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HEXOKINASE SYSTEM OF THE ERYTHROCYTE _____ E. S-Y. LO.

THE HEXOKINASE SYSTEM OF THE ERYTHROCYTE

by

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PREFACE

The work described in this thesis represents part of a large program of research on the preservation of blood. The investigation has been in continuous progress in the Department of Biochemistry under the direction of Dr. O.F. Denstedt since the outbreak of World War II in 1939.

The initial phases of the work in the McGill University Laboratories were concerned mainly with the various aspects of metabolism and the physical stability of erythrocytes during storage. Rochlin, Andrea, Pappius, Woodford, Fishman and Francoeur carried out an extensive study on the chemical changes of erythrocytes during storage. More recently, Rubinstein, Alivisatos, Brownstone and other workers in our McGill group have been activily engaged in the study of enzymology of the red blood cells during maturation and aging. The main object of these studies is to find out the changes responsible for the progressive metabolic failure of the red cells during storage with a view to developing methods of preventing or retarding the deterioration process. The role of co-enzymes in glycolysis and the means to improve the viability of stored blood such as the addition of purine nucleosides to the preservative medium also were investigated.

Until 1962, the evidence from work in our group and others support the view that hexokinase in the red cell is a labile enzyme, the progressive alteration of which accounted for the inability of the cells to utilize glucose and thus to maintain their energy metabolism and viability. However, Prevost and Hemphill , in this laboratory, have recently provided evidence to show that hexokinase activity is not diminished in red cells on storage for 30 days at $4^{o}C$.

The writer has undertaken the investigation of this controversial but highly significant view of the stability of the hexokinase system in the red cell during preservation and has endeavoured to explore the relationship of this enzyme to the metabolic failure of erythrocytes.The study of the hexokinase system inevitably neccessitates a concomitant study of the systems which utilize glucose-6-phosphate, the product of the hexokinase catalyzed reaction. The studies on the properties and characteristics of hexokinase and the hexose monophosphate shunt also represents a part of the investigation.

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LIST OF ABBREVIATIONS

AMP, ADP and ATP	Adenosine mono-, di-, and triphosphate
ACD, CD, C and CPD	acidified citrate-dextrose, citrate-dextrose, dextrose and citrate-phosphate-dextrose media.
DEAE cellulose	Diethylaminoethyl cellulose
2,3-DPG	2,3-diphosphoglycerate
DPN, DPNH	diphosphopyridine nucleotide and reduced DPN
G-6-P, G-6-P DH	glucose-6-phosphate, G-6-P dehydrogenase
6-PGA, 6-PGA DH	6-phosphogluconic acid, 6-PGA dehydrogenase
GSH	glutathione
Hb	hemoglobin
HMP	hexose monophosphate
M, mM	molar, millimolar concentration
MeB	Methylene blue
P_I P_L P_S	inorganic phosphate, labile phosphate, and stable phosphate
SFH	stroma-free hemolysate
SH	sulphydryl
TCA	trichloroacetic acid
TPN, TPNH	triphosphopyridine nucleotide, and reduced TPN

INTRODUCTION

Structure of the Erythrocyte and Permeability of the Cell Membrane

The human mature red blood corpuscles, the normocytes, are non-motile, highly differentiated cells which have lost their nucleus, Golgi apparatus, mitochondria and centrioles during the process of maturation. The erythrocyte has the form of a biconcave disk. Normal human erythrocytes are remarkably uniform in size, with a mean diameter of 8.5 ± 3 μ. The cross-section resembles an elongated dumb-bell. The thickness on the outer region is about 2.4 μ and in the constricted central part about 1 μ. The erythrocytes are readily deformable and resilient. The biconcave discoid shape permits moderate volume changes by becoming more spherical without stretching the cell membrane. These properties of adaptation enable the cells to pass through narrow capillaries without suffering undue mechanical distortion and injury.

Upon hemolysis of the red cells and subsequent high speed centrifugation in isotonic saline or potassium chloride, the hemolysate forms three distinct zones. The bottom precipitate consists mainly of the material of the membrane which persists as transparent shells or ghosts. The middle translucent layer probably is composed of stromal material from intracellular finely divided components of the red cells. The top clear supernatant is made up of largely hemoglobin in solution or

possibly in an extremely finely divided colloidal suspension. The presence of glycolytic enzymes can be readily demonstrated in the top layer and to a lesser extent in the stromal fraction.

Classically, the red cell was thought to be composed of two main parts; a retaining membrane and a highly concentrated solution of hemoglobin (1) (2). It is evident that the stroma comprises the main framework, or underlying architectural structure, of the cell (3). However, a sharp separation of stroma from hemoglobin cannot be achieved at all times, since hemoglobin probably is an essential structural component of the red cell. Harris (4) suggested that the membrane in turn, by means of the ultrastructure, may also determine the ordering and function of hemoglobin. Numerous studies have been made concerning the structure and thickness of the membrane (5) (6) (7) (8) (9). Although the detailed anatomy of the erythrocyte is still not completely understood, the lipoprotein nature of the membrane seems unquestionable. Ponder observed that the lipids have an intrinsic radial birefringence and the proteins a tangential birefringence, thus indicating that the protein molecules are arranged perpendicular to the lipids and parallel to the cell surface (10). The lipids are orientated with the hydrophilic acidic groups projecting outward and imparting a strong negative charge to the surface of the cell. It is the presence of these charges together with the lipoprotein complex of the membrane and the metabolic activity of the cell that play a major role in the permeability to various metabolites and regulate the energy metabolism of the red cells.

The red cells and the plasma are in a state of osmotic equilibrium. The high concentration of impermeable anions, notably the hemoglobin and organic phosphate within the cell, accounts for the distribution of the freely diffusible ions such as Cl^- and HCO_3^- in accordance with the law of Gibbs-Donnan equilibrium. Erythrocytes behave as good, but not perfect, osmometers (11). Observed deviations from osmotic laws have been explained on the basis of intracellular bound water and the anomalous molal coefficient of hemoglobin (12) (13).

The entry of inorganic phosphate ions into the cell is not a simple diffusion process such as that of Cl^- and HCO_3^- (4). Bartlett (15) and Jones et al (16) have shown with labelled P^{32} that there is little or no exchange between internal and external orthophosphate pools of the cell. However, if red cells are incubated with P^{32} or C^{14}-glucose, the radio-isotope is taken up and incorporated into organic esters within the cell. Both uptake and loss of the ion must therefore be linked to cell metabolism (17) (18). Prankerd and Altman (19) measured the specific activities of various cell components at different times after the addition of labelled inorganic phosphate. They concluded that phosphate is incorporated into the red cell during the interaction of triose phosphate and inorganic phosphate under the catalytic action of triose phosphate dehydrogenase, to form 1,3 diphospho-glycerate. This mechanism enables incorporation of phosphate ions into the red cell without admitting free phosphate. Alternatively the phosphate can also enter the cell by means of phosphorylation of purine nucleoside. The elimina-

tion of phosphate is presumably effected by the break down of phosphate esters in the cell membrane (17). Cl^- will exchange with organic anions such as lactate, acetoacetate β-hydroxybutyrate and oxalate though at a slower rate than HCO_3^- (20).

Permeability rates of other inorganic anions are found to be as follows: $CNS > I > NO_3 > Cl > SO_4 > H_2PO_4$ (14). They are all capable of exchanging for Cl^- in the red cell but are physiologically insignificant due to the low concentrations present in the cell. It is also evident that monovalent anions exchange more rapidly than polyvalent anions.

Maizels (14) has shown that the rate of penetration of aliphatic anions increases with the length of the hydrocarbon chain but decreases with the number of keto- and hydroxyl groups in the molecule. Salicylate is freely diffusible while citrate and tartrate cannot pass through the membrane. Monobasic amino acids appear to enter the cell by a simple process of diffusion.

The red cell membrane is readily permeable to cations. Fishman (21) and other investigators (22) (23) have shown that cation movements are intimately connected with the glycolytic activity of the cell, and are temperature and pH-dependent. Studies by Blostein (23) in our laboratory have established that the active metabolic control over cation transport of the red cells is negligibly small at 4^oC. On incubation at 37^oC, the restoration of the cation balance is found to be dependent on the metabolic activity and ATP reserve of the preserved erythrocytes.

Maizels (24) has demonstrated that all glycolytic inhibitors lead to disruption of cation balance against the concentration gradient (24). On the other hand, respiratory inhibitors such as cyanide and DNP are without effect. The correlation between ATP levels and the observations made by Skou (25) and Deul (26) on the inhibitory effect of cardiac glycosides on cation transport suggest the presence of an active carrier system. Indeed, Mg^{++}, Na^{+} and K^{+} activated ATPase system was later identified by Whitman (27) and others (28) as the system responsible for the metabolically coupled outflow of Na^{+} and inflow of K^{+} through the plasma membrane. The membrane is found to be spatially asymmetrical in its affinity for ions. Whitman (27) also obtained evidence for an intracellular localization of the site for combination with Na^{+} and the extracellular site for K^{+}. The system requires synergical stimulation of the movement of Na^{+} and K^{+} on the opposite sides of the membrane. Absence of either ion fails to bring about the translocation process. Mitchell (28) found that the hydrolysis of one molecule of Mg-ATP at the inner surface of the membrane is coupled with the transport of 3 Na^{+} ions outward and 2 K^{+} inward across the erythrocyte membrane. The stoichiometry and localization of the system has recently been confirmed (29).

Permeability of other cations also has been studied. Rubidium and caesium ions, like K^{+} ions, are pumped into human erythrocytes while lithium enters the cell only by passive diffusion (27). The red cell membrane is freely permeable to

the ammonium ion. Ca^{++} and Mg^{++} appear to be firmly bound by the cell membrane and do not readily escape from the cell (30).

The permeability of the red cell membrane to non-electrolytes is influenced by the presence of lipoprotein in the membrane as well as by the molecular volume and the specific groups on the non-electrolyte.

Widdas (31) found that with glucose the rate of entry exceeds the rate of utilization several fold. The equilibrium is rapidly established with low concentrations of glucose and the rate of penetration increases with the concentration of extracellular glucose up to 200 mg per cent.

Glucose tranport through the membrane of the intact human erythrocyte follows zero-order kinetics. The transport depends on the stereoconfiguration of the sugars and exhibits competitive and inhibitory effects. There is strong evidence that glucose crosses the cell membrane by a facilitated mechanism governed by specific sites in the cell surface (32) (33). Based on experimental data, a total of three possible mechanisms have been proposed: The Carrier Theory (34), Enzyme Theory (35) and the Pore Theory (36). Kinetic studies have failed to distinguish between the three types of mechanism (37), but, in all cases, the first stage in the transfer involves the formation of a complex between the hexose and a component of the membrane. A counter-transport mechanism also has been demonstrated unequivocally in the system which catalyses sugar translocates in red blood cells (34) (39).

Stein (40) interprets the glycerol and glucose transport as brought about by dimer formation with a membrane receptor. The coupled translocation is believed to be catalysed.

Le Fevre (41) has given an extensive review on the relation between structure and activity in red cell transport. He also showed that glucose metabolism maintains the transport capacity and postulated the presence of about 500,000 reaction sites in the membrane (42). A wide difference in permeability has been observed to sugars of similar molecular structure (43). A reciprocal inhibition of permeability is also found. to aldose and ketose sugars (44).

Glucose transport apparently is not an energy dependent process. It is inhibited by Hg^{++}, phloretin, and chloromercuribenzoate without simultaneous inhibition of glycolysis (44) (45). Furthermore, the outward passage of glucose is influenced much more than its inward passage by the same inhibitors. This indicates that different mechanisms or pathways may be involved in the movement of glucose in and out of the cell.

The red cell membrane is permeable also to certain metabolites. Lactate moves passively and freely across the membrane in either direction while permeability to pyruvate is controlled by the metabolic activity of the cell (46) (47). A concentration gradient of these metabolites exists <u>in vivo</u> but decrease <u>in vitro</u>. The membrane is impermeable to organic phosphate esters and nucleotides as well as to disaccharides, citrate and tartrate under normal conditions.

Metabolism of the Erythrocyte

It is established that a red cell possesses metabolic activity and must be regarded as a functional unit whose complexity is comparable to that of other cells. Though lacking a respiratory metabolism, the red cell is capable of maintaining the production of energy which enables it to survive circulation through the vascular system during a life span of about 120 days.

Since the content of glycogen in the red cell is negligible (48), a constant supply of glucose is necessary to maintain the steady supply of energy by glycolysis. Other sugars such as fructose, mannose and galactose also can serve as substrates (49), but the rate of utilization is less than with glucose. There is some evidence (50) that lactose and maltose also may be metabolized but not sucrose or pentose sugars. Dische (51), Gabrio *et al.* (52) and Rubinstein *et al.* (53) have demonstrated that purine nucleosides can be utilized by way of the pentose metabolic pathway in intact cells and hemolysates, while pyrimidine nucleosides are not utilized.

Since only remnants of the Krebs cycle are present in the mature erythrocyte, the cell must therefore derive its energy primarily from the anaerobic glycolysis *via* the Embden-Meyerhof pathway and, to a lesser extent, from the oxidation of glucose by way of hexose monophosphate shunt. These pathways are outlined in Fig. 1. page 10.

The penetration of glucose through the red cell membrane has already been discussed. The initial phosphorylation of glucose apparently occurs in the interior of the cell (54). The glucose-

FIGURE 1

The Glycolytic and Pentose Phosphate Metabolic Pathways of the Erythrocyte.

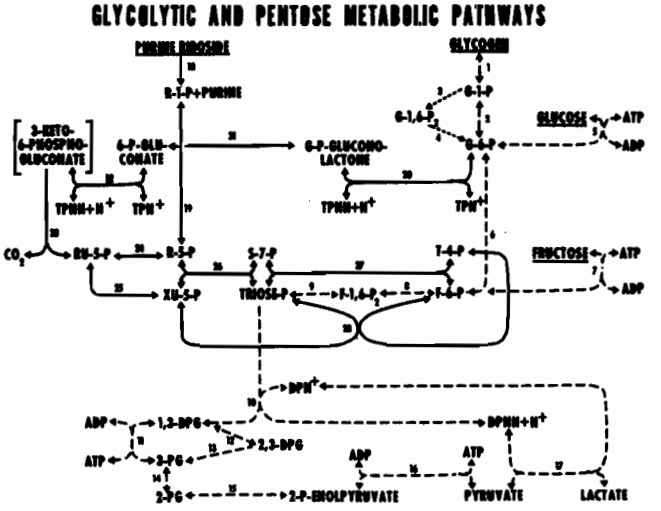
Glycolytic Pathway:

Reaction	Enzyme
1	Phosphorylase
2	Phosphoglucomutase
3	1-phosphoglucokinase
4	Glucose-1,6-diphosphatase
5,7	Hexokinase
6	Phosphoglucoisomerase
8	Phosphofructokinase
9	Aldolase
10	Triosephosphate dehydrogenase
11	Phosphoglyceric kinase
12	Diphosphoglyceric mutase
13	Glycerate-2,3-diphosphatase
14	Phosphoglyceromutase
15	Enolase
16	Pyruvic kinase
17	Lactic dehydrogenase

Pentose Phosphate Pathway:

Reaction	Enzyme
18	Nucleoside Phosphorylase
19	Phosphoribomutase
20	Glucose-6-phosphate dehydrogenase
21	Gluconolactonase
22,23	6-phosphogluconic dehydrogenase
24	Pentose phosphate isomerase
25	Ketopentose phosphate isomerase
26,28	Transketolase
27	Transaldolase

FIGURE I



Pentose metabolic pathway represented by solid lines and glycolytic pathway by broken lines.

6-phosphate (G-6-P) formed is then metabolized by anaerobic glycolysis. A net formation of two moles of ATP, with an energy equivalent of about 20,000 calories, occurs from the conversion of one mole of glucose to lactate. Alternatively, G-6-P may undergo oxidative decarboxylation by way of the hexose monophosphate shunt, with liberation of carbon dioxide.

The velocities of the reactions of glycolysis in the erythrocyte have been estimated by Chapman et al. (55), and Grignani and Löhr (56), They have shown that the enzymes concerned with ATP-utilization reactions have lower activity than those concerned with ATP-generating reactions. Hexokinase is found to have the lowest activity of all the enzymes and phosphofructokinase the next lowest.

In addition to ATP, other organic phosphate compounds are generated during the catabolism of glucose. The exact roles and contributions of the latter compounds to the energy balance of the erythrocyte are still obscure. The erythrocyte contains relatively high concentrations of diphosphate compounds such as 2,3-diphosphoglycerate (2,3-DPG), glucose-1,6-diphosphate (G-1,6-P_2), ribose-1,5-diphosphate (R-1,5-P_2). The last two compounds have been tentatively identified as the co-enzymes for phosphoglucomutase and phosphoribomutase respectively (57). Apparently 2,3-DPG is actively involved in the metabolism of glucose in erythrocyte (58) (59). It has a high rate of turnover, and after exhaustion in the stored red cells it can be resynthesized rapidly by the reconditioned red cells in the

presence of added nucleosides. In most cases, the concentration of 2,3-DPG rises and falls simultaneously with that of ATP in the erythrocyte (60).

Altman (54) and Vuopio (61) suggested that because the endergonic reactions utilizing ATP have become impaired in mature red cells and because of the inactivity of the ATPase system, there is a supplementary way for the accumulation of energy as 2,3-DPG *via* the Rapoport-Luebering cycle in the red cell (62) (63). This cycle is present in human red cells and some other mammals as a supplementary cycle at the triose-P level. 2,3-DPG makes up as much as 50% of the total acid soluble organic phosphate of erythrocytes. This compound therefore may represent the most important store of energy held in reserve and utilized only when the metabolism of glucose is inadequate (64). It should be mentioned that no direct evidence has yet been found regarding the conversion of 2,3-DPG back to the high energy compound 1,3-DPG in the red cells. Thus, the significance of the Rapoport-Leubering cycle in the regulation of glycolysis is still held in question.

Under normal conditions, human red cells utilize about 1.5 - 2.2 m.moles of glucose per litre of red cells per hour (58), which is equivalent to the liberation of 25 calories of energy. From the rate of production of $C^{14}O_2$ from G-1-C^{14}, it is estimated that 90% of the glucose utilized occurs by way of the glycolytic pathway and about 10% through the hexose monophosphate shunt (65) (66). Chapman *et al.* (55) and other workers (58) (67)

have demonstrated that each mole of glucose is quantitatively converted to 2 moles of lactate both in the intact human erythrocyte and in the hemolysate. The optimum of hydrogen ion concentration for glycolysis of human erythrocyte lies between 7.8 and 8.6, being highest at 8.1 (55). It has been observed by Murphy (68) that an increase in the hydrogen ion concentration or a high partial pressure of oxygen inhibits glycolysis. He suggested that influence of these conditions on the Embden-Meyerhof pathway is mainly on the sequence between the hexose-phosphate and the triose-phosphate levels.

The glycolytic system in the red cell also is susceptible to the influence of organic compounds which inhibit the glycolytic system in other tissues. The effect of these compounds on the hexokinase system is currently being studied by the author. Iodoacetate oxidizes thiol (SH) groups. Triose-P-dehydrogenase is especially sensitive to inhibition by this agent. The result is blockage of glycolysis and accumulation of fructose-1,6-diphosphate (F-1,6-P_2). Kashket demonstrated that fluoride has no effect on hexokinase but it inhibits glycolysis at the enolase level and leads to the accumulation of 2,3-DPG. Other agents such as N-ethylmaleimide and p-mercuribenzoate which react with the thiol groups, inhibit hexokinase and other SH dependent enzymes (31). Alivisatos and Denstedt (69) confirmed the observation of Quastel and Wheatly (70) that nicotinamide protects the co-enzyme DPN against the hydrolytic activity of DPNase

in the erythrocyte membrane, but the compound is also found to be a competitive inhibitor of the TPN- and DPN-link.dehydrogenase. Arsenate inhibits glycolysis by favoring the formation of 1-arseno-3-phosphoglyceric acid in place of triose-phosphate. Gabrio et al. have shown that Na^+, HPO_4^- ions or Tris buffer in concentration higher than 0.03M inhibits glycolysis both in intact cells and the hemolysate (55) (71). Prankerd (72) observed that a decrease in the concentration of intracellular potassium retards the glucose utilization and the formation of ATP. The cyanide ion reacts with keto-compounds such as pyruvate and thus inhibits the conversion of pyruvate to lactic acid while oxalate appears to interfere with the oxidation of reduced TPN (73).

The hexose monophosphate shunt is potentially operational in the red cells. However, due to the absence of a complete oxidative system for the reoxidation of TPNH, this pathway is relatively inactive in the mature red cells. Warburg (74) and Barron et al. (75) were the first to demonstrate the interesting phenomenon that the addition of methylene blue to red cells suspensions increases the oxygen uptake and the oxidation of glucose 20 - 25 fold. The methylene blue and certain other dyes act as electron carriers. Murphy (68) found that in an air-carbon dioxide atmosphere at pH 7.5, of the total potential energy derived from glucose metabolism in human erythrocyte, about 25% is in the form of TPNH and 75% in the form of ATP. It has been shown by Kashket and Denstedt (76) that the glycolytic system is progressively retarded with the fall in the hydrogen ion concentration and becomes inactive when the pH of the cell falls

to lower than 6.7. Brownstone (77) demonstrated that the enzymes of the pentose metabolic pathway, on the otherhand, remain active even at this increased hydrogen ion concentration. The significance of aerobic oxidation of glucose is therefore more apparent under conditions such as high pO_2, low pH or increased formation of methemoglobin (MHb) within the cell (68). Furthermore, the pentose phosphate pathway is the only known source of generation of TPNH in the erythrocyte. The main function of TPNH in red cells appears to be to donate hydrogen for maintaining glutathione (GSH) and hemoglobin in the reduced state.

Two important redox systems are present in the red cells. The first DPN $\rightleftharpoons$ DPNH system is involved in the synchronization of the triose-P-dehydrogenase and lactate dehydrogenases. The second TPN $\rightleftharpoons$ TPNH system, provides the co-enzyme for G-6-P dehydrogenase, which is coupled with the methemoglobin or glutathione reductases. DPNH is thought to be required also for methemoglobin reduction. In the presence of Methemoglobin, pyruvate rather than lactate becomes the end product of glucose catabolism (78). The postulated reactions are as follows:

1. $G\text{-}6\text{-}P + TPN^+ \xrightarrow{G\text{-}6\text{-}P\ DH} 6\text{-}PGA + TPNH + H^+$

2. $6\text{-}PGA + TPN^+ \xrightarrow{6\text{-}PGA\ DH} Ru\text{-}5\text{-}P + CO_2 + TPNH + H^+$

3. $MHb + TPNH^+ + H^+ \underset{\text{reductase}}{\overset{MHb^{+++}}{\rightleftharpoons}} Hb + TPN^+$

4. $GSSG + TPNH + H^+ \underset{\text{reductase}}{\overset{GSH}{\rightleftharpoons}} 2\ GSH + TPN^+$

5. G-3-P + DPN + P_I $\xrightleftharpoons{\text{Triose-P DH}}$ 1,3 diPG + DPNH + H^+

6. MHb + DPNH + H^+ $\xrightleftharpoons[\text{reductase}]{MHb^{+++}}$ Hb + DPN^+

7. Pyr + DPNH $\xrightleftharpoons{\text{Lactic DH}}$ Lactic Acid + DPN

In 1951, Dische (51) first demonstrated that adenosine, inosine, guanosine and xanthosine can serve as a source of R-5-P in the hemolysate. Shortly afterwards Gabrio et al. (52), and Rubinstein et al. (53) observed that the addition of purine nucleoside to stored blood induces the resynthesis of phosphate esters, restores the electrolyte and water balance, the energy potential in the form of ATP, and the metabolic viability of the preserved red cells. Gabrio and Finch (79) reported subsequently that the addition of ATP, ribose, ribose-phosphate, purines and red cell extracts are ineffective. The glycosidic linkage is therefore essential for the passage of ribose into the cell, and for the utilization of ribose in the pentose metabolic pathway. Rubinstein and Denstedt (53) showed that adenosine, when added to blood undergoes conversion to nucleotide. A considerable portion first is deaminated at the cell surface by a nucleoside deaminase with the formation of inosine. The latter compound, in the presence of inorganic phosphate, is phosphorylated by the action of nucleoside phosphorylase to yield hypoxanthine and Ribose-1-phosphate (R-1-P) (80) (81) The hypoxanthine is not further metabolized but the R-1-P undergoes isomerization to ribose-5-phosphate (R-5-P). The reaction is catalyzed by phosphoribomutase with R-1,5-P_2 as co-enzyme (57).

R-5-P is then utilized by way of the pentose metabolic pathway and the Embden-Meyerhof pathway and contributes to the formation of ATP and lactate. Bartlett and Shafer (82) pointed out that the action of nucleosides in reviving preserved red blood cells is achieved by providing ribose-5-P for utilization in the pentose metabolic system and also by providing purine base for the synthesis of ATP.

