP, found following incubation with adenine and inosine or glucose, Saito et al. (61) reported that phosphoglycerate kinase becomes rate limiting. In addition ATP also inhibits pyruvate kinase. It is however, unlikely that inhibition by ATP of phosphofructokinase plays a role in limiting the accumulation of ATP since the latter is twice that found in the fresh erythrocyte even in the presence of adenosine and inosine which by-pass the phosphofructokinase reaction. In order to pinpoint the inhibitory site of ATP action further experimentation must be performed. Estimation of the concentration of the intermediates of both glycolysis and the hexosemonophosphate shunt under various conditions of inorganic phosphate concentration with the idea of looking for accumulation of metabolic intermediates may be a fruitful approach to this problem. Controls monitoring the effect of inorganic phosphate concentration as well as duration of incubation must be employed to account for changes in the pattern of intermediates which are not a direct consequence of ATP elevation.

PART VI. - FUNCTION OF ELEVATED ERYTHROCYTE ATP CONCENTRATIONS

The effect of elevated ATP concentration on the metabolism of the erythrocyte has been only briefly studied by the author. No additional benefit to either glucose utilization or osmotic fragility could be attributed to the additional intracellular concentrations of ATP as compared to a control having a normal concentration of ATP and similarly incubated for 12 hours. Other investigators in our laboratory have been involved in the study of the effect of

elevated intracellular ATP concentration on fatty acid esterification into phospholipids (206), gross Na^+, K^+ changes,[1] and post transfusion viability.[2] None of these investigations have shown any change in the above parameters when compared to controls incubated for 12 hours with glucose alone. Further investigation of the biological turnover of ATP in cells containing both elevated and normal intracellular ATP concentrations is now in progress. The reason for the apparent lack of beneficial effect of the elevated ATP on these cells is not known. Two possibilities are suggested: first of all, incubation itself may cause a lesion which counteracts any possible positive effects of ATP elevation. Indirect evidence for this, is the observation of swelling of red blood cells upon incubation. Secondly, since elevation of ATP concentration does not increase the rate of the ATP dependent reactions it would suggest that the optimal ATP concentration required for metabolic function in red blood cells is that normally present. However, a possible function for the additional ATP may be as a reserve under conditions where ATP concentration is decreased; for example during preservation or physiological aging. The finding of increased post transfusion viability of subjects having elevated erythrocytic ATP concentrations (214) gives support to this idea.

Conclusion

Two different systems are capable of elevating adenine nucleotide

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[1] F. Novosad unpublished results.

[2] IBID

concentrations to twice that found in fresh cells. The first system involves adenine utilization, the mechanism of its incorporation has been previously described (81,82,187). The second system involves adenosine utilization at concentrations less than 12 mM. Adenosine elevates ATP concentration by directly being phosphorylated by erythrocyte adenosine kinase and by indirectly serving as a substrate for glycolysis.

The maximum level of ATP achieved under optimal conditions of substrate incubation could never reach more than twice the fresh cell value. Two possibilities are suggested as being responsible for limiting ATP synthesis: firstly, ATP itself may limit further nucleotide synthesis through feedback inhibition at the level of nucleotide synthesis from purine precursors and secondly, the provision of energy by ADP phosphorylation to ATP represents another method for limiting total ATP accumulation to twice that of fresh cells.

Concentrations of adenosine greater than 12 mM were found to inhibit ATP elevation produced upon glucose and adenine incubation. The most likely site of adenosine action is phosphoribosyl pyrophosphate synthetase.

SUMMARY

The cellular level of ATP can be doubled if freshly drawn human erythrocytes are incubated for a period of approximately 12 hours in the presence of glucose and adenine in Krebs Ringer phosphate (81,82). Addition of inorganic phosphate, alteration of hydrogen ion concentration or periodic change of the incubation medium followed by further incubation up to 24 hours, does not result in further elevation of the level of ATP. The effect of the nucleosides, inosine, guanosine and adenosine on the synthesis of nucleotide labile phosphorus during the incubation was studied. Inosine and guanosine had no effect, but adenosine, in concentrations between 10 and 20 mM, inhibited the increase in nucleotide labile phosphorus and the incorporation of adenine-8-^{14}C into ATP. The adenosine has no effect on the slight incorporation of adenine-8-^{14}C into, or the intracellular concentration of ADP or AMP. The inhibition is not due to the formation of ammonia and/or inosine resulting from the deamination of adenosine, nor is it due to the change of pH produced by the ammonia. Adenosine must be present to produce its inhibitory effect and the inhibition can be reversed by removal of the nucleoside, either by washing the cells or through the action of the erythrocyte adenosine deaminase. The inhibition cannot be accounted for by an elevation of IMP observed on adenosine incubation. Adenosine does not inhibit adenine phosphoribosyl transferase activity of a partial purified preparation from Ehrlich ascites tumor cells, although it exerts the same inhibitory action on the elevation of nucleotide