According to Gabrio et al. (83), the effectiveness of nucleosides in inducing regeneration of phosphorylated intermediates in preserved blood in descending order is as follows: inosine > adenosine > guanosine > xanthosine.
However, Lange et al. (84), Mollison and Robinson (85) compared the effects of inosine and adenosine on preserved red cells, and arrived at conflicting conclusions regarding the relative merits of these nucleosides. Rubinstein et al. (86) have suggested that adenosine, on deamination releases ammonia and lowers the hydrogen ion concentration in the preserved specimen, thus favoring the utilization of glucose. Inosine does not have this activity and thus is incapable of restoring the utilization of glucose in older specimens of blood; rather, it tends to increase the utilization of ribose. This explanation is in agreement with the findings reported by Shafer and Bartlett (60), that adenosine appears to be more effective than inosine in the restoration of the levels of the organic phosphate esters in preserved red cells. Nakao et al. (81) observed that the ATP concentration in 8-week old blood cannot be restored by inosine alone. However, when the system is supplemented

with adenine, the ATP level is restored. Using adenine-8-C^{14}, they have shown that the purine is actually incorporated into the nucleotide molecule. Thus the better preservation of glycolytic capacity exhibited by adenosine may also be attributable to its possible conversion to adenine through dephosphorylation. The replenishment of adenine for ATP synthesis is essential since no adenine synthesis de novo occurs in the erythrocyte and loss of the adenine moiety takes place continuously through the conversion of ATP to AMP; and finally by the irreversible deamination of the AMP to IMP (87). Although the complete pathway for purine formation is absent in the matured erythrocyte, it has been demonstrated that the erythrocyte can complete the final steps of nucleotide synthesis and utilize purines and the ribosyl derivative for the formation of nucleoside triphosphate. Evidently, mature red cells can utilize preformed purines for nucleotide synthesis but lack the capacity to convert inosinic acid to adenylic acid. It is not fully established that mature erythrocytes can synthesize DPN, TPN and GSH de novo although these compounds appear to be in a dynamic state in the mature cell (88) (89) (90).

One disadvantage in using adenosine for routine blood preservation is its toxic property. This nucleoside, through liberation of ammonia on deamination, produces hypotension and may cause cardiac arrest. Guanosine appears to be less toxic and inosine is non-toxic. The end product of metabolism of purine in the body is uric acid. While inosine is well tolerated in moderate

doses in normal subjects there is uncertainty as to whether the liver in sick patients, particularly after surgery, can metabolize the quantity of inosine administered in multiple transfusions and the kidney excrete the uric acid which may amount to more than 2 grams per transfusion.

Hexokinase

Hexokinase, in the broadest sense, includes any enzyme that catalyzes the transfer of the terminal phosphoryl group of ATP to any of the hydroxyl groups of a hexose monosaccharide. In practice, this type of enzyme activity involves the hydrolytic cleavage of P-O bond of the ATP molecule and the reaction with C-terminal group of the hexose.

Hexokinase was first discovered by Meyerhof (91) in an alcohol-precipitated fraction of yeast autolyzate which, when added to muscle extract, greatly increased the utilization of glucose and fructose. Subsequently the enzyme was found also in animal and plant tissues (92) (93), in microorganisms (94) and in a wide variety of invertebrate species. In 1932 the presence of hexokinase in a cell-free preparation from erythrocytes was demonstrated (95). The ubiquitous occurrence of the enzyme suggests an essential function in the energy producing system in most species. Embden and Meyerhof showed that hexokinase is the first step in the glycolytic conversion of glucose to lactate. The role of hexokinase in regulating the rate of glucose utilization in tissues was confirmed by various workers (96) (97). More recently, the rate limiting action of hexokinase

in anaerobic glycolysis in red blood cells was elucidated by Rapoport et al. (98) (99) in the following observations:

(a) There is a parallelism between hexokinase activity and glycolytic capacity in various tissues.

(b) The pH optimum of anaerobic glycolysis (about 8.1) corresponds closely to the optimum for hexokinase.

(c) The low activity of hexokinase compared to that of other glycolytic enzymes.

(d) The diminished activity of both glycolysis and hexokinase when reticulocyte matures to erythrocyte.

(e) The decrease in glycolytic activity in nutritionally induced Mg-deficient erythrocytes; hexokinase being a Mg-dependent enzyme.

Hexokinase is therefore established as the pace-maker of glycolysis in erythrocyte. Back (100) and Löhr et al. (101) confirmed these findings and showed that the enzyme controls also the rate of glycolysis in leucocytes and other formed elements of the blood.

Hexokinase shows a specific requirement for ATP and for magnesium ion as a co-factor. The reaction is shown as follows:

$$\text{Glucose} + \text{ATP} \xrightarrow[Mg^{++}]{\text{Hexokinase}} \text{Glucose-6-phosphate (G-6-P)} + \text{ADP}$$

In studies on the phosphorylation of yeast hexokinase, Martinez (102) found that nucleotides other than ATP and ITP are ineffective as the phosphate donor. The rate of phosphoration varies with the hexose acceptor. According to Kashket (43) the

optimal pH of hexokinase in red blood cell is 7.8. At this pH the affinity of the enzyme toward various sugars was found to decrease in the descending order: D-glucose > D-mannose > D-fructose > D-galactose. The activation energy for the phosphorylation of glucose by hexokinase is about 11,500 calories per mole. Gottschalk (103) reported that with yeast hexokinase, the α- and β- forms of D-glucose and D-mannose both react at the same speed without undergoing preliminary interconversion; however, D-fructose reacts only in the β-furanose form.

Crane (94), in a recent review, compared the characteristics of the hexokinase system in various tissues. The Km value of brain hexokinase was found to be close to that of the true enzyme-substrate dissociation constant. Thus the absence of a hydroxyl group on the sugar results in a great change in the Km value since it represents the loss of the site for the specific combination of hexokinase with its substrate. Following up this observation Crane studied the phosphorylation of 25 hydroxyl analogues of glucose and glucose-6-phosphate and concluded that the formation of the active hexose-hexokinase complex must involve the hydroxyl (OH) groups at carbons 1, 3,4 and 6. Thus to be a substrate for hexokinase, the pyranoid ring in the sugar and the OH groups on these carbon atoms of the hexose molecule are essential. Recently, Crane proposed further that the 3 bonds between D-glucose (β-D-glucopyranose-CE) and the specific sites on the surface of the enzyme-ATP (E-ATP) complex may contract; thus converting the glucose molecule to the β-D-glucopyranose-CA conformation with the C_6-OH lying

closer to the enzyme surface and the ATP molecule. Phosphorylation then occurs with the formation of β-D-glucopyranose-6-P-CA. Owing to the loss of an attaching hydroxyl group at C_6 the binding to the enzyme is greatly reduced. Separation of the product from the enzyme is further facilitated by charge repulsion. The resulting products of the reaction are β-D-glucopyranose-6-P-CA (+G-6-P) and ADP-E.

It has been observed in the yeast hexokinase system that an increase of Na^+ ion concentration, at constant ionic strength, stimulates the reaction rate several fold. The activation effect is believed to be due to interactions of the Na^+ ions with ATP and the ATP complex (104). Martinez and Trayser et al. (102) (105) have shown with the yeast enzyme that only one molecule of glucose or fructose is bound to a molecule of hexokinase and that the dissociation constant is the same as the Km values calculated from kinetic data. Various interpretations have been proposed for the mechanism of the reaction. However, studies from ultraviolet, fluorescence (105) or ATP^{32} labelling technique provide no evidence to indicate the presence of stable phospho-enzyme intermediate during the reaction. Fromm and Zewe (106) also have pointed out that any hypothesis involving enzyme-phosphate or enzyme-glucose intermediates is kinetically untenable. Hass et al. (107) further provide evidence favoring the view that the substrates, glucose and ATP, are added randomly to the enzyme in a system in which equilibrium kinetics prevail.

Hexokinase has been prepared in crystalline form from

bakers' yeast by Berger et al. (108), and by Knuetz and McDonald (109). Trayser and Colowick (110) recently have demonstrated that crystalline yeast hexokinase is separable into six active fractions by chromatography on DEAE cellulose, or into three active fractions by starch gel electrophoresis. Despite the apparent difference in the multiple molecular forms of hexokinase, these fractions were identical in their biochemical behaviour. Ramel and associates (111) found that hexokinase exhibits a reversible association and dissociation (without loss of activity) depending on the concentrations of glucose and hydrogen ion. The enzyme is split by sodium dodecyl sulphate (SDS) into subunits of molecular weight 2×10^4. Further studies on yeast hexokinase revealed that the enzyme is not a metallo-protein and contains no biotin nor any demonstrable prosthetic group. It has a weak but definite ATPase activity (105). The molecular weight was estimated to be about 96,600. Crystalline hexokinase, prepared from yeast and muscle, are now commercially available. However, the purification or isolation of hexokinase from erythrocyte has been fraught with many obstacles, the major one being the difficulty in getting rid of the hemoglobin. Hennessey et al. (112), using DEAE cellulose as exchanger, have prepared a hemoglobin-free protein fraction from erythrocytes.which contained hexokinase and other glycolytic enzymes.

The hexokinases from yeast and that from animal tissues are similar in their biochemical behaviour but differ in certain respects. The former is a glycoprotein (113) while the latter

appears to be a lipoprotein (114). The reversibility of the hexokinase system in yeast was demonstrated by the incorporation of P^{32}-labelled ADP into ATP. Crane et al.(114) reported that a maximum rate of the reversed reaction is about 20 per cent as fast as the forward reaction. On the other hand, the reaction is practically irreversible in the red blood cell. Rose et al. (115) and Hemphill (116) have shown that the red cell contains no functional glucose-6-phosphatase (G-6-Pase). While high concentrations of nucleotide and of the hexose monophosphates, G-6-P and G-1-P inactivate the hexokinase of brain (117) and of red blood cells (43), yeast hexokinase appears to be unaffected by the compounds. Crane believes that the difference between yeast and brain hexokinase with regard to product inhibition, may reflect a distinct role of G-6-P as part of a mechanism for the control of metabolism in plant and animal tissues.

The Km(Mg.ATP) and Km(glucose) values for the hexokinase of rabbit erythrocyte were in the order of 1.5×10^{-3}M and 2.8×10^{-4}M respectively (43). Different Km values of the hexokinase from different origins were found, thus reflecting the differences in the affinity of these enzymes towards their co-enzymes, substrates and the reversibility of the reaction in various species or tissues of a given species.

It has been shown by Cranes (94) (114) that brain hexokinase has at least three distinct sites of attachment respectively for the substrate, ATP and the product. Based on experimental data, he suggests further that the specific inhibition

of brain hexokinase by phosphorated compounds appears to involve the attachment of the enzyme through the hydroxyl groups at C_2 and C_4 of the sugar and the phosphoryl moiety at C_6. Sulfhydryl (SH) reagents, mercurial compounds and certain ions, e.g. Ca^{++}, Cu^{++}, inhibit the enzyme. Metal-binding agents (118) such as versene were found to protect the enzyme against inactivation by dilution whereas insulin and serum protein were less effective.

Studies on the inhibitory effects of strong SH-blocking reagents such as p-chloromercuribenzoate, alloxan and disulphide compounds (119) (120) (121) on hexokinase indicated that the activity of the enzyme may depend on some essential free thiol groups on or near the active centre of the protein molecule. Sols and Crane (118) found that cysteine or glutathione reverse the inhibition of hexokinase by p-chloromercuribenzoate but that they are ineffective against the inhibition produced by o-iodosobenzoate. Formanidine disulphide and tetrathionate both inactivate hexokinase as well as other SH-enzymes (122). Stromme (123) demonstrated that disulfiram is a potent inhibitor of yeast and brain hexokinase but the inhibition can be prevented by the presence of reduced glutathione. The fact that complete reactivation of hexokinase can be achieved by addition of cysteamine indicated that the inhibitory effect of disulfiram is due to the blockage of essential sulfhydryl groups on hexokinase. Eldjarn and Bremer (124) have shown that cystamine and cysteamine exert an inhibitory effect on hexokinase similar to that of disulfiram. Barnard and Ramel (125) studied the active centre of yeast hexokinase by titration of the SH-group in the

presence of various inhibitors. They concluded that 1 to 4 of the titratable SH-groups per molecule of the enzyme are required in the active centre (s). Fasella and Hammes (126) observed that while the activity of hexokinase decreases gradually during the titration in the presence of G-6-P as inhibitor, spontaneous denaturation of the enzyme does not occur until the titration is complete. It is therefore postulated that sulfhydryl groups may also be responsible for the stabilization of the active enzyme configuration.

Morphological and Enzymatic Changes during Maturation

The mean life span of human red blood cell is 120 days. About 0.8 percent of the circulating erythrocytes are eliminated from and replaced in the circulation daily. Ponder (1) and Wintrobe (129) attribute the normal red cell destruction to extra- and intra-corpuscular factors. The elimination of red cells from the circulation proceeds at a fairly constant rate. Apparently, due to the impairment of energy metabolism or changes in the cell membrane, destruction of the red cells ensued.

The metabolic activity of erythroid cells undergoes changes at various stages of development. The nucleated erythroid cells of the bone marrow are capable of performing most of the metabolic reactions that occur in other somatic cells, such as the synthesis of DNA and RNA for cellular proliferation, active anaerobic and aerobic metabolism and synthesis of lipids, protein and other materials. Nearly all the hemoglobin is

synthesized during the period of development in stem blast cells during transition from the erythroblast into the normoblast. As the normoblast matures to form a reticulocyte, the nucleus disappears. The anucleated reticulocyte contains a small amount of DNA but a nearly normal quantity of RNA. It derives the name from the reticulum in the cytoplasm which can be stained with brilliant cresyl blue. The reticulocyte still contains an active Krebs cycle, cytochrome system and electron-transfer system (130) (131). As the cell matures into the adult erythrocyte, the cytochrome system disappears. Little or no RNA remains and the Krebs cycle becomes inactive. Thus, the mature erythrocyte is incapable of carrying out oxidative processes. The capacity for lipid and protein synthesis is insignificant or null and the mature erythrocyte derives its energy entirely through anaerobic glycolysis. These metabolic changes are reflected in the observed alterations in the enzymatic activities of the red cells during the process of maturation. Valuable information has been provided by Marks et al., Rapoport et al., investigators in our own laboratory and others (139-143) concerning studies on the comparative enzymology of the reticulocyte and mature erythrocyte. A summary of the findings is presented in Table I.

Changes in the physical characteristics of the red cells are also observed during the process of maturation. Younger cells, in general, are more resistant to osmotic hemolysis than older cells. In addition, there is a reduction in the lipid and the water content resulting in an increase in the density

TABLE I

Enzymatic Changes during Maturation of Red Blood Cells.

Enzyme	Location in cells	Ratio: Activity of reticulocytes / Activity of normocytes		References
G-6-P DH	↑ Probably in both SFH and stroma (including cell membrane)	2	higher in young cells	132, 133,135,137
6-PGA DH		1-2		132, 137
Transketolase		2-5		136
P-Hexose isomerase				137
Aldolase		2		136, 138
Triose-P DH		1-2		
3PG kinase		10		133
Hexokinase		10		133, 135
DPG-mutase	↓	1-2	higher in old cells	140
Pyruvic kinase		1-2		140
Phosphofructokinase		1-2		130
Lactic DH		1	same in young and old cells	136,137,138,139
Glycerol-3-P DH		1		133
DPNase	Stroma outer surface of cell membrane	1		127

TABLE I (continued)

Enzyme	Location in cells	Ratio: Activity of reticulocytes / Activity of normocytes		References
ATPase	cell membrane	?		127, 195
Isocitric DH	both	2-3	higher in young cells	135
Malic DH	both	2-3		135
Succinic DH	stroma	0-10		133
Fumarase	both	10		135
Cytochrome reductase	stroma	8		133
Cytochrome oxidase	stroma	nil		135
Glyoxalase	both	2-3		142
Choline esterase	stroma	2-3		142
Catalase	stroma	1-2		140, 101, 142
Adenylate kinase	stroma	?		143
Pyrophosphatase	SFH	10		133, 134
Methaemoglobin reductase	both	1-2		141
Nucleoside phosphorylase	probably bound to structural components	1		137
Diaphorase		1	higher in young cells	133
GOT		1-2		133, 143
GPT		1-2		133
Glutaminase		10		133

of the cell during maturation. Diminution in the cell volume and membrane surface area also have been observed in the older cells (144). Thus, it is evident that during the maturation of reticulocyte, the size, as well as the complexity in subcellular structure of the cell, is reduced. The metabolic activity is somewhat suppressed and maintained at a lower level while the hemoglobin content increases almost to a saturated concentration. Such modifications in metabolic activity and structure in the mature red cell apparently is essential to its special functions in the circulation and possibly accounts for the prolongation of the life span of erythrocytes compared with that of other nucleated blood cells such as leucocytes, lymphocytes and others.

Changes during Aging of the Red Cells in vivo

The red cells present a convenient object for the study of the aging process in a tissue. They can be isolated in large quantities and fractionated into different age groups by various techniques. In addition, since the mature red cells are unable to synthesize new proteins, they are well suited for studies on the stability and activity of certain enzymes.

Gabrio and Finch (145) have claimed that there is limited similarity between the behaviour of red cells during aging *in vivo* and *in vitro*. However, some of the characteristic changes which occur in aged red cells in the circulation have also been observed in blood preserved *in vitro*. In order to understand how loss of cell viability occurs in preserved blood, it is helpful, therefore, to reveal the studies on the progressive changes observed in red cells during aging *in vivo*.

Löhr and Waller (101) studied the activity of blood cell enzymes during aging of red blood cells in the circulation. They observed a rapid exponential decrease in the activity of G-6-P dehydrogenase and G-3-P dehydrogenase. The half-life of the former enzyme was found to be 53 days, and of the latter, 34 days. A decrease of the energy-rich ATP along with a decline in the activity of the dehydrogenase was also observed. In their most recent reports (146) they emphasized the importance of the diminution in the hexokinase activity in that this enzyme appears to be the rate-limiting factor in the metabolic activity of aged cells, not withstanding the concurrent decrease in the activity of other enzymes including P-F-kinase, G-6-P dehydrogenase and 6-PGA dehydrogenase. In study of the kinetics of the hexokinase system of the red cell, Grignani et al. (56) observed that a decrease in the concentration of ATP in the red cells in the circulation from 9.1×10^{-4} to 5.1×10^{-4} moles in 90 days caused a reduction of 20 per cent in hexokinase activity. An additional decrease in 20 per cent in the activity was attributable to the inhibiting action of the increase in the concentration of extracellular ADP. Thus, after 90 days, the hexokinase activity in the circulating red cells had fallen to 40 per cent of the original.

The DPN content of red cell aging in vivo remains virtually unchanged during the first 50 days and decreases thereafter, being 80 per cent lower after 94 days. The cause of the fall is unknown but may be partly connected with a decreased production of R-5-P in the pentose shunt due to reduced G-6-PDH

activity. The methemoglobin content increases during aging of the red cell being increased 8 fold in 80 days. This is attributed to the diminution in the reducting activity owing to the deficiency of reduced DPN and TPN (147). Diminished activity of G-6-P DH, 6-PG DH, phosphohexose isomerase (132) and aldolase has been demonstrated in the oldest red cells separated by differential centrifugation or by the serial osmotic technique (138). The number of SH-groups and the concentration of lipids, cations and water also diminishes during the aging of red cells in vivo.

In studies with glucose-1-C^{14} Prankerd observed that the degree of glucose metabolism via the shunt is increased in the old cells. (148) He suggested that the large increase in the oxidation of TPNH by the increased Met-Hb in aged cells has probably overmasked the decrease of dehydrogenase activity. Concentrations of phosphate ester, glutathione and hemoglobin were found to remain remarkably constant throughout the life span of erythrocytes.

Recent investigations on the physical changes of red cells during aging in vivo (149) indicate that the cell membranes of aged erythrocytes are thinner, smoother and contain fewer concentric foldings than the membranes of young cells. The hemoglobin is also found to undergo changes during aging of the red blood cells (150).

It is believed that deterioration of energy metabolism of red cells leads to physical changes which favor sequestration and destruction of the cells in the reticulo-endothelial system, particularly in the liver and spleen. There is evidence in-

dicating that the decline in the metabolic activity of the red cells during aging is the condition which determines the life span of the cell (142). Marks et al. (132) observed that the rate of senescence is somewhat increased in red cells in which the hexosemonophosphate shunt is impaired as a result of a deficiency in the enzyme G-6-P dehydrogenase. Valentine et al. (151) also report that red cells defective in the glycolytic pathway, as by a deficiency of pyruvic kinase, shortens the life span of the erythrocytes considerably. Application of stress to such defective cells markedly accelerate the rate of cell damage and destruction. The mechanism by which defective or aged red cell is recognized, trapped and destroyed by the reticulo-endothelial cells is unknown. It is reasonable to assume that ultimately the red cell membrane must be altered since it is the surface of the cell that comes in contact with the erythroblastic cells.

Changes in the Erythrocyte during Storage in vitro

The chemical and physical changes which occur in preserved red blood cells during storage have been referred to as "storage lesions" (152). The main biochemical defect of stored blood is an intrinsic metabolic failure of the red cell since the alteration of extracorpuscular factors such as plasma, leucocyte, reticulocyte, hemolysate, citrate and gas phase were ineffective in accelerating or retarding the process of degeneration. The storage lesion, therefore, appears to be different from senescence of red cells in vivo (153). The findings from our group and

others on the metabolism of stored blood are consistent with the view that an intact functional apparatus is indispensable for the survival of erythrocytes preserved at low temperatures.

The changes that occur during storage are in greater or less degree reversed when the stored cell is reintroduced into the circulation. The restoration is accompanied by an increase in the ATP concentration and hence in the metabolic potential of the cells. The phenomenon of reconstitution appears to be an all-or-none process. If the storage damage has not reached a critical point, whereby the cells would be lost from circulation within 24 hours after transfusion, then the phosphate partition and other attendant systems of the remaining cells would return to normal within a few hours.

1. Preservation of Blood at $4^{o}C$.

In view of the danger of hemolysis at below zero temperatures and the advantage of reducing cell metabolism in refrigeration, most efforts were directed towards storage at low temperature without formation of ice, i.e. at 0^{o} - $5^{o}C$. Among the anticoagulates such as heparin, EDTA and citrate, the latter is most satisfactory for blood storage. Citrate ions cannot pass through the cell membrane and thus it increases the osmotic pressure of the extracellular solution; thus cell swelling is reduced.

Blood stored without substrate or preservative deteriorates rapidly. The commonly adopted preservative media include heparin, CD, ACD, and CPD. Dextrose serves as the substrate for glycolysis

of erythrocytes while the increased hydrogen ion concentration retards the break-down of ATP. The ACD and CD media, and especially the former, have been proved to be the most efficient for preservation of red cells during storage in the cold. Bowman (154) and Bishop (155) have shown that erythrocytes stored in CPD retained viability even better than those in ACD on storage for 28 days at 4°C. Gibson (156) believed that the initial damage to the erythrocytes occurred in blood collected in ACD due to the hypotonicity and acidity of the medium (pH 5). On the other hand, CPD provides phosphate ions but less citrate and hydrogen ions (pH 6). These factors probably account for the superiority of CPD to maintain the viability of stored blood. It should be mentioned that storage of blood in various preservative media merely retards, but does not prevent, the deterioration of red cells.

Denstedt (131) and London (157) have presented reviews on the biochemical changes that occur during blood storage. A summary of the important observations from the studies of stored blood in this laboratory and elsewhere is shown in Table II (158-165). The glycolytic capacity of erythrocytes diminishes during storage as evidenced by the inability of glucose metabolism to maintain the normal concentration of ATP and other metabolic intermediates in the cells. In addition, an increase in cell fragility and a disturbance in the electrolyte balance were also observed. The time when these changes occur during storage varies with the preservative medium and other conditions such as temperature etc. The progressive decrease in viability

TABLE II

Chemical Changes in Blood during Preservation at 4°C

I. Enzymes and Metabolites	Observed Changes	Reference
(1) Hexokinase system	Decrease in activity	43
(2) Phosphofructokinase	Sharp initial decrease in activity	158
(3) Fructose-1,6-diphosphate	Decrease due to decrease in (2)	159
(4) Triosephosphate DH	Decrease possibly due to denaturation	148
(5) DPN	Decrease due to decrease in (3) and (4)	
(6) 2,3-DPG, ATP, AMP ADP	Decrease Slight decrease	
(7) Inorganic phosphate	Steady increase due to breakdown of 2,3-DPG and other phosphate esters	89
(8) Lactic DH	Decrease activity due to lack of DPN (5)	89
(9) Pyruvic acid	Accumulates with decrease in 2,3-DPG (6) and change in (8)	89
(10) Transketolase	No change in activity	160
(11) Transaldolase	No change in activity	
(12) Change on addition of purine nucleoside	Increase in ATP and DPG synthesis and improvement in cell viability	52,53,60

TABLE II (continued)

I. Enzymes and Metabolites (cont'd.)	Observed Changes	Reference
(13) Amino acid uptake	Little change except decrease in rate of uptake of methionine	161
(14) Lipids	Phospholipids and cholesterol largely from cell membrane released; decrease of 1/2% in cell lipid in 2 weeks at 4°C.	4, 148
(15) Glutathione	Little change as long as glucose is present	88
(16) Water	Cell water and hence cell volume steadily increased owing to increase in osmotic pressure from accumulation of inorganic phosphorus, lactate and other substances in the cell	4, 144, 157
(17) Cations:		
Mg^{++}	No significant change	21
K^{+}	Cellular K^{+} falls steadily	4, 21
Na^{+}	Cellular Na^{+} increases	4, 21

TABLE II (continued)

II. Metabolic system	Change in Activity during Storage of Blood	Reference
(1) Glycolysis	Progressive diminution	4, 131, 153
(2) Pentose shunt	Enzymes remain stable but utilization of glucose and intermediates of the pathway, on addition of methylene blue, progressively decreased	77, 162

III. Cell components	Alteration during Storage	Reference
Membrane	No detectable damage observable with electron microscope, but membrane known to lose choline esterase and lipids, including phospholipid and cholesterol	131, 163, 164

of blood preserved in ACD at 4°C., i.e. under blood bank conditions, is slow in the first week of storage, but becomes faster after three or four weeks. The acceleration of aging during preservation might be attributable to the increased degenerative changes including the break-down of high energy phosphate compounds, or to the inhibition of processes tending to restore the endogenous level of these compounds. Further discussion of the degenerative changes and the possible causes of storage defects will be presented later. (See discussion.)

2. Storage of Red Blood Cells at Sub-zero Temperature

The research of blood storage in the frozen state has been carried out actively in the last ten years. In view of its significance and promising prospect to the preservation of blood, a review of the literature regarding the development in this aspect of research is included.

Plasma separated from red blood cells can be kept frozen at low temperatures for long periods without significant deterioration. The corpuscles, on the otherhand hemolyse when frozen to, or thawed from, temperatures below -3°C and were destroyed by desiccation. Hemolysis is believed to be induced by the rise in concentration produced during formation of ice.crystals which, in turn, may disrupt the lipoprotein cell membrane.

It was observed by Strumia (166) that little improvement in viability of erythrocyte occurs when blood is stored at -3°C instead of at +4°C. Inspired by the protective effect of glycerol in avian spermatozoa against damage during freezing and thawing processes, Smith (167) subsequently demonstrated

that in the presence of 15 per cent glycerol, citrated red cells could be frozen and thawed without lysis. Lovelock (168) found that the protective action of glycerol was shared with a number of other neutral solutes of low molecular weight including methanol, acetamide and glycerol monoacetate. These compounds, however, are inferior to glycerol as protective additives owing to their toxicity and the difficulties involved in the removal of these compounds. The principle action of glycerol and the neutral solutes is to prevent the excessive concentration of electrolytes and other substances that otherwise occurs on freezing. Studies with the aid of the microscope suggest that another effect of glycerol is to alter the mode of growth of the ice crystals. It is also believed that glycerol competes with water molecules in the formation of hydrogen bonds, consequently the ice formation in the cells is prevented or greatly diminished. Several other compounds with different degrees of effectiveness against freezing-thawing lysis of the red cells have been used as additives in the preservation of mammalian red cells in the frozen state. These include dextrose, lactose, sucrose, maltose, polyvinyl pyrrolidone (PVP) (169).

Before transfusion glycerol must be removed, otherwise, water will enter the red cells faster than the glycerol to equilibrate the high intracellular osmotic pressure and would then result in swelling and rupture of the cell. The deglycerization may be achieved by dialysis or centrifugal washing technique, but the procedure still presents a major problem

since these methods are time consuming especially when a large volume of blood is involved.

Further studies in the optimum concentration of glycerol and other constituents in human blood below zero temperatures were carried out by Chaplin and associates (171). Prolonged storage of glycerol-treated cells at -15°C or -20°C has proved unsatisfactory for preservation. However, encouraging results were obtained with blood stored at -79°C. The capacity of preserved cells to become reconditioned when placed in the circulation and their subsequent life span were not diminished on prolonged storage at this temperature up to two years (172).

Among the most important factors affecting cells stored in the frozen state are the salt and glycerol concentrations of the medium. The former maintains a suitable osmotic pressure of the external medium and minimizes the harmful effect of diffusion (173). The ratio of the volume of blood to that of the suspending fluid also can be increased, and the rate of cooling and rewarming thus reduced without causing hemolysis when the cells are frozen in high concentration of glycerol. Unfortunately, very high concentrations of glycerol may damage the red cells and also lead to a fall in ATP content. Jones *et al.* (172) also observed that red cells exposed to glycerol at concentrations higher than 5 *M* do not survive well after the deglycerolization and transfusion.

The object of storing cells in the frozen state is to minimize the biological changes which proceed at relatively rapid rates in specimens preserved at temperatures above zero.

For example, blood stored at 4°C in ACD solution utilizes about 0.4 mM of glucose per litre of red cells per day. At -20°C, in the absence of, or in the presence of, low concentrations of glycerol, the rate of utilization of glucose is reduced to 1/80th of the rate at 4°C. Also, at -20°C the ATP content falls more slowly, reaching half the normal level of fresh blood after one and a half years. Jones **et al.** (172) observed that in blood specimens preserved in citrated medium containing 30 per cent of glycerol and stored at -20°C and at -45°C, about 70 per cent of intracellular potassium was lost during the first 6 weeks of storage. Oxygenation of the specimen accelerates the rate of loss of the cation. The use of a buffer medium or of an antioxidant is found to abolish this undesirable effect. Hemolysis and the loss of potassium were reduced during storage at -45°C but the deterioration was still progressive. At -75°C, however, these effects were much reduced.

It is generally believed that even at low temperatures, where metabolism in the cell is virtually arrested, the loss of cell substance continues (notably the lipid of the cell membrane) though very slowly. (174). This type of deterioration, unlike the metabolic and chemical changes, is relatively unaffected by a lowering of the temperature. It follows that successful cold storage of blood for periods of one year or longer is likely to be possible only under conditions in which the rate of loss of ions or substances has been reduced to a minimum. Thus the structural integrity of the cell may be preserved. In practice, this can be achieved only at temperatures

lower than $-70^{o}C$.

The development of the methods in the preservation of blood at very low temperatures has provided hope for extending the life span of erythrocytes *in vitro*. At the present time these methods are too costly to be economical for routine use in hospitals. The cost would be justified, however, if it were ever necessary to "stockpile" blood in a national emergency.

EXPERIMENTAL

I. Collection and Preservation of Blood

Blood was collected aseptically by venipuncture from student donors into sterile bottles containing the preservative solutions. Occasionally, discarded blood was obtained through the courtesy of the Montreal Depot of the Canadian Red Cross Blood Transfusion Service.

The composition of preservative media and the proportion of blood to medium were as follows:

1. Citrate (C): In experiments in which isotonic trisodium citrate (3.2%) alone was used, the proportion of blood to citrate solution was 5:1 (V/V). The final pH of the specimen was about 7.5.

2. Citrate-dextrose (CD): The medium contained 1.8% (W/V) sodium citrate and 1.47% dextrose. The proportion of blood to CD was 4:1. The final pH of the specimen was about 7.5.

3. Acidified citrate-dextrose (ACD): The solution, as marketed by Abbott Laboratories for use in hospital blood banks, contained 1.32% trisodium citrate, 10.44% citrate and 1.32% dextrose. The pH of the medium alone was 5.6. The proportion of blood to medium was 4:1 and the final pH of the specimen was 7.2.

Bottles containing these preservative solutions were autoclaved before being used for collecting blood. In the case of CD, the constituent solutions were autoclaved separately to avoid caramelization.

II. Materials and Reagents

The chemicals and reagents used throughout this investigation were commercial preparations purchased from Nutritional Biochemical Corporation, Sigma Chemical Company and other sources.

Substrates, TPN, magnesium and nucleotide solutions were prepared in isotonic KCl and kept frozen until used. The ATP and TPN solutions were neutralized and stored for not longer than five days at -15°C.

Enzymes such as lipase, pepsin and G-6-P dehydrogenase were reconstituted with isotonic KCl and kept at 5°C.

III. Preparation of Blood Samples

1. Preparation of red cells:

A sample of blood was removed aseptically from the preserved blood specimen and centrifuged at 3000 r.p.m. for 20 minutes in a refrigerated centrifuge (4°C). The plasma and white cell layer were then siphoned off. An equal volume of cold isotonic KCl was added to the packed red cells and the suspension carefully mixed. This preparation is referred to as an "unwashed" sample of red cells.

"Washed" cell preparations were prepared by centrifuging the specimen referred to in the preceeding paragraph, removing the supernatant and resuspending the cells in cold isotonic KCl to bring the specimen to the original volume. The washing procedure was repeated four times.

2. Preparation of hemolysate

The washed erythrocytes, suspended in isotonic KCl, were lysed by freezing them in an ethanol-dry ice mixture (-78°C) and then thawing the specimen in cold water at 10°C. This procedure was repeated three times to ensure hemolysis of all the cells and taking care to maintain the same volume as the original.

3. Preparation of stroma-free hemolysate (SFH) and stroma

The hemolysed sample was centrifuged in an International high-speed refrigerated centrifuge, Model HR I, at 10,000 x g for 30 minutes at 4°C. The sparkling clear stroma-free top layer of hemolysate was removed carefully. This represents the SFH sample obtained after four washings.

The lower cloudy zone of the hemolysate along with the precipitate contains the stroma and membrane residue. The precipitate (membrane residue) was removed and washed and centrifuged at least six times with isotonic KCl. The material in the final precipitate was pale pink in colour.

4. Fractionation of red cells into various age groups by differential centrifugation

A sample of blood was removed aseptically from the stored specimen and centrifuged at 500 x g for 20 minutes. After the removal of plasma and the white cell layer, the packed red cells were suspended in cold isotonic KCl to make a 40% suspension. After standing for 10 minutes at 4°C, the suspension was centrifuged at 100 x g for 10 minutes and then at 2000 x g for 45 minutes.

Portions of the top, middle and the bottom 10% fractions of centrifuged cells were removed and the SFH prepared for assay. These three layers or fractions represent respectively the youngest, intermediate and the oldest cell group in the blood specimen.

IV. Chemical Analysis

1. Estimation of glucose

Glucose was estimated by the glucose oxidase ("Glucostat") method as described in the technical bulletin published by Worthington Biochemical Corporation.

2. Estimation of pyruvate

Pyruvate was determined by the method of Lu (175) as modified by Bueding and Wortis (176) and Nelson et al. (177).

3. Estimation of lactic acid

Lactic acid was determined by the method of Barker and Summerson (178) as modified by LePage (179).

4. Estimate of phosphate fractions

Inorganic phosphate (P_I) was determined by the Fiske and Subbarow method (180). Fractional analysis of organic phosphate esters was performed by the differential hydrolysis procedure as described by Pappius et al. (181).

The phosphate fractions are symbolized and calculated as follows:

P_I represents the concentration of inorganic phosphate initially present in the blood specimen.

P_L, the 'labile' phosphate, represents the phosphate esters that undergo hydrolysis in 2N HCl at 100°C in 7 minutes. During this interval, the two terminal phosphate groups of ATP and one of ADP and the phosphate on C_1 of hexoses and pentoses undergo hydrolysis. Thus the P_L represents the increase in the total inorganic phosphate at the end of the 7 minutes hydrolysis, that is

$$P_L = P_I \text{ at 7 min.} - \text{initial } P_I$$

P_S represents the 'stable' phosphate esters, (that which resisted hydrolysis on heating with 2N HCl at 100°C for 100 minutes). The stable phosphate esters consist mainly of 2,3-diphosphoglycerate (DPG).

The fraction P_S represents the inorganic phosphate concentration after hydrolysis with 70% perchloric acid for 10 minutes at boiling temperature minus inorganic phosphate present after 100 minutes hydrolysis.

The inorganic phosphate liberated between 7 and 100 minutes hydrolysis is derived from the phosphate attached to the terminal carbon of hexoses and pentoses.

5. Estimation of hemoglobin

A modification of King's method (182) as described by Brownstone (183) was used for hemoglobin determination.

V. Hexokinase Assay

1. Assay of hexokinase activity in intact red cells: Method A.

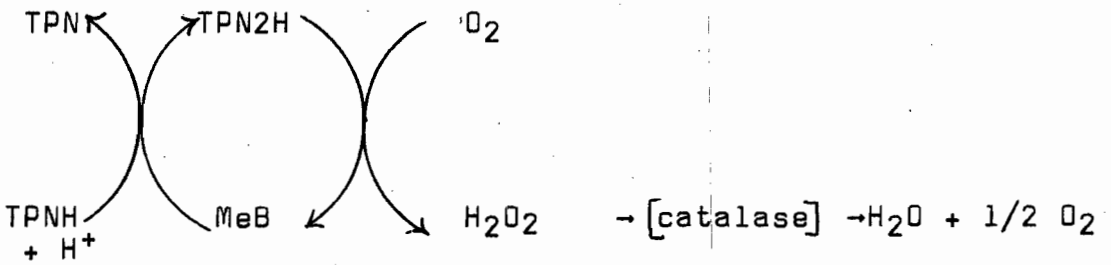
This method has been developed recently by Bishop (184). The assay involves the measurement of the oxygen uptake by intact red cells in the presence of methylene blue (MeB) under appropriate conditions.

Principle of Method A: The method is based on the fact that MeB stimulates oxygen uptake of red cells via the shunt pathway. The sequence of reaction is as follows:

i) $\text{Glucose} + \text{ATP} \xrightarrow[Mg^{++}]{\text{Hexokinase}} \text{G-6-P} + \text{ADP}$

ii) $\text{G-6-P} + \text{TPN}^{+} \xrightarrow[\text{dehydrogenase}]{\text{G-6-P}} \text{6-PGA} + \text{TPNH} + \text{H}^{+}$

iii) $\text{6-PGA} + \text{TPN}^{+} \xrightarrow[\text{dehydrogenase}]{\text{6-PGA}} \text{R}\Psi\text{-5-P} + \text{TPNH} + \text{H}^{+} + \text{CO}_2$



The reduced dye itself is oxidized by molecular oxygen. Thus the rate of oxygen uptake in intact red cells depends on the rate of formation of G-6-P which, in turn, is controlled by the hexokinase reaction.

Procedure: The reaction was carried out in a Warburg vessel. 2.0 ml of blood and 0.6 ml of Krebs-Ringer-phosphate buffer at pH 7.4 were placed in the main chamber of the vessel with 0.2 ml of 20% KOH in the centre well. In the side bulb were placed 0.2 ml of 0.05% MeB solution and 0.2 ml of either 3% glucose or 2.25% inosine solution. The reaction was carried out at 37°C.

The manometer reading was taken every 10 minutes and the level of the fluid in the manometer reset every half hour for a period of 3 hours. Hemoglobin was estimated at the end of the assay period. The hexokinase activity was expressed as ml of oxygen taken up per minute per 100 gm Hb.

2. Assay of hexokinase activity in SFH: Method B.

This method, with the SFH as the source of the enzyme, was first developed by Gabrio et al. (112) and modified by Hemphill (116). The reaction sequence is the same as given in Method A without MeB. The TPNH formed in the reaction was measured spectrometrically at 340 mμ. It was found that the first reaction catalysed by hexokinase is the slowest and the second reaction is the fastest of the three in the sequence. The rate of the hexokinase catalysed step is at least 1/10th that of the succeeding steps catalysed by G-6-P dehydrogenase and 6-PGA dehydrogenase under the same assay conditions. Hence, the rate of glucose utilization, as measured by the rate of TPNH production reflects the activity of hexokinase in the SFH.

The activity of G-6-P dehydrogenase and 6-PGA dehydrogenase similarly can be measured by using equivalent amounts of G-6-P or 6-PGA respectively, instead of glucose as the substrate.

Procedure: The composition of the assay medium was as follows:

Ingredient	Concentration of Stock Solution	Volume of stock reagent added	Final concentration of Ingredient
Glycyl-Glycine buffer(pH 7.80)	0.4	0.4	53.2
Phosphate buffer (pH 7.80)	0.20	0.20	13.3
ATP	0.075	0.40	10.0
TPN	0.003	0.30	0.34
KOH	0.154	0.93	-
KCl	0.154		
SFH*	-	0.12	-
$MgCl_2$	0.075	0.40	10.0
Glucose* or G-6-P* or 6-PGA*	0.029	o.25	2.4

*Solutions were prepared in isotonic KCl.

The pH of the assay medium was adjusted to 7.8 by addition of the appropriate amount of KOH, and the final volume was made up to 3 ml with isotonic KCl. The prepared SFH was first diluted with isotonic KCl to a concentration of about 30 mg Hb/ml. A final concentration of approximately 1.2 mg Hb/ml in the assay medium was therefore obtained. The ingredients were added with the exception of glucose to the quartz cuvettes in the order listed. The cuvettes were maintained at 30°C in a thermospacer and the optical density was measured at 340 mμ with a Beckman DU spectrophotometer. After establishing that no reaction takes place in the absence of substrate, 0.25 ml of glucose (or G-6-P

or 6-PGA) was added to the assay medium while an equal volume of isotonic KCl was added to the blank. Readings were taken at 10 minute intervals for at least 120 minutes. The system usually took about 20 minutes to equilibrate but a longer time was required for stored SFH. At the end of the assay period, the Hb concentration in the medium was determined and the final pH checked.

Calculation:

Taking the value of 6.22×10^6* as the molar extinction coefficient of TPNH at 340 mμ and the ratio

$$\frac{\text{TPNH production}}{\text{Glucose (G) or G-6-P utilization}} = 2,$$

$$\frac{\text{TPNH production}}{\text{6-PGA utilization}} = 1$$

the activity of the enzymes was calculated according to the equation:

Hexokinase activity =

$$\frac{\text{RATE OF CHANGE OF O.D.} \times \text{M.W. of G} \times 100}{6.22 \times 2 \times \text{Hb concentration (mg/ml)}} \text{ mg G/100mg Hb/hr.}$$

G-6-P dehydrogenase activity was calculated similarly by replacing the molecular weight of glucose with that of G-6-P in the equation.

6-PGA dehydrogenase activity was calculated by replacing the molecular weight of glucose with that of 6-PGA and omitting the factor 2 in the denominator.

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* Horecker, B.L. and Kornberg, A., J.Biol.Chem.175,385, 1948.

The rate of change of O.D. was determined graphically from the plot of O.D. against the duration (in minutes) of the assay.

In view of the non-specific action of MeB as electron carrier or as a stimulant both the shunt pathway and to glycolysis, the assay Method A has been subjected to much criticism. On the other hand the assay conditions in Method B can be precisely controlled and thus the effect of various substances on hexokinase activity can be tested without having to take their permeabilities into consideration. Furthermore, the addition of G-6-P or 6-PGA to the assay medium as substrate can serve to measure activities of other enzymes during the assay of hexokinase. Misleading conclusions on the hexokinase activity due to possible alterations in the activities of other enzymes can therefore be avoided. Consequently Method B was adopted for the assay of hexokinase activity in the greater part of this investigation.

EXPERIMENTAL RESULTS

A. The Stability of Hexokinase Activity in Red Cells during Storage

1. Hexokinase activity of intact erythrocyte during storage in ACD at 4°C.

In the studies of the hexokinase activity in the erythrocyte, conflicting conclusions have been reached by various investigators regarding the stability of the red cell enzymes during storage of blood at 4°C. (43) (82) (116). In view of the possibility that the contradictory results may be attributed to differences in the assay method and in the conditions in which the hexokinase activity was measured, experiments were undertaken to assay the enzyme activity both in the intact cells and the SFH.

In the following experiments the assay Method A as described on p.46 was used to measure the hexokinase activity in the intact cells. The assay involves the estimation of the rate of oxygen uptake by the red cells in the presence of methylene blue.

A sample of blood was collected in ACD and stored at 4°C. On the first and fifteenth day of storage, aliquots of blood removed aseptically and the hexokinase activity determined in duplicate. In all, 4 Warburg flasks were used to measure the oxygen uptake of the blood: one pair containing glucose and the other containing inosine. The purpose of using inosine as a substrate was to ensure that sufficient

amounts of G-6-P DH and TPN were present with the intact cells. The results of the experiment are represented in Figure 2. Evidently, with inosine as the substrate the oxygen uptake remained essentially constant in both blood samples. Also, in the 1 day blood using glucose as the substrate, the rate of oxygen uptake was comparable with that using inosine as the substrate. With the 15-day-old preserved blood, however, the rate of oxygen consumption decreased with glucose as the substrate while no decrease was observed when inosine was used. The result suggests that the glucose utilization of erythrocytes tends to decline in blood specimens after 15 days of storage. At the same time, the hexose monophosphate shunt, which utilizes the inosine, remains essentially intact. Accordingly, a second experiment was set up as follows.

A specimen of blood was stored in ACD medium at $4^{o}C$ for 40 days. At intervals during the storage period an aliquot of blood was removed and the rate of oxygen uptake determined. However, in addition to the estimation of hexokinase activity, the ATP content of the samples also was measured. In blood specimens stored for more than 15 days, additional samples were removed and treated with adenine and inosine to restore the ATP level of the red cells. Subsequently, the purines were removed by washing. Accordingly, three additional pairs of flasks were set up for these reconditioned blood samples: One pair had no substrate - a control ensuring the complete removal of the purines after the reconditioning

FIGURE 2

Oxygen Uptake of Fresh and Stored Blood in the Presence of Methylene Blue.

Legend

A. 1-day-old blood with inosine as substrate.

B. 15-day-old blood with inosine as substrate.

C. 1-day-old blood with glucose as substrate.

D. 15-day-old blood with glucose as substrate.

FIGURE 3

Change in ATP Concentration in Blood Preserved in ACD, CD and C Media at $4^{o}C$.

Legend

A. Blood preserved in isotonic citrate medium.

B. Blood preserved in isotonic citrate-dextrose.

C. Blood preserved in acidified citrate-dextrose.

FIGURE 2

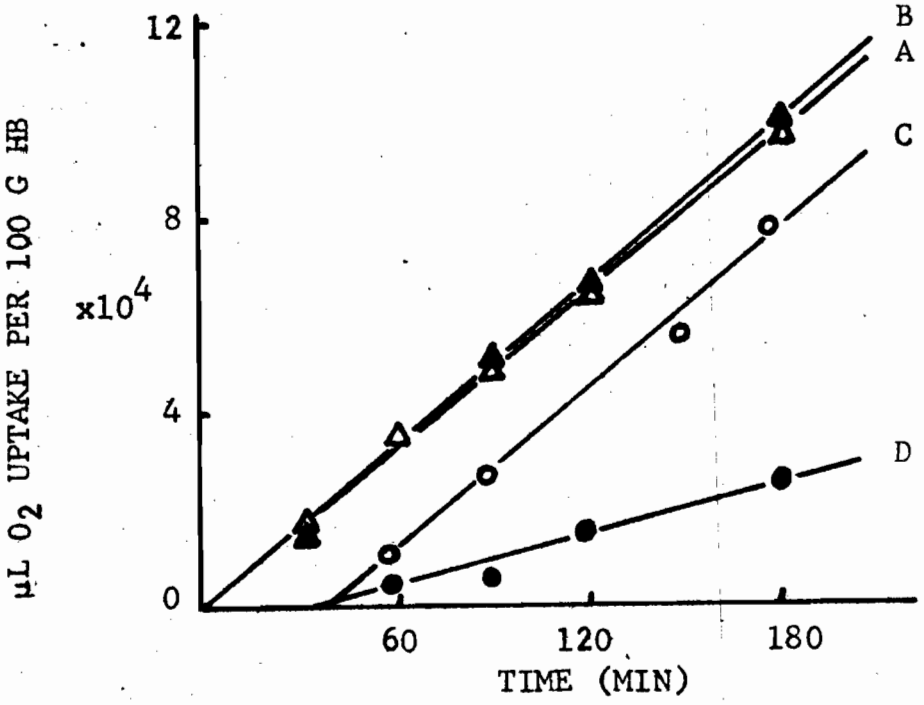
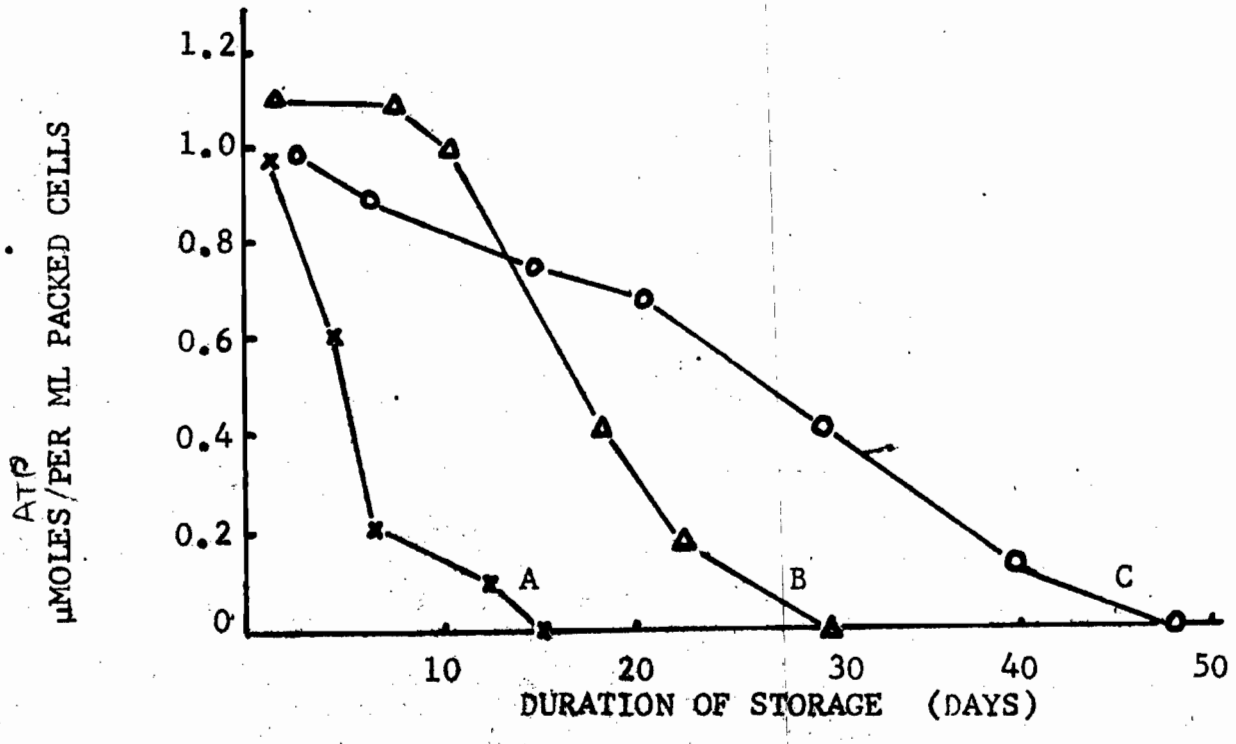


FIGURE 3



process. The other two pairs contained glucose or inosine as the substrate. The results are illustrated in Table III.