labile phosphorus in intact cells. Confirmation of this finding was also obtained with the same enzyme from human red cell hemolysate. It appears that adenosine produces its inhibitory effect when it is present in concentrations too great to be completely deaminated. It is suggested that adenosine acts by inhibiting adenosine kinase and probably phosphoribosyl pyrophosphate synthetase.

Incubation of freshly drawn human erythrocytes with 8 mM adenosine elevated nucleotide labile phosphorus concentration to twice the preincubation level. The increase in nucleotide could be accounted for by adenosine incorporation into adenine nucleotides by adenosine kinase. The existence of this enzyme in erythrocytes was demonstrated directly by the incorporation of [γ - ^{32}P] ATP into AMP in the presence of adenosine. Incorporation of adenosine-8-^{14}C could not be diluted by the presence of cold adenine, inosine or hypoxanthine, suggesting that adenosine is incorporated directly and not through one of its catabolic products. The erythrocyte enzyme is inhibited by 6-methyl mercapto ribonucleoside. The enzyme has optimum activity when the molar ratio of ATP : Mg^{2+} is 1. Elevated levels of ATP inhibit the kinase, especially at physiological Mg^{2+} found in the erythrocyte. The Km value of adenosine kinase for ATP was found to be 4.4×10^{-4}M. An optimum for adenosine was found at 4.5×10^{-5} M with higher concentrations proving inhibitory. The fate of varying concentrations of adenosine, in the hemolysates used to study adenosine kinase was investigated; at low concentrations most of

the adenosine is incorporated into adenine nucleotides. At higher concentrations the deamination becomes the predominant feature.

The role of adenine and adenosine as precursors of adenine nucleotides following 12 hours incubation was studied with fresh and preserved human erythrocytes. Adenine and glucose doubles the intracellular ATP level in both fresh and preserved erythrocytes although in the latter the actual level of ATP after incubation is less due to the initial decrease during preservation at 4°C. Adenosine can also elevate the level of ATP to twice that of freshly drawn cells and maintain this level when incubated with preserved blood. The maximum level of ATP obtained with any cells or substrates was twice that of the fresh cells. The incorporation of [8-^{14}C] adenine or [8-^{14}C] adenosine paralleled the increment in ATP levels. When adenine and adenosine were present with fresh cells both were incorporated into nucleotides at approximately the same rate. As the period of preservation was prolonged the incorporation of [8-^{14}C] adenosine was more rapid and accounted for most of the ATP synthesized. However no further incorporation of either precursor occurred when the level of nucleotide labile phosphate reached twice that of the fresh cells. It is suggested that ATP itself may limit further adenine nucleotide synthesis through feedback inhibition at the level of nucleotide synthesis from purine precursors or through the inhibition of ADP phosphorylation to ATP.

The relative ineffectiveness of adenine in preserved blood was not due to a decrease in phosphoribosyl pyrophosphate synthetase or adenine phosphoribosyl

transferase, since the activity of these enzymes, as well as that of adenosine kinase, remained stable throughout the 6 week preservation period; but was due to a decrease in glucose utilization which could be overcome by the use of inosine in the incubation medium.

Physiologically aged cells (oldest 5% of the freshly drawn cells) contained a lower ATP level than that of the rest of the population but this level could be doubled by incubation with adenine and glucose.

Elevated levels of ATP were found to have no effect on glucose utilization or osmotic fragility of human erythrocytes.

The significance of these findings are discussed.

CLAIMS OF ORIGINALITY

The author considers all the results outlined in the summary, with the exception of the finding presented in the first sentence, original contributions to the understanding of nucleotide metabolism in erythrocytes.

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