TABLE III

Hexokinase Activity of Intact Red Cells from Blood Specimens during Storage in ACD at 4°C and the Activity after Reconditioning.

Duration of Storage (days)	ATP Concentration		Hexokinase activity in presence of specified substrate.			
			Preserved blood		Preserved and reconditioned blood	
	μ mol/100g Hb	μ mol/ml packed cells	glucose	inosine	glucose	inosine
			(ml O_2 taken up/min./100g Hb.)			
2	312	1.06	550	548	-	-
5	250	0.82	502	535	-	-
15	220	0.75	458	546	543	540
28	86	0.25	195	530	501	531
40	30	0.10	trace	535	480	552

Conditions: Blood samples were washed three times with isotonic KCl before assay of Hexokinase activity. The figures represent the mean of duplicate test results. Reconditioned blood represents stored blood which had been washed and suspended in Krebs-Ringer phosphate buffer, pH 7.4, containing 40 mg inosine and 10 mg adenine per 10 ml buffer. The mixture is incubated at 37°C for 2 hours. The samples were then washed 3 times before assay.

Again, in specimens with inosine as the substrate, the rate of oxygen consumption was observed to be the same in the

fresh and in the stored specimens. The glucose utilization, however, was observed to decrease rapidly in stored blood, especially in blood samples stored for longer than 15 days. For instance, the oxygen uptake in the 28 day old blood was found to be only about 35% of that observed in fresh blood. A continuous diminution of ATP content in the stored blood also was evident from the results. In the reconditioned blood in which the ATP content is augmented, the glucose utilization in outdated blood was markedly increased compared to that of the untreated samples.

It was observed also in this experiment that there was a lag period in the oxygen uptake in the cases where glucose was used as the substrate. The lag period was found to be prolonged with increase in the duration of storage of blood. This observation may be explained, at least in part, by the increase in the length of time required for the reactivation and equilibration of the enzyme system in stored red cells.

The significance of ATP in maintaining the viability of preserved erythrocytes has been stressed by various investigators (74) (82) (85). The importance of ATP in maintaining the utilization of glucose in preserved red cells was evident from the aforementioned experiments. The abilities of various preservative media to maintain the ATP level of blood was compared as follows:

Blood samples preserved in ACD, CD and C media were stored at $4^{o}C$. The ATP concentration of the samples was then followed until the supply of nucleotide was depleted. The ACD sample was used also for determination of the various enzyme activities

presented on p. 60. It is evident from Figure 3 that the ATP level was best maintained in ACD, CD and C in this order of effectiveness. The result is consistent with the finding that ATP is relatively stable in blood in an acidic medium (63) (131). The result also shows that the ATP decreased progressively on storage and disappeared from the ACD, CD and C blood after 47, 30 and 15 days respectively.

2. Hexokinase activity of SFH prepared from blood stored in ACD or CD at $4^{o}C$.

It was evident from previously described experiments that the diminution of hexokinase activity in intact cells during storage can be accounted for, at least partially by the decrease in availability of ATP. However, no conclusion can be reached regarding the stability of the enzyme itself during blood preservation. For instance, if the hexokinase were to suffer slight denaturation or partial inactivation during storage, the results would have been similar. To clarify this point, it was necessary to estimate the actual enzyme activity in a complete assay medium. Such a system can conveniently be devised with SFH as the source of the enzyme.

Specimens of ACD and CD blood were stored at $4^{o}C$ for 50 days. Samples were withdrawn at intervals during this period. After removal of the plasma and the buffy coat layer the SFH was prepared from the washed and unwashed red cells from the specimen preserved in ACD. Similarly, SFH was prepared from washed cells from a specimen of blood preserved in the CD medium. The preparation of SFH; the washing of red

FIGURE 4

Hexokinase Activity of SFH from Blood Preserved ar $4^{o}C$.

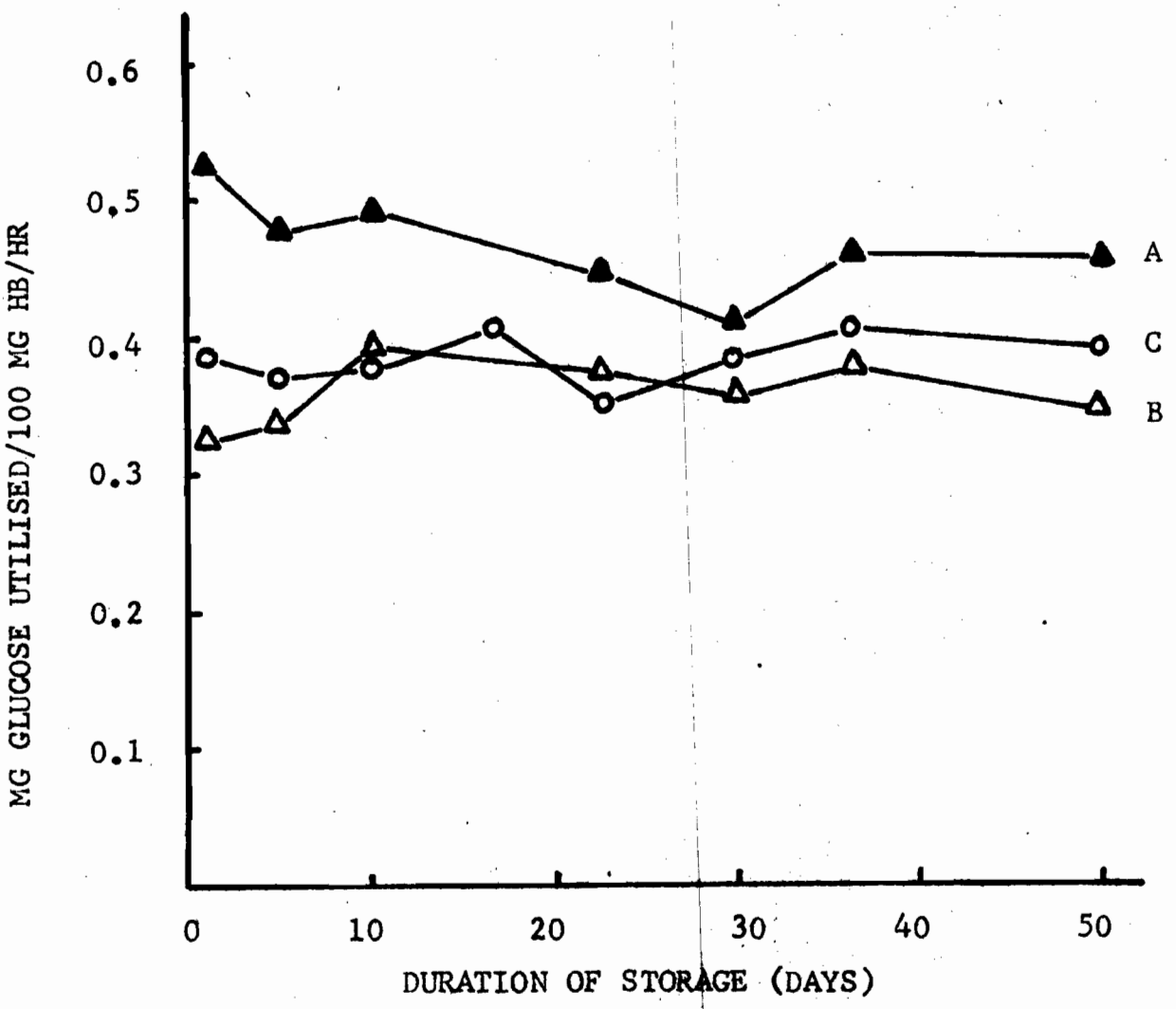
Legend

A. SFH from unwashed red cells stored in ACD.

B. SFH from washed red cells stored in ACD.

C. SFH from washed red cells stored in CD.

FIGURE 4


MG GLUCOSE UTILISED/100 MG HB/HR
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
DURATION OF STORAGE (DAYS)
A
C
B

cells and assay of the hexokinase activity of SFH were carried out as described on p. 43. The results are presented in Figure 4. Variation in the hexokinase activity was observed from day to day, ranging from about 0.3 to 0.4 units in the washed samples of ACD and CD blood. However, no significant difference in the activity of the enzyme was observed in either case during storage at 4°C for 50 days. The SFH prepared from washed cells of the ACD preserved blood displayed a similar pattern of activity, but the hexokinase activity was found to be considerably higher than that of the unwashed counterpart. The significance of the washing of the cells was investigated in more elaborate experiments and will be discussed later.

The observed fluctuation of hexokinase activity reflects the difficulty encountered in reproducing identical conditions for the assay of hexokinase activity. Experimental differences in the extent of washing red cells, errors in the preparation of given concentrations and delivery of the components constituting the assay medium, as well as in the determination of hemoglobin also may account for the fluctuation. Nevertheless, the observed hexokinase activity in SFH from ACD or CD blood during storage at 4°C was consistent with the results reported by Prevost (185) and Hemphill (116).

The same sample of ACD blood as was used to follow the ATP content of the red cells was used in the following experiment. SFH was prepared from washed cell aliquots of ACD blood at regular intervals during the storage period and the activities of the enzymes, hexokinase, G-6-P DH and 6-PGA DH were then

estimated using the method described on p. 48.

It is evident from Table IV that there was no significant decrease in the activity of these enzymes in the cells during storage of blood in ACD at 4°C for 50 days or longer. The activity of these enzymes was found to be respectively in the ratio 1:10:6.2.

TABLE IV

The Stability of Hexokinase, G-6-P Dehydrogenase and 6-PGA Dehydrogenase Activity during Storage of Blood in ACD at 4°C.

PRESERVATION	ENZYME ACTIVITY		
Duration of Storage	Hexokinase	G-6-P DH	6-PGA DH
(days)	(mg substrate utilized/100mg Hb/hr.)		
2	0.37	5.7	3.5
15	0.36	5.9	3.8
26	0.38	5.6	3.5
38	0.34	5.3	3.9
50	0.35	5.0	3.6

For the sake of comparison the enzyme activity and the ATP content were expressed as the percentage of the initial values in fresh ACD blood in Figure 5. The capacity of the ACD preserved cells to survive after transfusion as reported by Mollison and Robinson (85) also is included in the group. It is evident that the reduction in viability of blood during storage is related to ATP level of the red cells and that the primary defect cannot be attributed to the incompetence of the enzymes hexokinase, G-6-P DH or 6-PGA DH.

FIGURE 5

Activity of Hexokinase, Glucose-6-phosphate Dehydrogenase and 6-Phosphogluconic Acid Dehydrogenase in Erythrocytes Preserved in ACD Medium at $4^{o}C$.

Legend

A. 6-PGA DH activity of SFH*

B. Hexokinase activity of SFH*

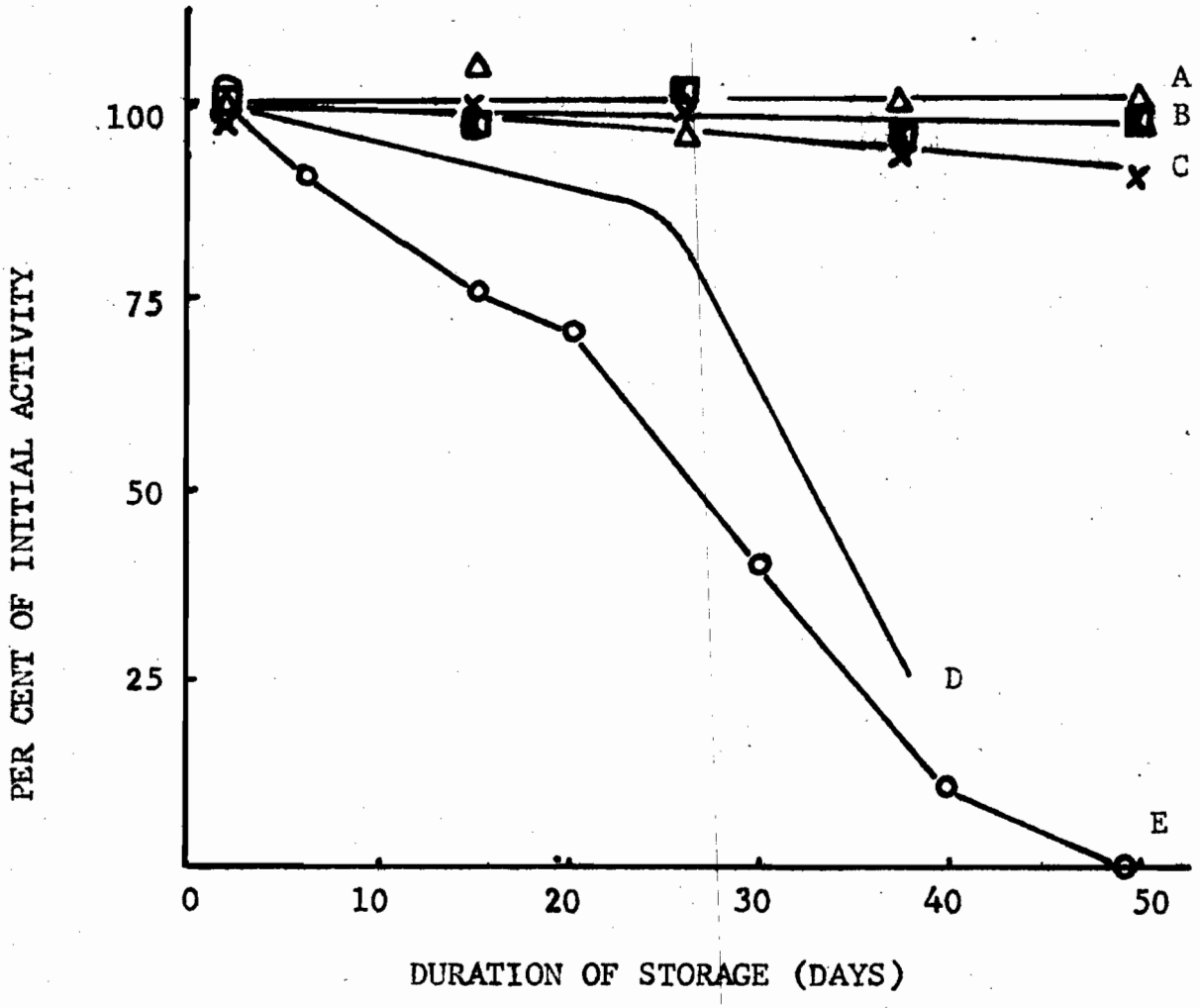
C. G-6-P DH activity of SFH*

*Values are expressed as per cent of initial activity.

D. Viability of red cells as measured by the per cent of post-transfusion survival. [After Mollinson et al.(86)]

E. ATP concentration of whole blood.

FIGURE 5


PER CENT OF INITIAL ACTIVITY
100
75
50
25
0
10
20
30
40
50
DURATION OF STORAGE (DAYS)
A
B
C
D
E

B. Changes in the Glycolytic Activity of Erythrocytes during Incubation at 30°C and during Storage at 5°C.

1. Hexokinase activity and the rate of glycolysis of erythrocytes during incubation.

The relationship between hexokinase activity in relatively younger and older red cell fractions and their glycolytic activity during incubation was investigated in the following experiments.

Erythrocytes from fresh blood preserved in ACD, was fractionated into three age groups by differential centrifugation (P.43). Since the validity of this method has been established by isotopic techniques (186) (187), We can designate the top, middle and bottom layers of the sedimented red cells respectively as containing the youngest, the intermediate and the oldest red cells in the specimen. The three cell fractions were removed separately and each was resuspended in isotonic Krebs-Ringer phosphate buffer with added glucose (final concentration 500 mg%). The three cell suspensions, in 25 ml conical flasks, were then incubated at 30°C with a gentle agitation in a mechanical device. A sample from each specimen was removed at the start, and at the end of the first and third hour during the incubation. The hexokinase activity (method B) and the glucose, lactate, hemoglobin concentration and the pH were determined on all the samples. The rate of glycolysis was measured by the amount of glucose utilized and of lactic acid formed during the interval between

the first to third hour of the incubation period. The estimations were made again at the end of 24 hours of incubation. The results are presented in Table V. It is apparent that the glucose utilization and lactate formation were greatest in the top layer specimen and least in the lowest layer and after incubation. This indicates a greater rate of glycolysis in the younger red cells than in the older ones. There is a relative decrease in the rate of glycolysis as well as in the percentage of conversion from glucose to lactate in all three specimens at the termination of incubation. The result shows also that the top fraction appeared to be less efficient in the quantitative conversion of glucose to lactate compared to the bottom cell fraction. This is true both before and after the incubation period. The seemingly unexpected observation may be perhaps explained by the increased proportion of glucose oxidized via the pentose shunt or the tendency for various glycolytic intermediates to accumulate in the youngest cell fraction.

In spite of the observed diminution in glycolysis, the hexokinase and G-6-P DH activity was found to remain constant in all the fractions. These findings confirm that the glycolytic activity tends to decrease progressively with aging of the red cells and that instability of hexokinase is not responsible for the decline of the glycolytic activity during incubation of red cells <u>in vitro</u>. Since identical changes are found during incubation of red cells <u>in vitro</u> and in stored blood (2) (138), we can reasonably conclude

TABLE V

The Glycolytic and Hexokinase Activity of Fractionated Red Cells during Incubation *in vitro* at 30°C.

Duration of incubation	Glycolytic Activity	Top layer (youngest)	Middle layer (intermediate)	Bottom layer (oldest)
hour				
0	Glucose utilization (μ mole/g Hb/hr)	6.10	4.30	4.00
	Lactic acid production (μ mole/g Hb/hr)	9.50	7.40	7.30
	Conversion %	78	86	92
	Hexokinase activity (mg glucose utilized/100mg Hb/hr)	0.40	0.32	0.71
	G-6-P DH activity (mg glucose utilized/100 mg Hb/hr)	5.40	5.00	5.30
24	Glucose utilization (μ mole/g Hb/hr)	4.0	3.8	3.2
	Lactic acid production (μ mole/g Hb/hr)	5.6	5.9	5.5
	Conversion %	69	78	86
	Hexokinase activity (mg glucose utilized/100 mg Hb/hr)	0.39	0.32	0.30
	G-6-P DH activity (mg G-6-P utilized/100 mg Hb/hr)	5.00	4.98	4.80

that instability of hexokinase is not primarily responsible for the deterioration of blood during storage.

In another experiment, an attempt was made to investigate the influence of added glucose and glutathione on the maintenance of the hexokinase activity in red blood cells during incubation at 30°C for 24 hours. A sample of 18-day-old blood in ACD medium was centrifuged and washed four times. The erythrocytes were suspended in Krebs-Ringer buffer in four 25 ml conical flasks. Flask I with no added reagent served as the control. Glucose and glutathione were added to the buffer solution in flasks II and III respectively and both substances were added to flask IV. The suspensions were then incubated at 30°C and the enzyme activities estimated under the conditions described in the previously mentioned experiment. The results presented in Table VI indicate that there was a marked decrease in the hexokinase activity in the control flask which contained no glucose. On the other hand, the presence of glucose or glutathione in the suspension medium appeared to have a protective influence in maintaining the activity of both hexokinase and G-6-P DH. With both reagents present in the incubated suspensions, the activity of the enzymes was well preserved for longer than 21 hours. The results obtained here were consistent with the previously made observation that the hexokinase activity is well preserved during incubation at 30°C for 24 hours in the presence of glucose. It was concluded, therefore, that the integrity of the enzymes may depend upon the energy production of the cell, which in turn, requires the presence of the substrate

TABLE VI

Influence of Added Glucose and Glutathione in the Activity of Hexokinase of Erythrocyte during Incubation.

Duration of incubation	REAGENTS ADDED							
	FLASK I Control		FLASK II Glucose		FLASK III Gluththione		FLASK IV Glucose + glutathione	
(hour)	HK.	G-6-P DH	HK.	G-6-P DH	HK.	G-6-P DH	HK.	G-6-P DH
	(mg substrate utilized per 100 mg Hb per hour.)							
0	0.36	7.0	0.41	6.8	0.36	6.7	0.40	-
9	0.34	6.0	0.38	-	0.34	6.5	0.41	6.8
21	0.28	6.3	0.40	8.7	0.33	6.0	0.43	6.5

Conditions:

(1) Washed red cells from 18-day-old blood in ACD.

(2) Glucose added to give final concentration of 6 mg per ml; Glutathione 5 mg per ml.

glucose. It is interesting to observe that the addition of GSH to the suspension medium also is beneficial in maintaining the activity of the enzymes. A study of the function of glutathione in red blood cells is being carried on in our laboratory. It is likely that the protective effect of glutathione is attributable to its protective action on the SH groups of the enzymes.

2. Changes in the concentration of metabolites and the partition of metabolites between the red cells and plasma during storage.

Three specimens of blood were collected respectively in C, CD and ACD medium and stored at 4°C for 50 days. At intervals during the period the cells were carefully resuspended and were withdrawn for the estimation of glucose, lactate and pyruvate.

The concentration of glucose in the red cells preserved in ACD and CD and the partition of glucose between cells and plasma during storage are indicated in Table VII. The intracellular glucose concentration was found to be slightly lower than that in the plasma throughout the storage period. However, if correction is made for the cell water which amounts to about 65% of the cell volume, the distribution of this substrate is approximately equal in the cells and in the medium. The glucose utilization was somewhat depressed in the ACD specimen compared to that in the CD (neutral) specimen. There was a significant reduction in glucose utilization after about 25 days of storage in both cases.

TABLE VII

Glucose Concentration during Storage in ACD and CD Media.

Duration of Storage	ACD Medium			CD Medium		
	Red Cells	Whole Blood	Plasma	Red Cells	Whole Blood	Plasma
(days)			(μ moles/ml blood)			
0	20.2	20.1	21.9	11.1	13.0	13.3
3	20.1	21.9	23.8	-	-	-
5	18.2	18.3	20.6	10.2	9.8	11.7
10	15.7	16.1	17.0	8.0	8.7	9.5
17	-	14.7	15.2	3.9	-	4.2
25	13.0	13.2	14.4	3.7	3.6	4.1
30	12.1	12.0	14.0	2.0	2.5	3.3
50	10.1	10.9	11.0	2.3	2.1	2.9

In Figure 6 it is evident that the lactic acid accumulated rapidly during the first two weeks of storage, and that concurrently with the decline in the rate of utilization of glucose, the rate of lactate production slowed down after the third week. The amount of the intracellular water accounts for the higher concentration of lactate in the plasma than in the red cells. The even distribution of this end product of glycolysis between the cells and the plasma is in accordance with the view that lactate can move freely across the cell membrane by simple diffusion.

FIGURE 6

Changes in the Concentration of Lactic Acid and its Distribution between Cells and Plasma during Storage of Blood in Acidified Citrate Dextrose at 4°C.

Legend

A. Whole blood.

B. Red cells.

C. Plasma

FIGURE 7

Changes in the Concentration of Pyruvic Acid in Blood Preserved in Various Media at 4°C.

Legend

A. In isotonic citrate.

B. In citrate-dextrose.

C. In acidified citrate-dextrose.

FIGURE 6

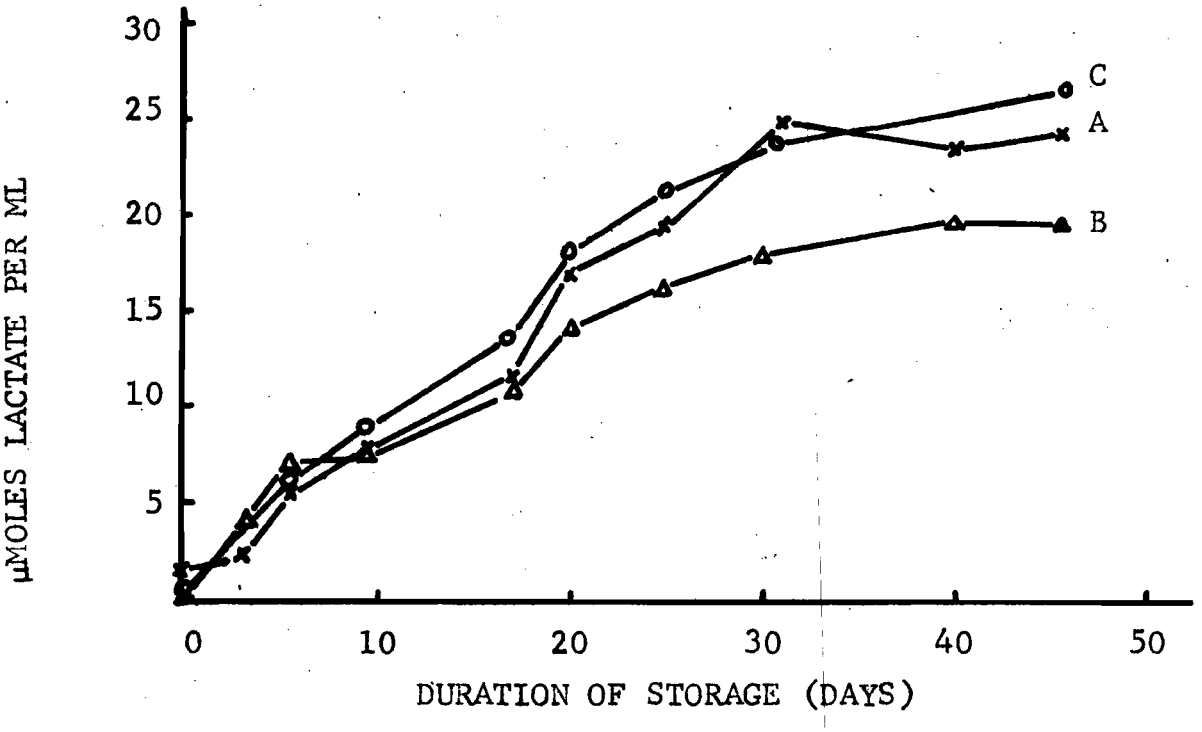


FIGURE 7

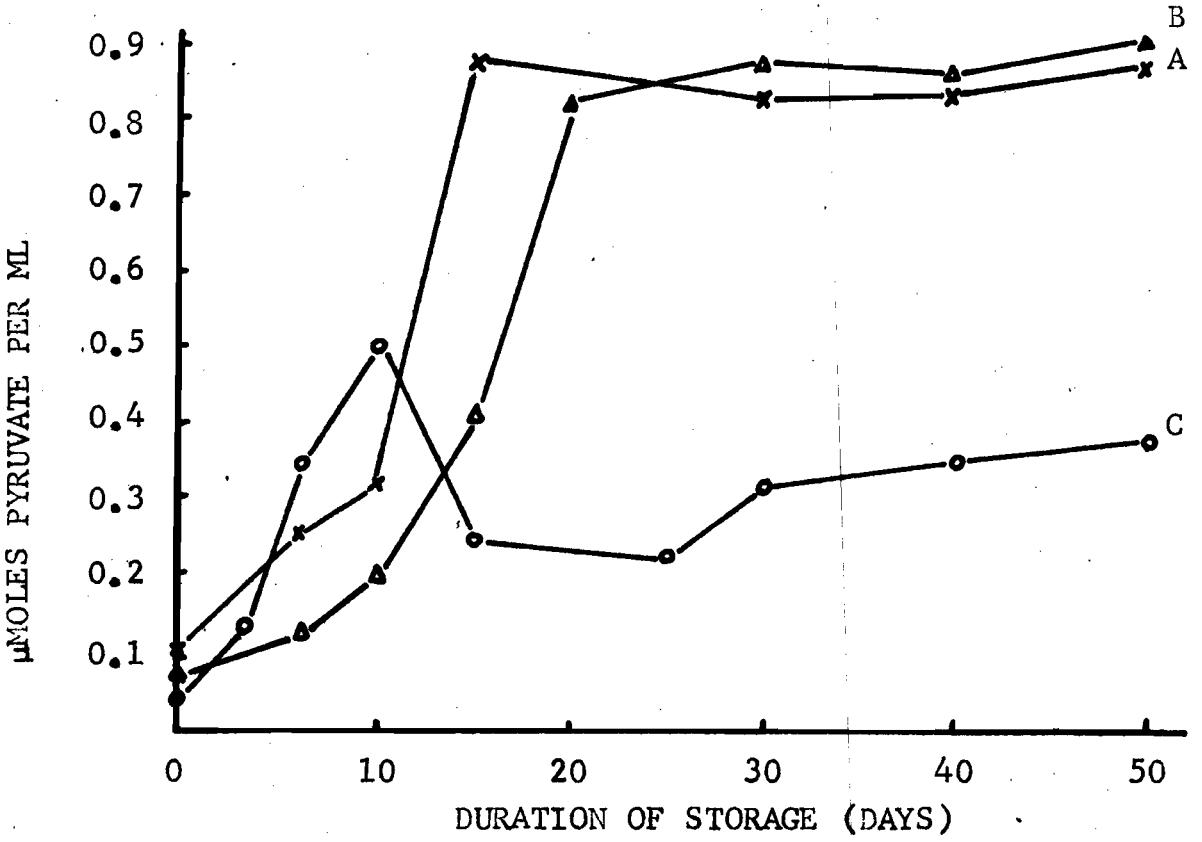


Figure 7 shows the change in concentration of pyruvic acid in the erythrocytes stored in ACD, CD and C media at 4°C. The behaviour of this metabolite was found to be as described by Pappius (89) and Andreae (181) and many other workers.

The pyruvic acid accumulation in the ACD blood increased steadily and rapidly after the third day of storage reaching a concentration of 0.5 μ moles per ml by the 10th day. Thereafter the concentration decreased slightly and levelled off. This observation is in agreement with Prevost's finding that the pyruvate produced in the cells during storage in ACD during the first 2 weeks passes rapidly into the external medium (185). In the C specimen (in citrate alone) the pyruvic acid was found to increase abruptly after about 10 days of storage presumably due to the depletion of glucose. The acidity of the ACD sample activates the 2,3-DPGase which causes breakdown of 2,3-DPG and the accumulation of pyruvate compared to that with the neutral preservative media CD or C (89). The accumulation of pyruvate therefore reflects the increased breakdown of this phosphate intermediate.

In another experiment analysis of the various phosphate fractions was made periodically on the blood samples preserved in ACD and CD media at 4°C for 50 days. The results are illustrated in Table VIII. The partition of the inorganic and the 'stable' phosphate was studied in another set of blood specimens preserved in ACD, CD and C media (Figure 8). The following observations were made:-

FIGURE 8

Behaviour of Phosphate Fractions in Blood Specimens Preserved in Various Media at $4^{o}C$.

(a) Changes in stable phosphate fraction in blood preserved in citrate, citrate-dextrose, and acidified citrate-dextrose media at $4^{o}C$.

Legend

A. In isotonic citrate.

B. In citrate-dextrose.

C. In acidified citrate-dextrose.

(b) Distribution of inorganic phosphate between cells and plasma in blood preserved in acidified citrate-dextrose medium at $4^{o}C$.

Legend

A. In the red cells.

B. In whole blood.

C. In plasma.

(c) Changes in the concentration of inorganic phosphate fraction and its distribution between cells and plasma in blood preserved in citrate-dextrose at $4^{o}C$.

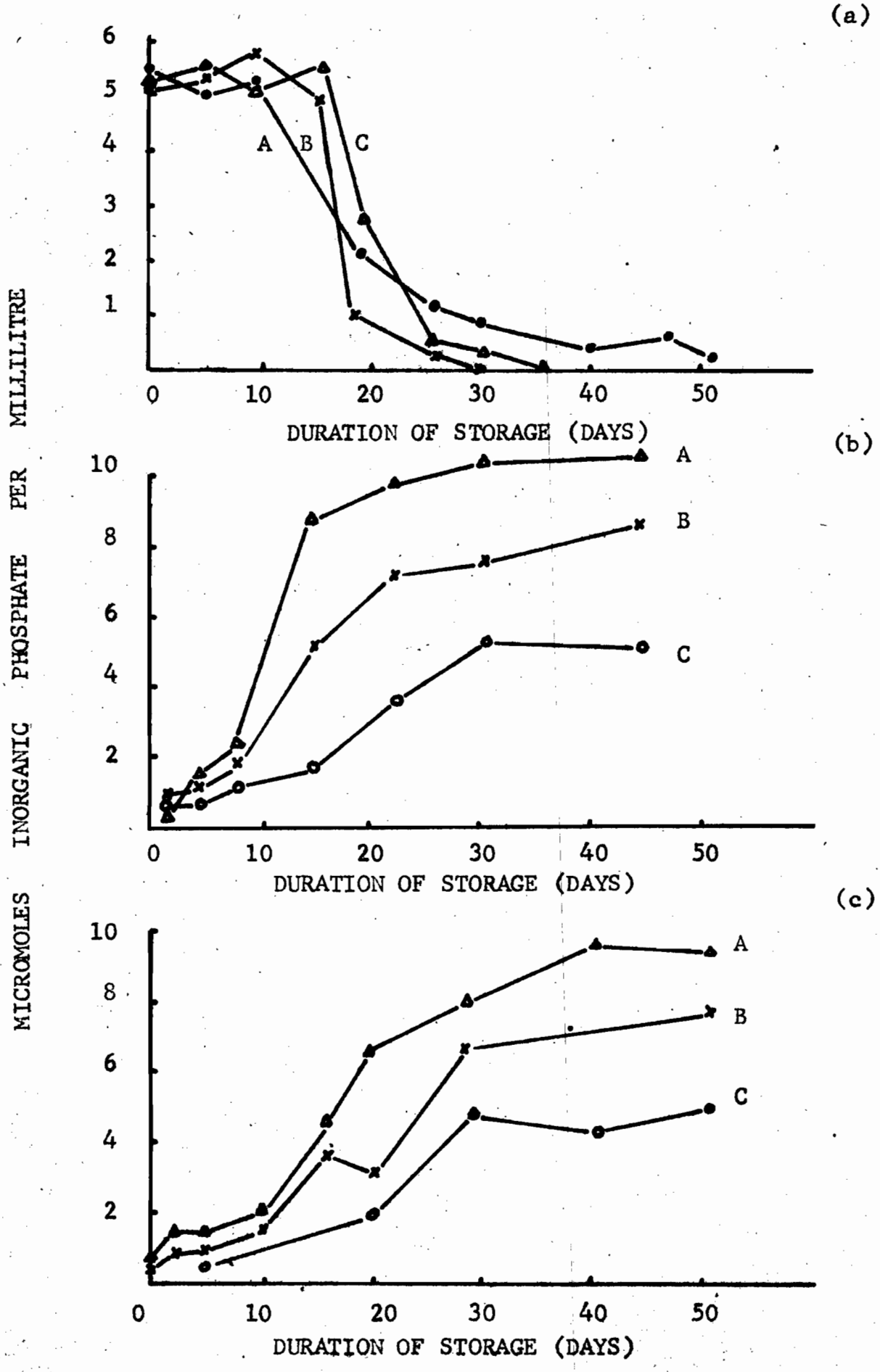
Legend

A. In the red cells.

B. In whole blood.

C. In plasma.

FIGURE 8


(a)
MILLILITRE
6
5
4
3
2
1
A
B
C
0
10
20
30
40
50
DURATION OF STORAGE (DAYS)
(b)
INORGANIC PHOSPHATE PER
10
8
6
4
2
A
B
C
0
10
20
30
40
50
DURATION OF STORAGE (DAYS)
(c)
MICROMOLES
10
8
6
4
2
A
B
C
0
10
20
30
40
50
DURATION OF STORAGE (DAYS)

The changes in ATP level, represented by the labile phosphate (P_L) follows a pattern similar to that previously described (p. 56). The fall in ATP was slower than that of 2,3-DPG at first, but proceeded more rapidly after the latter was depleted. The stable phosphate (representing mainly the 2,3-DPG and minute amounts of hexose-6-phosphate and adenylic acid) fell steadily during storage in both the ACD and the CD specimens (Figure 8a). Hydrolysis of 2,3-DPG proceeded more rapidly in the ACD than in the CD specimen.

The inorganic phosphate increased slowly during the first week of storage in ACD (Table VIII, Figure 8b). Thereafter, the increase was more rapid. At the end of the third week, the inorganic phosphate concentration was about five times that in the original blood specimen. The results from Table VIII and Figure 8c also indicated that the changes in P_I level in CD specimen was similar to that of the ACD specimen but occurred considerably later. The inorganic phosphate concentration reflects tha rate of break-down of 2,3-DPG. The fall in the latter compound and the concomitant rise in inorganic phosphate reached completion in about 3 weeks. The concentration of inorganic phosphate was about twice that in the plasma in the ACD and the CD specimens. The accumulation of inorganic phosphate within the cells indicated that the membrane is impermeable to the phosphoric anion. Hence the ion does not escape from the cell by simple diffusion.

TABLE VIII

Changes in the Concentration of Phosphate Fractions during Storage of Blood in ACD and CD Media at 5°C.

Duration of Storage	ACD Medium				CD Medium			
	P_I	P_L	P_S	2,3-DPG	P_I	P_L	P_S	2,3-DPG
(days)	(μ moles/ml whole blood)				(μ moles/ml whole blood)			
0	0.8	1.1	5.2	2.6	0.6	1.0	5.0	2.5
3	0.5	0.9	4.8	2.4	0.8	0.9	5.1	2.5
5	0.9	0.9	4.1	2.0	0.9	0.8	4.8	2.4
8	2.2	0.8	1.5	0.8	1.5	0.8	4.6	2.3
13	5.3	0.7	0.8	0.4	2.6	0.7	4.0	2.0
15	5.7	0.6	0.6	0.3	-	-	2.5	2.3
18	5.4	0.6	0.6	0.3	4.3	0.4	2.1	1.1
22	5.6	0.5	0.5	0.3	5.0	0.2	1.8	0.9
28	5.8	0.3	0.3	0.2	5.7	0.1	1.0	0.5
35	-	-	0.3	0.2	5.8	0.1	0.3	0.2
40	5.8	0.1	0.2	0.1	5.8	0	0.2	0.1
50	5.7	0	0.2	0.1	5.9	0	0.2	0.1

P_I = Inorganic phosphate P_L = Labile phosphate, e.g. ATP. P_S = Stable phosphate.

The H^+ ion concentration during storage of blood at 4^oC was followed in specimens preserved in ACD and CD. The hexokinase activity, and the concentration of glucose, lactate and hemoglobin, were followed also in ACD specimens. The results are indicated in Table IX. It was observed that the pH of the ACD specimen fell from 7.2 to about 6.6 in 30 days while that of the CD specimen fell from 7.4 to about 6.9 during the same period. The pH of the C specimen decreased from 7.5 to about 7.2 in 16 days and thereafter remained constant.

The extent of hemolysis in the ACD specimen was measured by determining the concentration of hemoglobin in the supernatant of the lightly centrifuged samples. The results in Table IX indicated that hemolysis proceeded very slowly during the first two weeks of storage. The results are presented also in Figure 9 in which the change in glucose and lactate as well as in the hexokinase activity of the ACD blood also are included. It is apparent that the diminution in the glucose utilization and accumulation of lactate, and the significant increase in the degree of hemolysis occurred about the 25th day of storage. Presumably the decline in glycolytic activity and fall in the concentration of the various metabolic intermediates are responsible for the progressive failure in energy production which is essential for the maintenance in the structural and metabolic integrity of the erythrocyte. Hemolysis of the cell subsequently occurs. However, it is noteworthy that the hexokinase activity of the SFH prepared at various intervals during the storage period remained stable in spite of the changes described.

TABLE IX

Hydrogen Ion Concentration of Blood during Storage in ACD, CD and C Media at 5°C.

Duration of Storage	Hb.	ACD	CD	C
(days)	(µg/ml plasma)	pH	pH	pH
0	0	7.20	7.43	7.55
3	5	7.05	7.32	7.40
7	6	6.89	7.19	7.29
10	8	6.85	7.14	7.21
12	7	6.82	7.13	7.21
14	9	6.83	7.08	7.20
16	-	6.75	7.03	7.18
20	9	6.80	7.01	7.16
22	-	6.79	6.98	7.21
27	19	6.76	6.92	7.12
33	28	6.61	6.86	-
40	-	6.60	6.75	7.11
50	62	6.50	6.76	7.12

FIGURE 9

Chemical Changes in ACD Blood during Storage at $4^{o}C$.

Legend

A. Hexokinase activity of SFH.

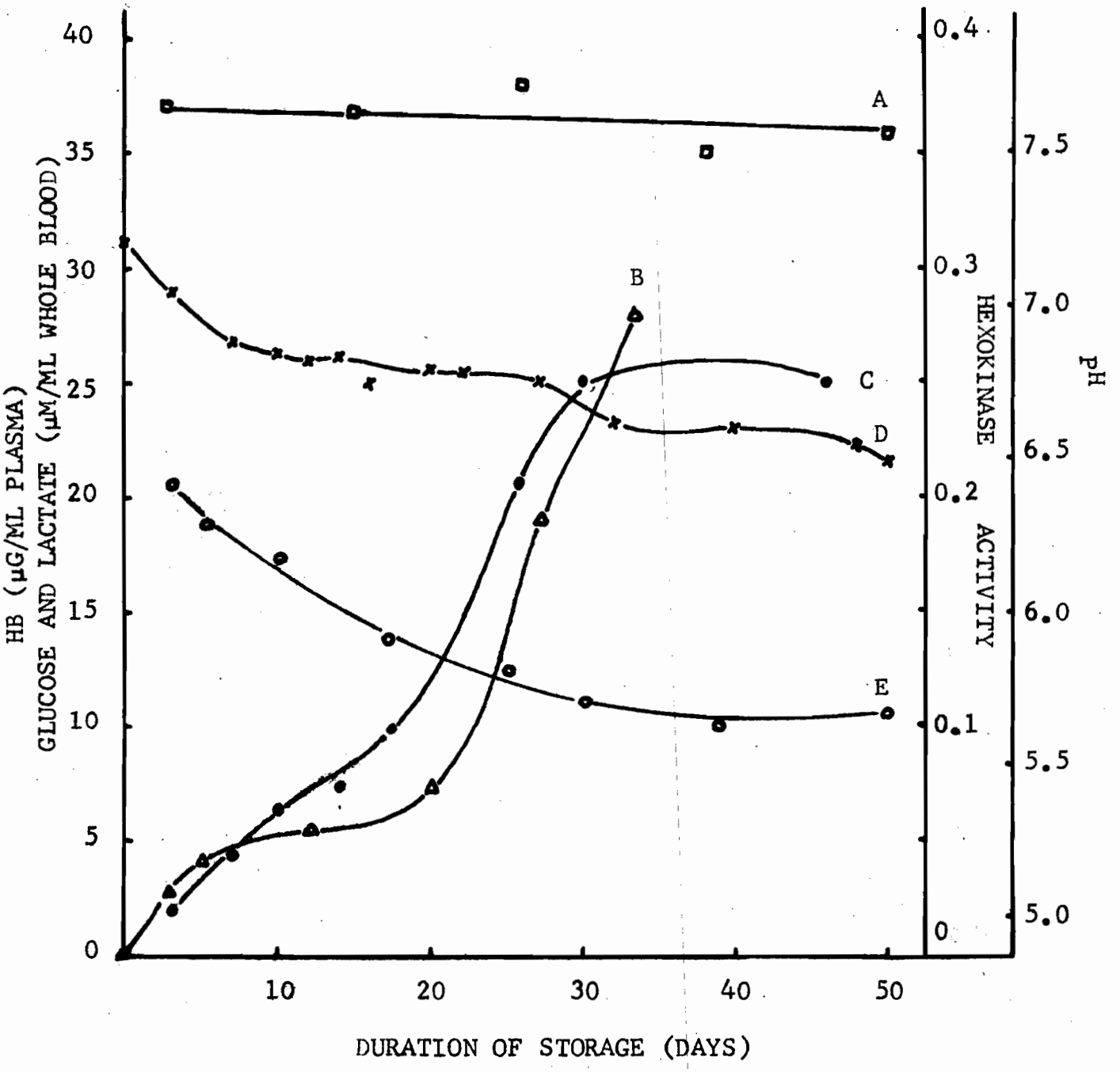
B. Hemoglobin in plasma.

C. Lactic acid concentrations of whole blood.

D. pH of whole blood.

E. Glucose concentration of whole blood.

FIGURE 9


HB (µG/ML PLASMA)
GLUCOSE AND LACTATE (µM/ML WHOLE BLOOD)
HEXOKINASE ACTIVITY
pH
DURATION OF STORAGE (DAYS)
A
B
C
D
E
0
5
10
15
20
25
30
35
40
0
0.1
0.2
0.3
0.4
5.0
5.5
6.0
6.5
7.0
7.5
10
20
30
40
50

C. Behaviour of Hexokinase in Preserved Red Blood Cells.

1. The effect of hydrogen ion concentration on hexokinase activity.

The influence of the hydrogen ion concentration on the hexokinase activity is indicated in Figure 10. The greatest activity of the enzyme lies between pH 7.2 and 8.2 being optimum at about 7.8. Within this range of pH, the variation in the hexokinase activity is less than 5%. The activity of the enzyme decreases rapidly on the acid side and approaches zero at pH 5.5. The assay mixture becomes turbid at hydrogen ion concentrations lower than pH 5. No significant change in the behaviour of hexokinase with change in hydrogen ion concentration was observed even in the out-dated blood specimens. The optimum pH corresponds closely to that of glycolysis as reported by Rapoport et al. (98) and Gabrio et al. (55). This affords evidence for the finding that the hexokinase reaction is the rate-limiting step of glycolysis.

2. The influence of adenine nucleotides on the activity of hexokinase.

It is well known that hexokinase requires ATP as a specific cofactor. The following experiment was designed to test the influence of other adenine nucleotides on the hexokinase activity. It is evident from Table X that no phosphoration of glucose occurred without the addition of

FIGURE 10

Influence of Hydrogen Ion Concentration on Hexokinase Activity of Erythrocyte.

Legend

A. SFH prepared from 28-day-old blood preserved in ACD medium at $4^{o}C$.

B. SFH prepared from fresh ACD blood.

Conditions: Hexokinase assayed by Method B.

FIGURE 10

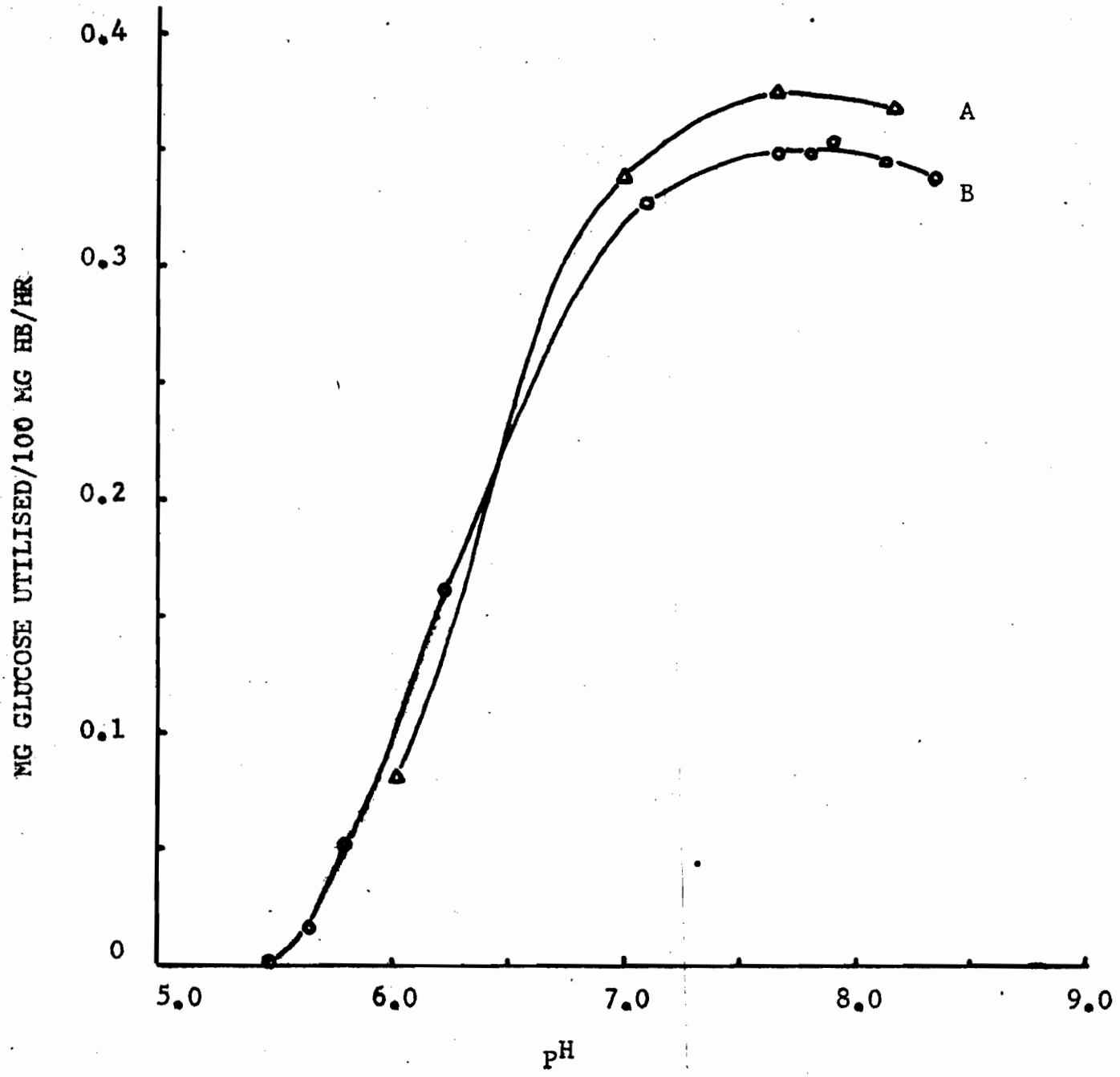


TABLE X

The Influence of Adenine Nucleotides on Hexokinase Activity.

A.

Nucleotides	Hexokinase Activity
(10mM)	(mg glucose/100 mg Hb/hr.)
Control	0
ATP	0.42
ADP	0.12
AMP	0

B.

Nucleotide Concentration (mM.)		Hexokinase Activity	Inhibition
ATP	ADP	(mg glucose/ 100mg Hb/hr.)	per cent
10	-	0.36	-
10	1	0.37	0
10	5	0.32	12.4
10	10	0.23	38.0

C.

Nucleotide Concentration (mM.)		Hexokinase Activity	Inhibition per cent
ATP	AMP	(mg glucose/100 mg Hb/hr.)	
10	-	0.42	-
10	1	0.33	8.7
10	5	0.22	26.0
10	10	0.12	72.0

adenine nucleotides. The result is as expected since the endogenous ATP in the erythrocytes undergoes hydrolysis rapidly during hemolysis of the red cells. It is interesting to note that addition of ADP alone to the assay system brought about the phosphorylation process although the hexokinase activity estimated was only about 25% of that measured with the ATP-fortified system. On the other hand, addition of an equivalent amount of AMP was not effective. The phenomenon can be explained in terms of Kashket's findings that adenylate kinase which catalyses the conversion of 2 moles of ADP to one mole each of AMP and ATP is present both in the membrane and the cytoplasm of the red cells. Consequently the ATP necessary for the phosphorylation of glucose may be generated from added ADP but not from AMP.

Addition of ADP or AMP to the ATP-containing assay system inhibited the hexokinase reaction, as indicated in Tables X-B and X-C. A marked inhibitory effect was observed when the ratio of ATP to ADP or to AMP in the system exceeded 2:1 and 10:1 respectively. The percentage of inhibition of the hexokinase activity was found to be increased with increase in the concentration of added ADP or AMP. The inhibition apparently is of the competitive type. It will be evident from the results of a later experiment (see Fig. 17) that ATP itself at high concentration also produces the inhibitory effect on the phosphorylation of glucose. Various mechanisms may be involved in the inhibitory phenomenon. It is possible also that these nucleotides in higher concentrations cause

steric hindrance or that the ionic strength may become increasingly unfavourable to the hexokinase reaction.

3. The specific requirement of hexokinase for metal cations.

Hexokinase is not a metal-enzyme, yet, like most of the phosphate transfer reactions, the enzyme requires Mg^{++} as the cofactor of the reaction. Kashket (143) has demonstrated the dependence of red cell hexokinase activity on the concentration of Mg^{++}.

The specificity of hexokinase for metallic ions (as cofactor) is presently under investigation. A preliminary experiment was carried out to ensure that the Cl^{-} anion does not affect the activity of the hexokinase system. Various chloride salts were then added to the assay medium in a final concentration of 0.075 mM in the system. Since analysis for the endogenous inorganic ions was not carried out, the figure represents only the minimum value of the cations present. The result with 13 types of cation are indicated in Table XI. It is evident that no reaction occurred in the absence of added Mg^{++}. Of the bivalent ions tested, only Mn^{++} and Co^{++} could take the place of Mg^{++} in the reaction and with these ions the reaction rate was less than 30, and 15 per cent of the maximum rate with Mg^{++}. Monovalent ions such as Na^{+} and K^{+}, and trivalent ions such as Al^{+++} were ineffective. Presumably, the complex formation between the enzyme (E), ATP and the bivalent ion, (Me^{++}) either in the form of E-Me^{++}-ATP or E-ATP-Me^{++} is required

TABLE XI

Ion Specificity of Hexokinase

Ion (0.075mM Cl^- salts)	Hexokinase Activity (mg glucose/100 mg Hb/hr.)	Remarks
Control	0	-
Mg^{++}	0.35 0.33	-
Mn^{++}	0.09 0.10	-
Co^{++}	0.03 0.04	-
Ca^{++}	0	Assay medium turns turbid - turbidity increases during assay
Hg^{++}	0	
Zn^{++}	0	
Cd^{++}	0	-
Al^{+++}	0	-
Na^{+}	0	-
K^{+}	0	-
Fe^{++}	-	Yellowish green solution*
Cu^{++}	-	Blue solution*
Ni^{++}	-	Green solution*

* Colour interference developed in the assay medium.

for the reaction. The specificity of hexokinase for metal ions does not seem to depend on the capability of complex formation of various metallic ions. However, the stability of the substrate-enzyme complex of each metal ion may be significant in the determination of the specificity of hexokinase towards its cofactor.

4. Affinity of hexokinase for various hexose sugars.

A comparison was made of the utilization of glucose, fructose, mannose and galactose by the SFH. It is clear from the results in Table XII that at pH 7.8, the utilization of the hexose decreased in the following sequence: glucose, galactose, fructose, mannose. Mannose was found to inhibit the phosphorylation of glucose while other hexoses present in the same concentration had no effect. Kashket, using a different method for assay of hexokinase activity, has reported that the rate of utilization of hexoses by rabbit erythrocytes, at pH 7.8 was of the following order: glucose, mannose, fructose, galactose (smallest).

It is not certain whether the phosphorylation of different hexoses by human erythrocyte involved the same hexokinase system. The situation will be clarified only when the isolation of the pure hexokinase system from erythrocyte will be achieved.

5. Hexokinase activity in young and aged red cells.

The hexokinase activity of erythrocytes of various age groups in fresh and stored blood specimens was studied.

TABLE XII

Affinity of Hexokinase in SFH towards Various Hexoses.

Hexose (mM.)	Hexokinase Activity (mg glucose/100 mg Hb/hr.)	Percent of Activity compared to that with glucose.
Glucose (2.4)	0.40	100
Fructose (2.4)	0.13	33
Fructose (2.4) + Glucose (2.4)	0.39	99
Mannose (2.4)	0	0
Mannose (2.4) + Glucose (2.4)	0.22	54
Galactose (2.4)	0.24	61
Galactose (2.4) + Glucose (2.4)	0.41	100

Specimens in the ACD medium at 4°C were removed at various times during storage and subjected to differential sedimentation by means of the centrifugal treatment described on p. 43. The hexokinase assay was performed on the SFH prepared from the 10% fractions of the uppermost, middle and lowest layers of the packed red cells. The activities of G-6-P DH were assayed at the same time in each case to ensure that any difference observed in the hexokinase activity cannot be attributed to the changes in the activity of the G-6-P DH. The results are given in Table XIII.

TABLE XIII

Hexokinase Activity of SFH Prepared from Red Cells of Various Age Groups.

Duration of Storage of Blood in ACD at 5°C.	ENZYME ACTIVITY CELL AGE GROUPS						
	Youngest		Intermediate		Oldest		Youngest-Oldest
	HK	G-6-P DH	HK	G-6-P DH	HK	G-6-P DH	HK (difference in activity)
(days)	(mg substrate utilized per 100 mg Hb per hour)						
5	0.37	4.9	0.34	4.9	0.29	4.7	0.08
10	0.35	5.1	0.32	4.8	0.30	5.0	0.05
18	0.34	4.6	0.31	-	0.30	4.9	0.04
30	0.36	4.8	0.36	4.6	0.35	4.7	0.01
50	0.32	4.6	0.31	4.7	0.32	4.5	0

We find from the results in Table XIII that there was no significant change in the hexokinase or G-6-P DH activity in a given population of red cells on storage of the blood in ACD medium at $4^{o}C$. However, a small but consistent difference in hexokinase activity was observed between the youngest and oldest cell fractions of fresh blood. This difference cannot be attributed to the presence and activity of leucocytes since most, if not all of the white cells were removed with the buffy coat before the preparation of SFH from the red cell samples. Furthermore, it was found that the difference in hexokinase activity between the youngest and oldest cell fraction diminished with duration of storage of the blood. After 50 days, no detectable difference was observed between the hexokinase activity of the two cell populations. The result of the experiment can be readily reproduced with specimens of ACD blood. In all cases the difference in hexokinase activity between oldest and youngest groups of cells in fresh blood was found to be about 0.1 units. The difference became negligibly small in blood stored for more than 30 days. These findings are in contrast to Hemphill's observation that a difference in hexokinase was detectable only in preserved specimens but not in fresh blood. In her study the fractionation of cells into the three age groups was done by graded osmotic hemolysis. This method provides a relatively small quantity of red cells for the test and the results are open to greater experimental variation than with the differential centrifugation method.

FIGURE 11

Osmotic Resistance of Red Cells in Preserved Specimens.

I. Comparative osmotic fragility of red cells of three age groups.

Legend

A. Top layer (youngest) red blood cells.

B. Middle layer (intermediate) red blood cells.

C. Bottom layer (oldest) red blood cells.

II. Increase in total red cell osmotic fragility during storage in ACD at 4° C.

Legend

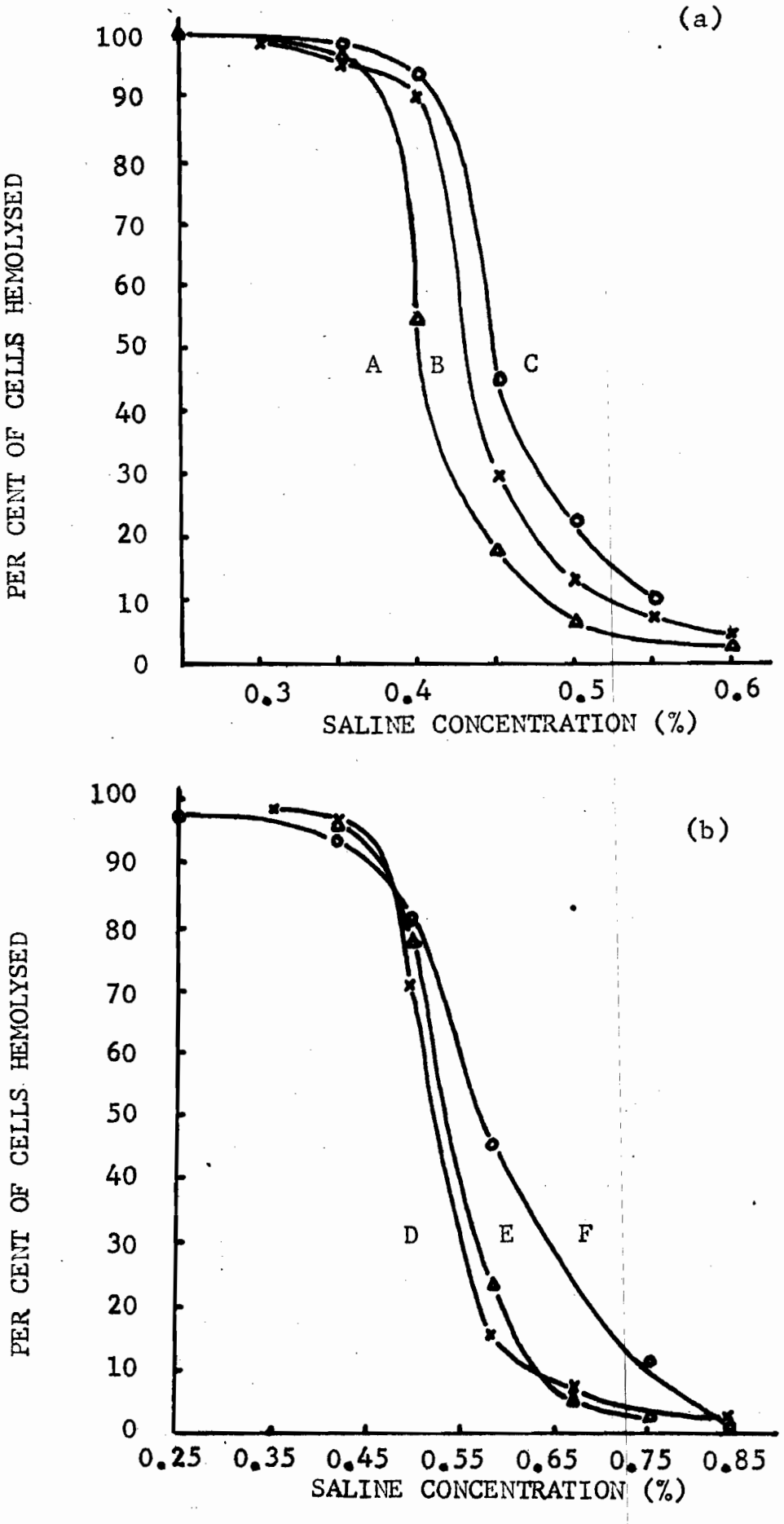
D. After 1 day of storage.

E. On the 15th day of storage.

F. On the 30th day of storage.

Conditions: 0.5 ml of blood and 5 ml of saline were mixed gently and allowed to stand for 15 minutes at room temperature. The suspension was centrifuged at 4°C. Hemoglobin concentration of the supernatant was then estimated.

FIGURE 11


(a)
PER CENT OF CELLS HEMOLYSED
100
90
80
70
60
50
40
30
20
10
0
A
B
C
0.3
0.4
0.5
0.6
SALINE CONCENTRATION (%)
(b)
PER CENT OF CELLS HEMOLYSED
100
90
80
70
60
50
40
30
20
10
0
D
E
F
0.25
0.35
0.45
0.55
0.65
0.75
0.85
SALINE CONCENTRATION (%)

In Figure 11(a) a comparison is given of the osmotic fragility of erythrocytes of various age groups that had been separated by differential centrifugation. The top layer - presumably youngest red cell fraction - was evidently the most osmotically resistant group and the bottom layer - presumably the fraction containing the oldest calls of the given population - was the least resistant.

Figure 11(b) indicates the change in osmotic fragility of the red cells during storage in ACD at $4^{o}C$. The result is consistent with the observation that stored erythrocytes are generally more susceptible to osmotic hemolysis than fresh cells.

ø. Kinetic studies of the hexokinase of the red cells.

(a) The effect of temperature on hexokinase activity in fresh and stored erythrocytes.

The SFH from a sample of blood (sample A) preserved in ACD medium was prepared at intervals during storage at $4^{o}C$. The hexokinase activity was assayed over a temperature range from 20^{o} to 60^{o} C.

The results recorded in Figure 12(a) show that in all the SFH preparations, the hexokinase activity increased with the temperature up to about $40^{o}C$. However, the rate of the reaction began to fall off at higher temperatures. For instance, the hexokinase activity was lost when the SFH was incubated at $60^{o}C$ for 5 minutes, $55^{o}C$ for 15 minutes, or at $50^{o}C$ for about 30 minutes. Furthermore, it is evident that the peak of the curves representing the temperature at which thermal denaturation occurred, shifted towards lower temperatures as the period

FIGURE 12

Influence of Temperature on Hexokinase Activity of Fresh and Preserved Red Cells Stored in ACD at $4^{o}C$.

(a) Hexokinase activity in relation to temperature.

(b) Log_{10} Reaction Velocity vs $\frac{1}{T_A} \times 10^{-3}$

Legend

ACD Blood - Sample I

A SFH prepared from blood preserved for 10 days.

B SFH prepared from blood preserved for 15 days.

C SFH prepared from blood preserved for 30 days.

D SFH prepared from blood preserved for 50 days.

ACD Blood - Sample II

E SFH prepared from blood preserved for 1 day.

F SFH prepared from blood preserved for 22 days.

G SFH prepared from blood preserved for 40 days.

Conditions: Hexokinase activity of SFH was estimated by Method B at the temperature as indicated in the figure.

FIGURE 12

(a)

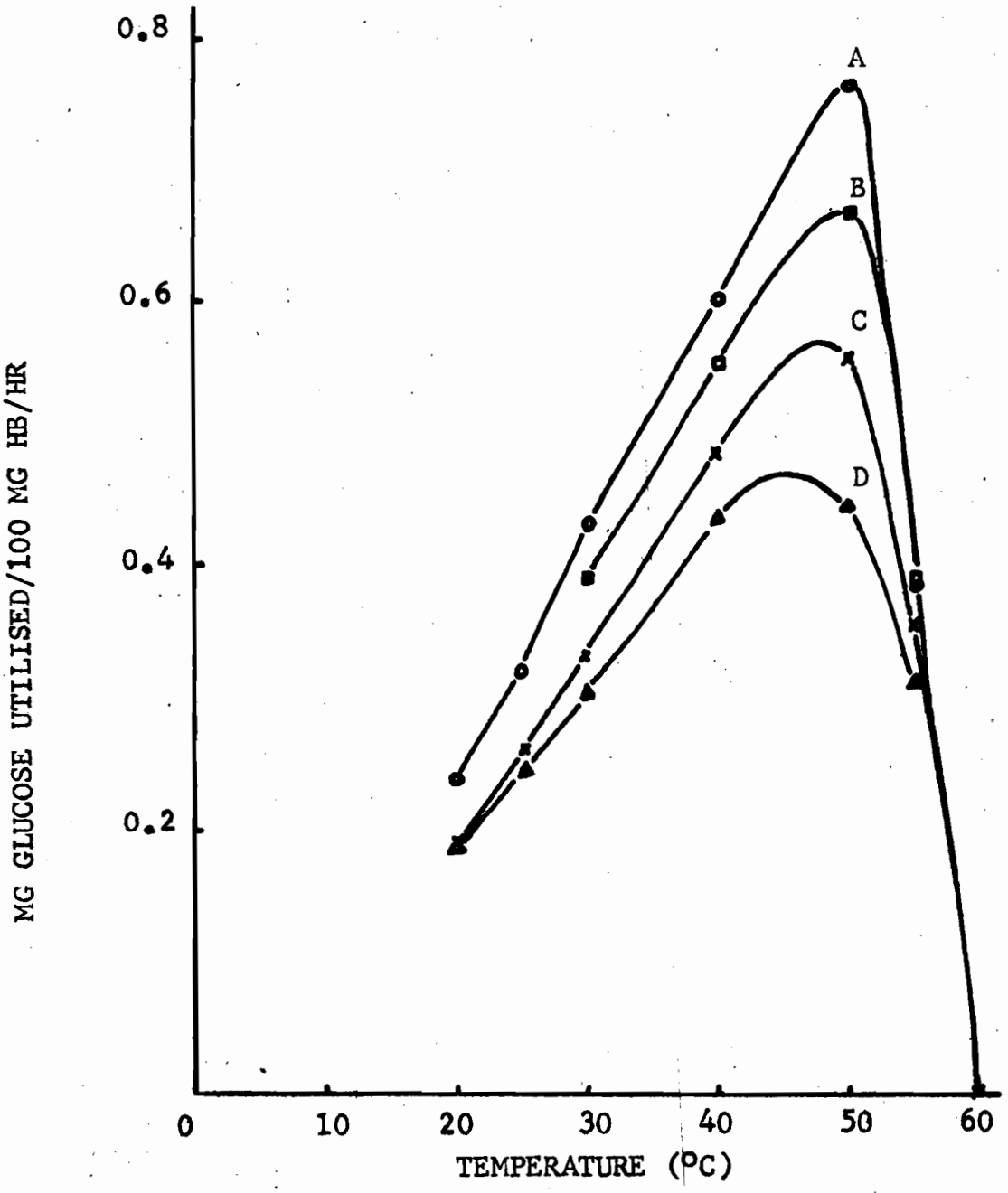
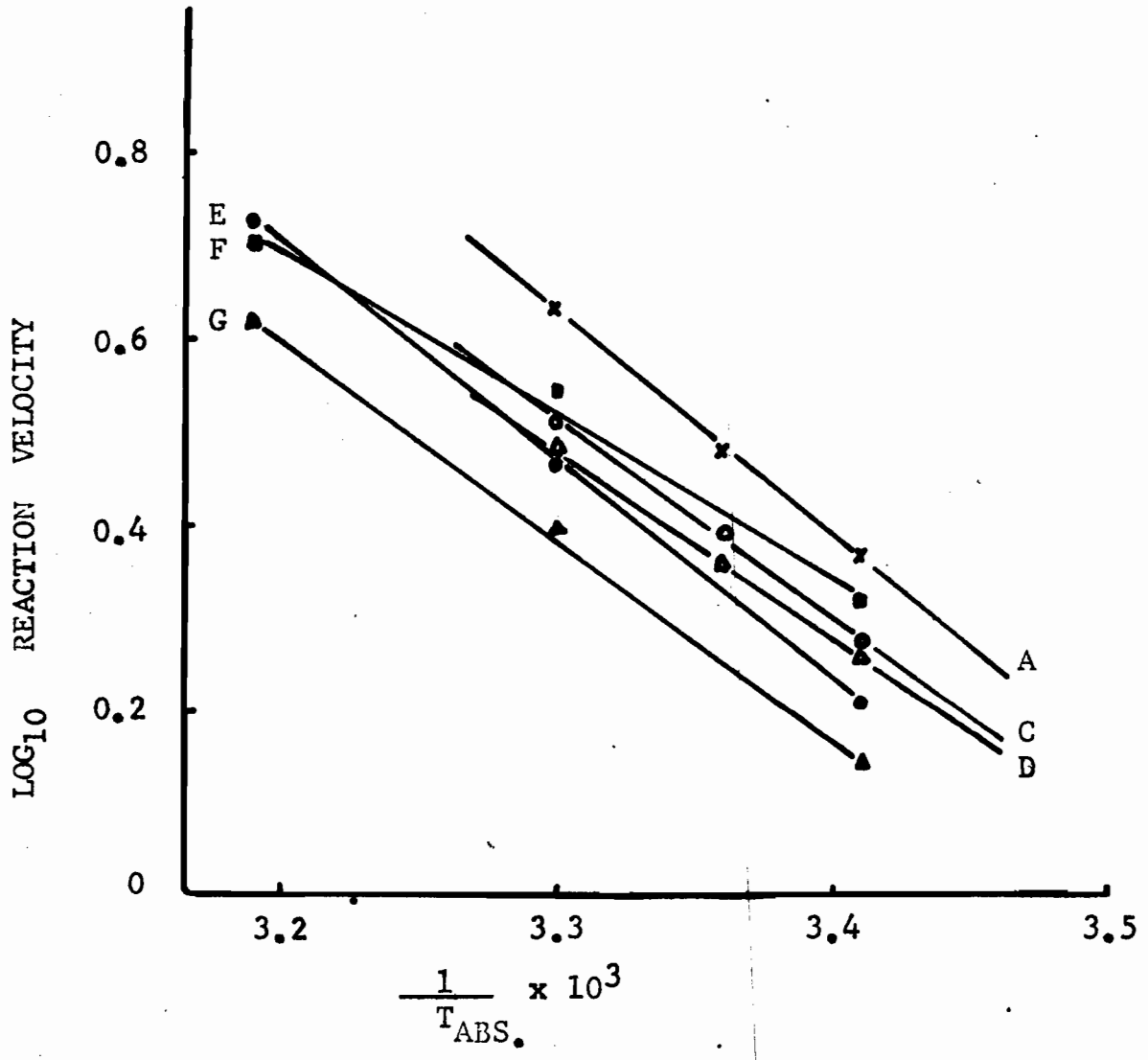

0.8
0.6
0.4
0.2
A
B
C
D
0
10
20
30
40
50
60
MG GLUCOSE UTILISED/100 MG HB/HR
TEMPERATURE (°C)

FIGURE 12

(b)


0.8
0.6
0.4
0.2
0
E
F
G
A
C
D
3.2
3.3
3.4
3.5
LOG_{10} REACTION VELOCITY
$\frac{1}{T_{ABS.}} \times 10^3$

of storage was prolonged. The increase in hexokinase activity with temperature (slope of the graphs) in fresh blood also was found to be greater than the stored samples. The experiment was repeated with another sample of ACD blood (Sample B). The results of the two experiments were plotted with the $\log_{10}$ reaction velocity against the reciprocal of the absolute temperature in Figure 12(b). Since the hexokinase reaction follows zero order kinetics in the lower temperature range (20^{o} - 30^{o}C), the activation energy (μ) of the reaction can be calculated according to the equation,

$$\mu = \frac{4.6(\log_{10}K_2 - \log_{10}K_1)}{\frac{1}{T_1} - \frac{1}{T_2}}$$

where K_1 and K_2 represent the reaction rates at the absolute temperatures T_1 and T_2 respectively.

The activation energy for the phosphorylation of glucose by the hexokinase in the SFH was found to be 11,280 calories/mole for specimen A and 10,900 units for specimen B. These figures represent an average of 3 determinations on the blood sample during a storage period of 50 days. It was observed further the activation energy of the reaction (μ) is independent of the length of the storage period of the blood.

(b) <u>The influence of substrate (glucose and ATP) concentration on hexokinase reaction</u>.

The hexokinase activity of the red blood cells depends on the concentration of glucose and ATP in the system. In Figure 13 are shown Lineweaver-Burk plots of the data obtained

FIGURE 13

Lineweaver-Burk Plots.

(a) Plot of the reciprocal of ATP concentration versus the reciprocal of the initial velocity rate of hexokinase reaction at various concentrations of glucose. p.101.

Legend

A 0.24 m$\underline{M}$ glucose

B 0.40 m$\underline{M}$ glucose

C 2.40 m$\underline{M}$ glucose

(b) Plot of the reciprocal of glucose concentration versus the reciprocal of the initial velocity rate of hexokinase reaction at different concentrations of ATP. p.101.

Legend

D $1 \times 10^{-3}\underline{M}$

E $2.2 \times 10^{-3}\underline{M}$

F $1 \times 10^{-2}\underline{M}$

(c) Plot of the reciprocal of substrate (ATP,glucose) concentration against the reciprocal of the maximum velocity of the hexokinase reaction. p.102.

Legend

A ATP

B Glucose

FIGURE 13

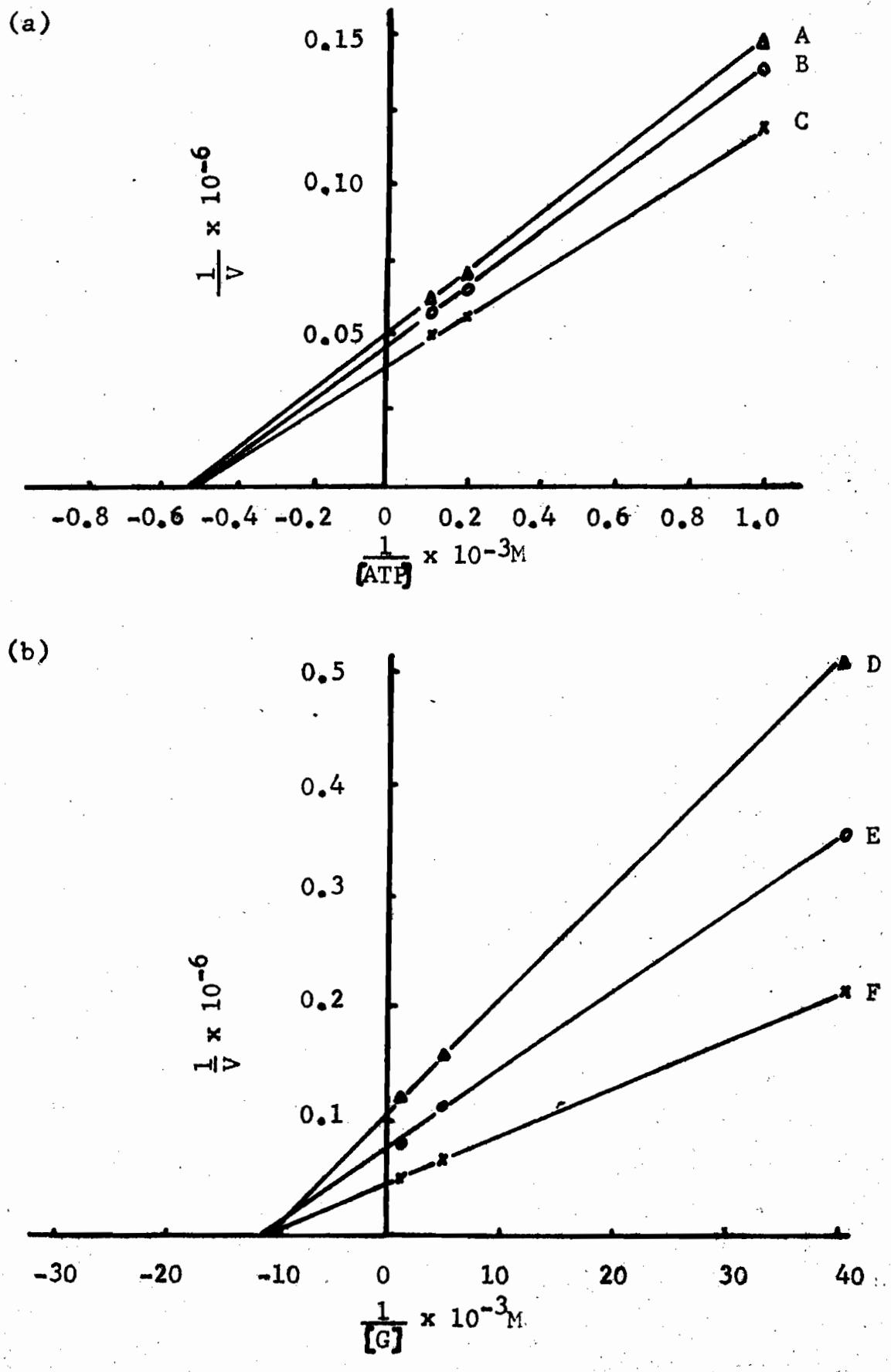
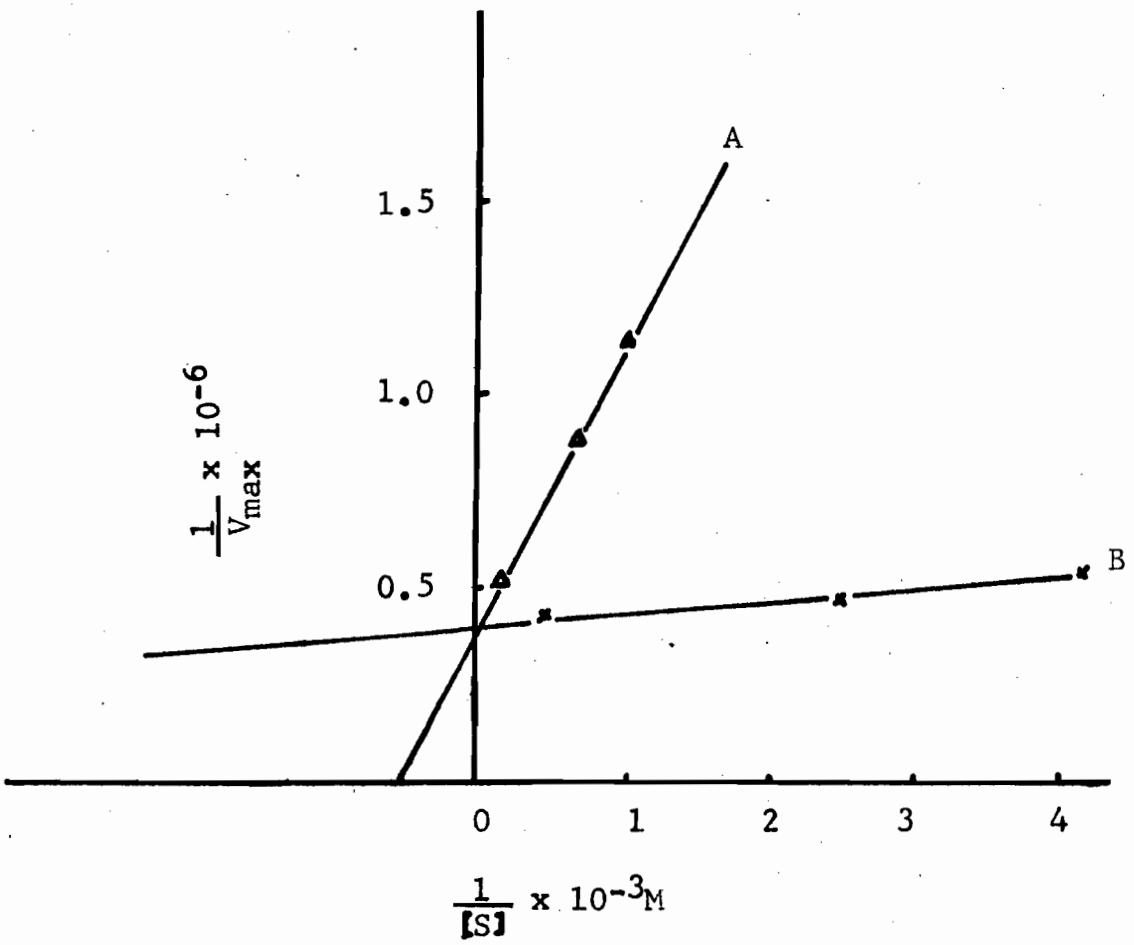

(a)
0.15
0.10
0.05
A
B
C
$\frac{1}{V} \times 10^{-6}$
-0.8 -0.6 -0.4 -0.2 0 0.2 0.4 0.6 0.8 1.0
$\frac{1}{[ATP]} \times 10^{-3}$M
(b)
0.5
0.4
0.3
0.2
0.1
D
E
F
$\frac{1}{V} \times 10^{-6}$
-30 -20 -10 0 10 20 30 40
$\frac{1}{[G]} \times 10^{-3}$M

FIGURE 13

(c)



with hexokinase from the SFH preparation. It is evident from the graphs that the curves are all linear and converge at a common point in the 1/substrate axis. Also from Figure 13(a) it is clear that an increase in ATP concentration increases the reaction rate of hexokinase. The initial velocities of the reaction were determined as a function of the ATP concentration which was varied in the range from 1×10^{-3} M to 10×10^{-3} M. There is no change in the slope and intercept at all 3 levels of glucose. Figure 13(b) represents the change of initial velocity rate with increase in the glucose concentration from 0.24 to 2.40 mM. From the intercepts in both graphs, K_{ATP} and K_G were calculated to be 1.75×10^{-3} M and 6.9×10^{-5} M respectively. The values are remarkably close to those reported by other investigators. Thus, Rose (115) with G-1-^{14}C as the isotope, estimated the K_{ATP} of red cell hexokinase to be in the order of 1.5×10^{-3} M. Weil-Malherbe (188) reported that the value of K_G is 5×10^{-5} M for the hexokinase of rat brain. Slein (189) found K_G for yeast hexokinase to be 9.5×10^{-5} M.

Figure (c) shows the relationship between the maximum reaction velocities of hexokinase and the concentration of glucose and ATP in the assay system. The data were obtained from the previous figures. The intersections with the y axis evaluate $\frac{1}{V_f}$ - the final maximum velocity under optimal assay conditions of hexokinase. The fact that both intersections coincide in the y axis indicates the accuracy of the kinetic results obtained from these experiments.

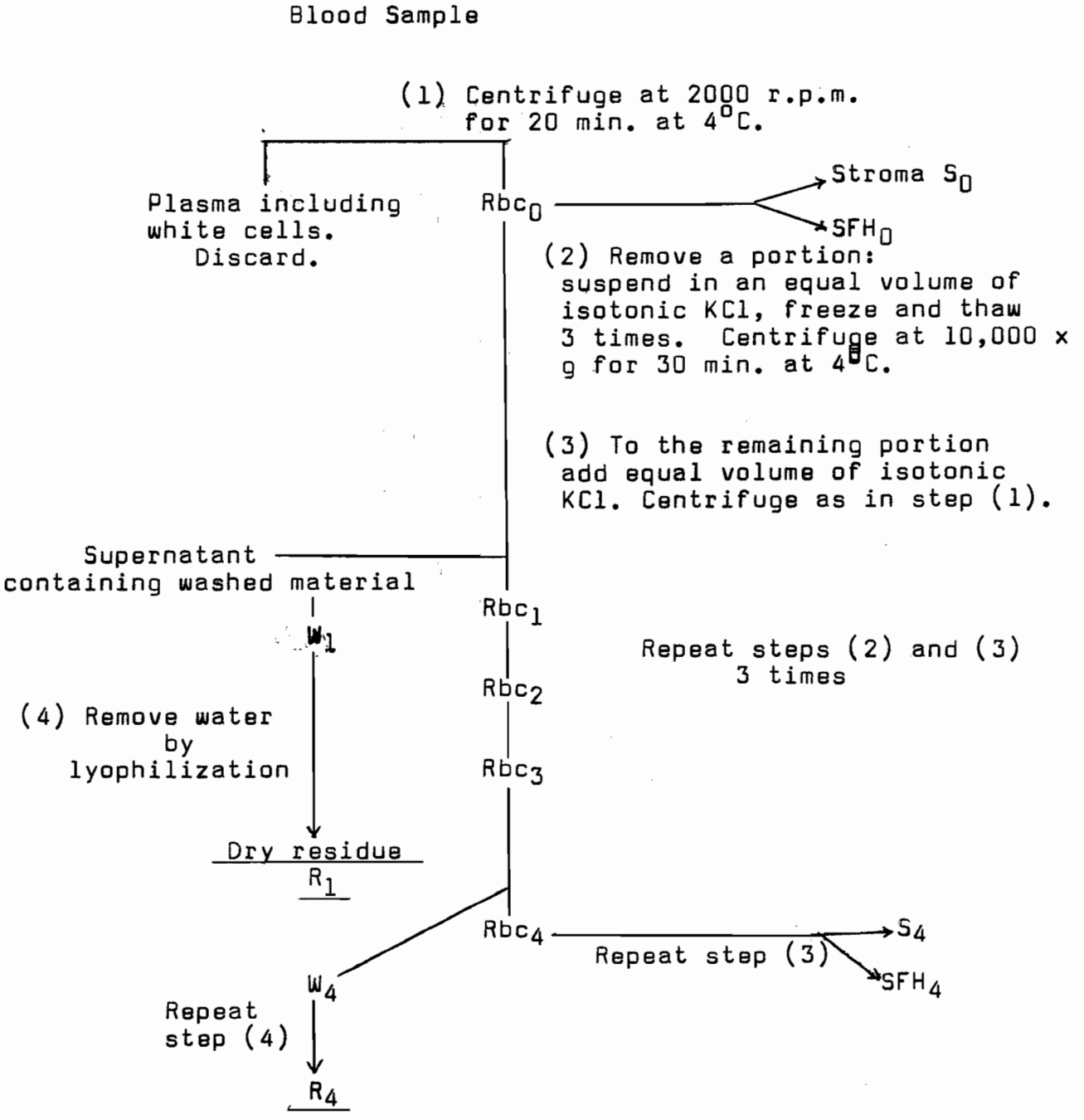
7. The effect of washing of red cells on the hexokinase activity.

It was observed in previous experiments that hexokinase activity of unwashed red cells is consistently higher than that of the washed cells from the same blood sample (see fig. 4). Accordingly, an effort was made to compare the hexokinase activity of SFH prepared from red cells which had been subjected to a number of washings. The experiment was performed with a sample of ACD blood. The procedure is summarized in Table XIV(a). The hexokinase and the G-6-P DH activity was measured; the latter serving as a control for the former enzyme.

It is evident from the results presented in Figure 14 that the hexokinase activity decreased markedly in the erythrocytes with successive washings with isotonic KCl. The decrease in the enzyme activity was more pronounced after the first and second washings. With further washing the decrease in the activity was relatively small. The observed differences in hexokinase activity could not be attributed to the presence of white blood cells in the specimen as most, if not all, of these had been removed. Nor could the effect of hemolysis of the sample produce the observed differences in hexokinase activity since the assay of hexokinase itself is based on hemoglobin concentration in the SFH. One is obliged to conclude, therefore, that red cells or the cell membrane appear to contain a factor which has a stabilizing or activating influence on hexokinase and which is at least partially removable by repeated washing of the cells with isotonic KCl.

TABLE XIV(A)

Procedure for preparation of Red Cell Fraction.



Abbreviations:

Rbc = red blood cell
SFH = stroma free hemolysate
S = stroma fraction
Suffix indicates the number of washings

The experiment was repeated with a specimen of blood in ACD medium. This time, the first and the fourth washings of the red cells were concentrated by lyophilization. The residues then were dissolved in isotonic KCl. The fractions of stroma and the dissolved residue were added one at a time to the hexokinase assay system and the preparation assayed as indicated in Table XIV(b). The results represent the average of duplicate determinations. It is apparent that the hexokinase activity of the SFH (SFH_0) prepared from the unwashed sample was considerably higher than that of the washed samples (SFH_1, SFH_4). However, when the material in the first washing was reintroduced into the system, the hexokinase activity was restored almost to the value of the unwashed red cells. The residue fraction obtained by concentration of the materials of the fourth washing did not have this effect. Presumably, the first and second washing had already removed a large portion of the substance which has a pronounced effect on the activity of hexokinase in the cell. Addition of dried stroma residue prepared from unwashed and washed (four times) cells to the assay system produced no change in the activity, thus indicating that the factor is not bound to stroma.

The results strongly suggest the presence of some kind of protector or activator for hexokinase in the red cell. Further investigation would be warranted to determine the nature of the factor. Lovelock (174) has shown that washing of red cells with salt solutions removes lipids. Ponder (1)

FIGURE 14

Influence of Repeated Washing of Erythrocytes on the Hexokinase Activity.

Legend

A G-6-P DH activity of SFH prepared from 1-day-old ACD preserved blood.

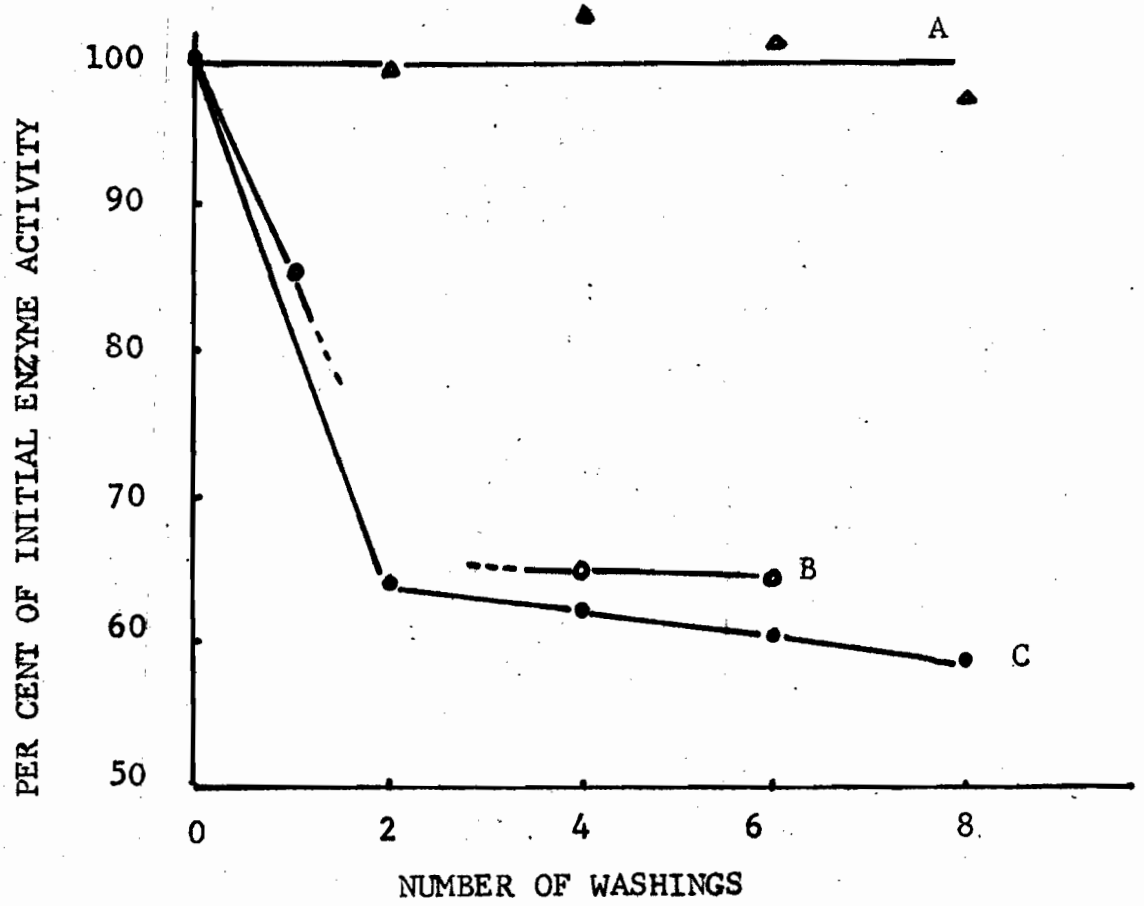
B Hexokinase activity of SFH prepared from 5-day-old ACD preserved blood.

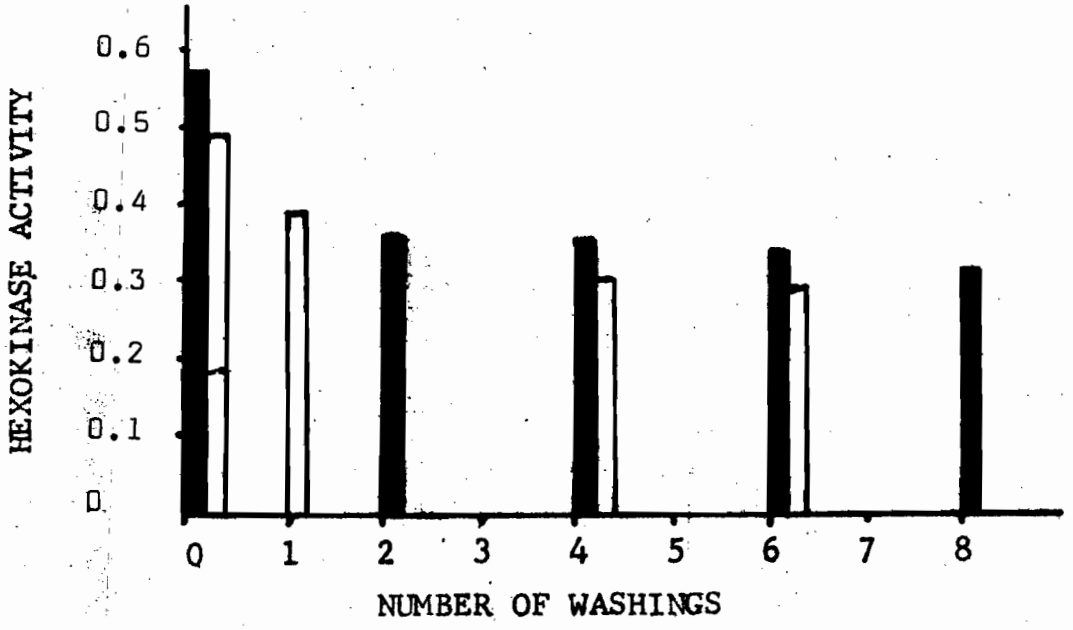
C Hexokinase activity of SFH prepared from 1-day-old ACD preserved blood.

Enzyme Activities: Milligrams of substrate utilized per 100 mg hemoglobin per hour. Enzyme activity was estimated by Method B.

Conditions: Plasma and white cell layer were removed completely by aspiration after each centrifugation. The washing procedure was carried out as described in the Method B p.47. SFH was prepared from red cells which had been given a number of washings as indicated in the figure.

FIGURE 14


PER CENT OF INITIAL ENZYME ACTIVITY
100
90
80
70
60
50
A
B
C
0
2
4
6
8
NUMBER OF WASHINGS


HEXOKINASE ACTIVITY
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
7
8
NUMBER OF WASHINGS

also found that "substances other than hemoglobin are lost in the washing process; these substances include lipids as well as protein such as the anti-sphering substance and an unidentified globulin." Weil-Malherbe and Bone (188) also obtained some evidence for the existence of an activating factor for hexokinase in the erythrocyte membrane.

TABLE XIV(b)

The Effect of Washing Fractions on Hexokinase Activity

Stroma free Hemolysate	Fractions Added	Hexokinase Activity
		(mg glucose/100 mg Hb/hour)
SFH_0	-	0.46
SFH_1	-	0.39
SFH_4	-	0.31
SFH_4	-	0.31
SFH_4	R_1	0.40
SFH_4	S_0	0.30
SFH_4	S_4	0.28
SFH_4	R_4	0.31

8. The effects of inhibitors on the hexokinase activity.

(a) The influence of alloxan, taurocholate, lipase and pepsin.

The proteolytic enzymes and alloxan were prepared fresh in isotonic KCl. The SFH was incubated with each of these reagents separately for 30 minutes. The hexokinase

and G-6-P DH activity were then estimated and the results compared with that of the control which contained none of the special reagents mentioned.

As indicated in Figure 15, alloxan in concentrations lower than 0.06×10^{-3} M produced no effect on the reaction, but in higher concentrations exerted a strong inhibitory action. At 0.5×10^{-3} M, the glucose utilization was completely inhibited. The inhibition with alloxan on G-6-P DH was found to be weaker than that on hexokinase. Alloxan was known to inactivate many enzymes through the oxidation of the essential SH-groups. Taurocholate, at the concentrations indicated in the figure, produce no inhibitory effect on G-6-P DH. However, at concentrations higher than 1.33×10^{-3} M, taurocholate produced about 12% inhibition on the hexokinase activity. Pepsin inhibited the enzyme at concentrations higher than 33.3 mg per cent while lipase was found to have no effect on the enzyme at the same concentration (Figure 16). The results essentially agree with previous finding that hexokinase is of the nature of a lipoprotein. (43) (104).

(b) The influence of various organic inhibitors.

The organic inhibitors used in the experiment may be classified into 3 groups: A,B and C as indicated in Table XV.

The group A inhibitors in the form of sodium salts were used in a final concentration of 1×10^{-2} M in the SFH assay system. The Na^+ and Cl^- ions did not produce any inhibitory

FIGURE 15

Influence of Alloxan and Taurocholate on the Activity of Hexokinase and G-6-P DH.

Legend

A Hexokinase activity in the presence of alloxan.

A_1 G-6-P DH activity in the presence of alloxan.

B Hexokinase activity in the presence of taurocholate.

B_1 G-6-P DH activity in the presence of taurocholate.

FIGURE 16

Influence of Lipase and Pepsin on the Activity of Hexokinase and G-6-P DH.

Legend

C Hexokinase activity in the presence of pepsin.

C_1 G-6-P DH activity in the presence of pepsin.

D Hexokinase activity in the presence of lipase.

D_1 G-6-P DH activity in the presence of lipase.

Hexokinase activity expressed in mg glucose utilized/100 mg Hb/hr.
G-6-P DH activity expressed in mg G-6-P utilized /100 mg Hb/hr.

FIGURE 15

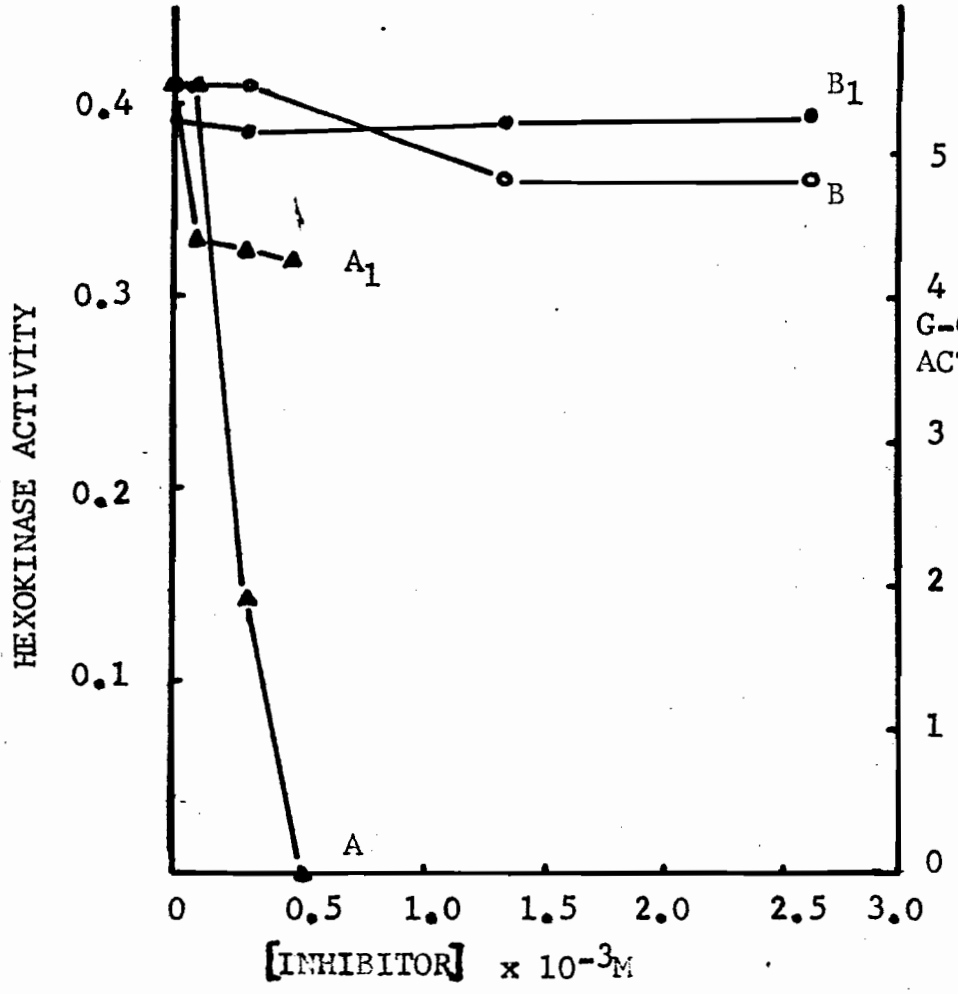
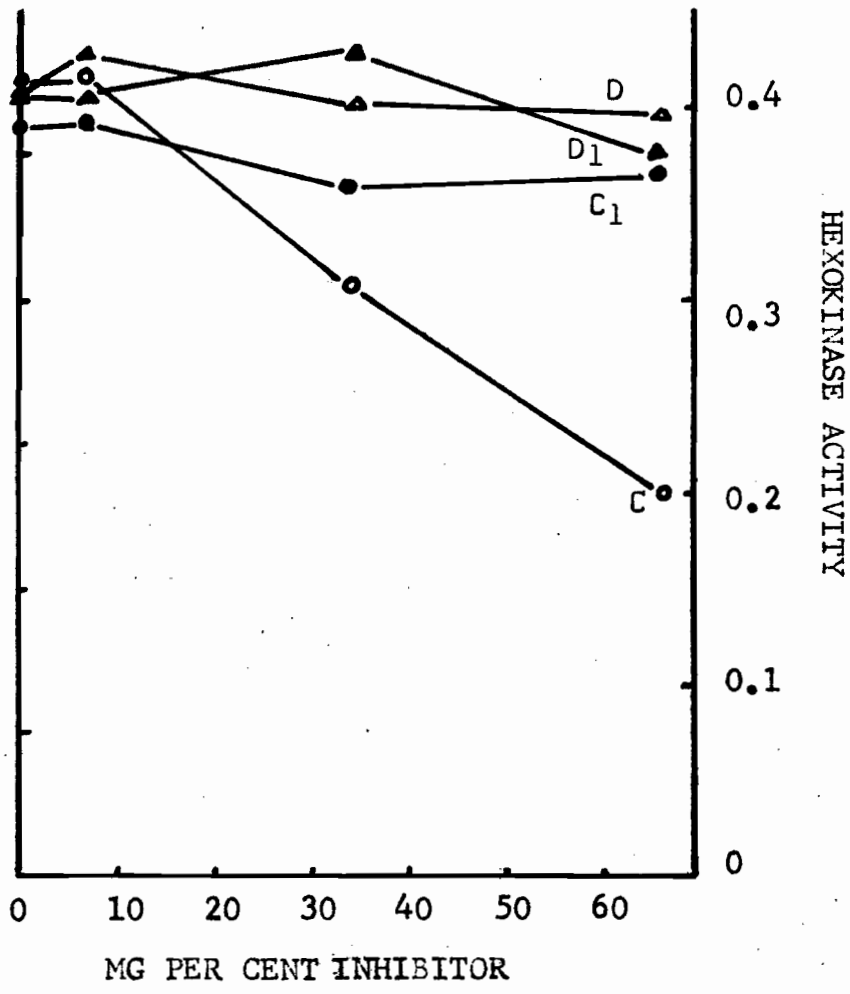


FIGURE 16



action at concentrations as high as 5×10^{-2} M. Hence, any observed change in the hexokinase activity must be attributed to the cationic form of the inhibitors tested.

It was observed that both fluoride and arsenate had no inhibitory action on hexokinase or on G-6-P DH. Oxalate inhibited hexokinase slightly ($>$10%) but was ineffective with G-6-P DH. On the other hand, iodoacetate caused strong inhibition of both enzymes.

The group B reagents were used in a final concentration of 1×10^{-3} M. Ouabin, known to inhibit the active transport of ions across the cell membrane, was found to have no effect on the two enzymes. The SH-inhibitors such as Chloromercuribenzoate (p-CMB) and N-ethylmaleimide (NEM) at 1×10^{-3} were found to cause complete inhibition of the hexokinase and above 40% inhibition of G-6-P DH activity. The addition of G-6-P to the SFH system in which the glucose utilization was completely inhibited by SH-blocking reagents demonstrated that the SFH still could metabolize the substrate (G-6-P) though at a diminished rate. The results signified the importance of the SH-groups in relation to the activity of hexokinase.

In group C, nicotinamide was found to have no effect on hexokinase but it inhibited the TPN-linked G-6-P DH. Pyruvate and lactate, the metabolic products of glycolysis, produced no observable inhibitory effect on the activity of either hexokinase or G-6-P DH.

TABLE XV

The Influence of Various Organic Inhibitors on the Activity of Hexokinase.

Metabolic Inhibitor	Hexokinase Activity (mg glucose/100 Hb/hr)	G-6-P DH Activity (mg G-6-P/100mg Hb/hr)
A (1×10^{-2}M)		
Control	0.36	5.2
NaCl (5×10^{-2}M)	0.36	4.9
NaCl (1×10^{-2}M)	0.36	5.1
Cyanide	0.31	4.3
Oxalate	0.33	5.2
Fluoride	0.36	5.2
Arsenate	0.36	5.2
Iodoacetate	0.14	4.0
B (1×10^{-3}M)		
Control	0.36	4.8
Ouabain	0.36	4.7
Phloretin*	Assay medium turns yellow	
Dinitrofluorobenzene	Colour interference occurs	
Chloromercuribenzoate	0	2.9
"	0 + G-6-P	2.6
N-ethylmaleimide	0	3.6
C (1		
Co		
Ni		
Py		
La		
Co		

(c) Effect of G-6-P on the hexokinase activity.

The inhibitory effect of G-6-P on hexokinase from various sources has been reported by various investigators (43) (44) (116). In the present investigation, the estimation of hexokinase activity in the SFH is based on the rate of production of TPNH in the assay system with glucose as the substrate. It is thus impossible to measure the inhibitory effect of G-6-P on the activity of hexokinase of SFH by simply adding various amounts of G-6-P to the system. A more indirect approach was adopted namely, study of the effect of removal of the G-6-P initially present by adding various quantities of G-6-P DH to the system and measuring the hexokinase activity. The quantity of G-6-P DH added is expressed in Kornberg units/ml.

It is evident from Table XVI that introduction of G-6-P DH to the system markedly increased the rate of reduction of TPN and accumulation of TPNH. Thus, the addition of 6.5 units of the enzyme doubled the rate of glucose utilization by the SFH. The rate of the increase was proportional to the amount of G-6-P DH added. The reaction of G-6-P DH proceeded so rapidly that the activity of the enzyme could not be measured by the assay method as used. It was shown previously that the hexokinase catalyzed reaction proceeds at less than 1/10th the rate of G-6-P DH catalyzed reaction and the succeeding reaction in the system under the conditions of the assay. Thus hexokinase constitutes the rate limiting step in the SFH system. The increased hexokinase activity upon the addition of G-6-P DH to the assay system is explainable by the removal of the accumulated G-6-P DH which was responsible for the inhibition of the hexokinase catalyzed reaction.

TABLE XVI

Influence of Addition of Yeast G-6-P DH on Hexokinase Activity of SFH.

Concentration of G-6-P DH* (units/ml)	Hexokinase Activity (mg glucose utilized per 100 mg Hb/hr)	G-6-P DH Activity (mg G-6-P utilized per 100 mg Hb/hr)
0	0.34	5.2
0.05	0.40	75.0
1.5	0.47	∞
3.0	0.59	could not be measured by this assay method.
6.5	0.71	
8.0	0.75	
10.0	0.78	

Conditions: SFH was prepared from fresh blood.

* G-6-P DH was the type V variety obtained from Sigma Chemical Company of St. Louis, Mo.

(d) <u>The effect of high concentrations of ATP, Mg^{++} and inorganic phosphate on hexokinase activity</u>.

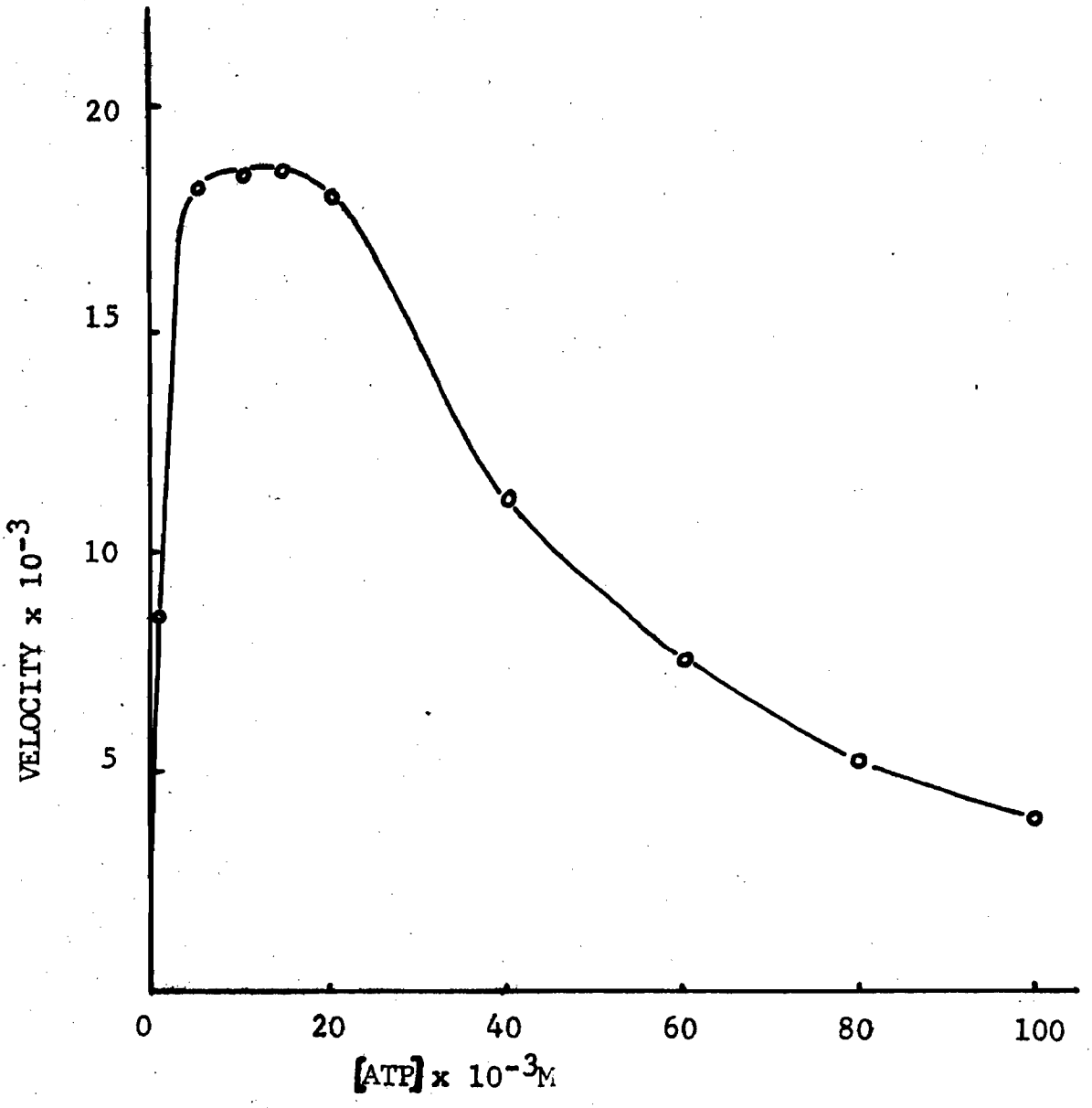
It was observed in previous experiments that the optimal concentration of ATP for the hexokinase assay system of SFH is 1×10^{-2} M. The results represented in Figure 17 show that the initial velocity of the hexokinase reaction increased with the amount of ATP added at constant Mg^{++} concentration (1×10^{-2} M) of the system. Further increase in the

FIGURE 17

Influence of ATP Concentration on the Initial Reaction Rate of Hexokinase in the SFH from Fresh Erythrocytes.

Conditions: Hexokinase activity was estimated by Method B with ATP present in concentrations as indicated in the Figure.

FIGURE 17



ATP concentration ($>2 \times 10^{-2}$ M) resulted in a tremendous decrease in the velocity of the reaction. Thus 1×10^{-1} M of ATP produced greater than 75% inhibition on the glucose utilization. Furthermore, it was found that this inhibition by ATP in higher concentration cannot be reversed by addition of excess (>5 x optimum concentration) Mg^{++} or glucose to the system. Purity test on the ATP sample by paper electrophoresis show that the preparation is free from contamination with other nucleotides such as AMP or ADP which also may inhibit the hexokinase reaction.

A similar inhibitory effect of muscle P-F-kinase by high ATP concentration has been previously reported by Passonneau et al. (190). The inhibition phenomenon has been also observed with yeast hexokinase (104). These authors further suggested that the inhibitory action of ATP possibly may play a regulatory role in the metabolism of the cell.

The effect of various levels of Mg^{++} upon the hexokinase activity with various concentrations of glucose and ATP is indicated in Table XVII. The data were obtained in four experiments (A,B,C,D) using the same sample of SFH prepared from fresh blood preserved in ACD medium. In experiments C and D the inhibition of hexokinase by high ATP concentration was observed. Mg^{++} in high concentration as represented in A and B, also was found to inactivate the reaction. Increasing the ATP concentration failed to reverse the inhibition produced by the metallic ion. (Compare A B with C D). On the other hand, the G-6-P DH activity was unaffected by the changes in ATP, Mg^{++}

TABLE XVII

Influence of Magnesium Ion Concentration on Hexokinase Activity.

	ATP (Molar concentration)	$MgCl_2$ (1×10^{-2} M)	Glucose (mM)	Hexokinase Activity	G-6-P DH Activity
				(mg substrate/100mg Hb/hr)	
A.	0.01	10.0	2.4	0.40	5.2
	0.01	5.0	2.4	0.50	5.3
	0.01	1.0	2.4	0.36*	5.3
B.	0.01	10.0	0.02	0.15	5.1
	0.01	5.0	0.02	0.17	5.0
	0.01	1.0	0.02	0.14	5.1
C.	0.01	1.0	2.4	0.37*	5.0
	0.05	1.0	2.4	0.33	5.1
	0.10	1.0	2.4	0.24	5.2
D.	0.01	10.0	2.4	0.36*	5.3
	0.05	10.0	2.4	0.31	5.3
	0.10	10.0	2.4	0.20	5.0

*Concentration of constituents corresponding to that in standard assay medium.

and glucose concentration in the system. Melchior and Melchior (104) found that excess Mg^{++} was not inhibitory to yeast hexokinase. The result presented here agrees well with Brintzinger et al. (191) who reported that yeast hexokinase was inhibited by many metallic ions including Mg^{++}.

The effect of inorganic phosphate ion (at concentrations ranging from 0 to 50mM) on the hexokinase activity was studied with the SFH assay system. The enzyme activity was estimated as described on p.47, with the exception that phosphate buffer was omitted in the assay system. Tri-sodium phosphate was used as the source of phosphate ions. At the pH of the medium (7.8), the phosphate ions exist almost entirely in dibasic form. A preliminary test with NaCl shows that the activity of hexokinase was unaffected by these ions in concentrations as high as 60mM. Thus, any alterations in the enzyme activity in the assay must be attributable to the increase in the concentration of $PO_4^{=}$ ions. The results presented in Figure 18(a) show that the hexokinase activity increased with the concentration of phosphate ions added. The optimum concentration was 1.25mM. Above this concentration, however, the phosphate exhibited an inhibitory effect on hexokinase. Thus, at a concentration of 50mM the phosphate caused 70% inhibition in the reaction. The G-6-P DH activity was unaffected by the change in phosphate ion concentration. It was observed that the addition of phosphate buffer as previously used in hexokinase assay systems, reduced the activity by about 5%. However, the presence of phosphate strengthens the buffering capacity

FIGURE 18

Influence of Inorganic Phosphate Concentration on the Hexokinase Activity.

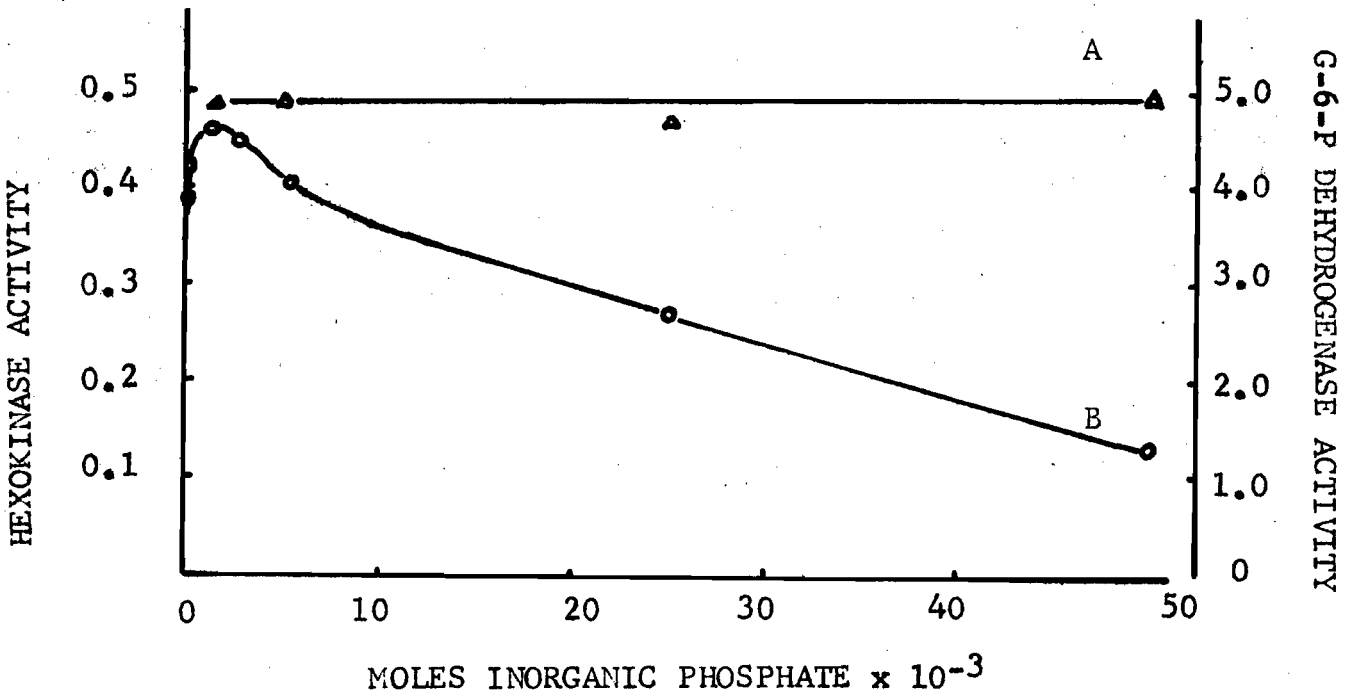
(a) Plot of the inorganic phosphate concentration against the hexokinase and G-6-P DH activity.

(b) Plot of the inorganic phosphate concentration against the reciprocal of initial reaction velocity of the hexokinase reaction.

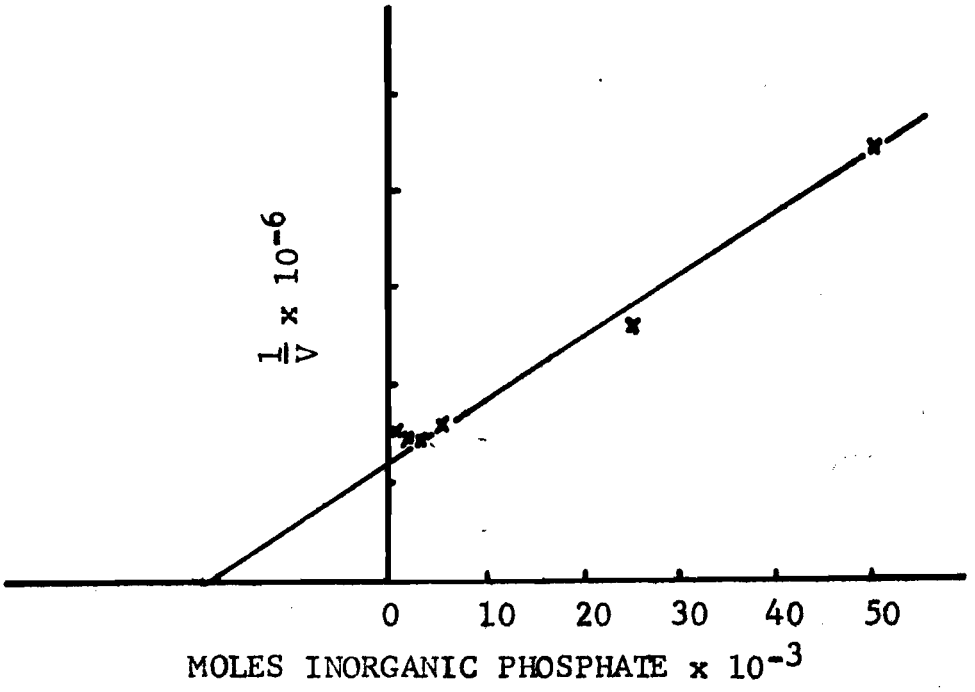
Enzyme activities are expressed as mg of substrate utilized per 100 mg Hb per hour.

FIGURE 18

(a)


HEXOKINASE ACTIVITY
0.5
0.4
0.3
0.2
0.1
A
B
5.0
4.0
3.0
2.0
1.0
0
G-6-P DEHYDROGENASE ACTIVITY
0
10
20
30
40
50
MOLES INORGANIC PHOSPHATE x 10^{-3}

(b)


$\frac{1}{V} \times 10^{-6}$
0
10
20
30
40
50
MOLES INORGANIC PHOSPHATE x 10^{-3}

of the hexokinase assay system in which glycyglycine is the principal buffer. It was also reported that inorganic phosphate ions can prevent the inactivation of muscle hexokinase (192). Thus phosphate buffer was used in the assay system in all other experiments. In Figure 18(b) the inorganic phosphate ion concentration is plotted against the reciprocal of the initial velocity of the hexokinase reaction. Since the phosphate ion concentration increases with the hydrolysis of organic phosphates during the storage of blood, the inhibitory effect of this ion on hexokinase (and hence on glycolysis) may be in some measure responsible for the decline in the energy metabolism of the preserved red cells. (See Discussion)

9. Hexokinase activity in preserved SFH.

It was observed previously that there was no significant decline in the hexokinase activity of the SFH prepared from blood samples preserved at $5^{o}C$ for a period of 50 days. On the other hand, when the SFH itself was preserved at 5^{o}, a rapid decline in the activity of the preparation was observed. Accordingly, experiments were undertaken to investigate the nature of this decline in activity.

SFH preparations from a blood sample were stored at 25^{o}, 5^{o} and $-5^{o}C$. The ability of the SFH to utilize glucose was followed by measuring the increase in the TPNH in the assay medium (p.47). It is evident from Figure 19 that the capacity to utilize glucose decreased rapidly in the SFH kept at room temperature. Within less than 3 days the activity was lost.

FIGURE 19

Influence of the Duration and Temperature of Preservation on the Glucose Utilization by the SFH.

Conditions:

SFH was prepared from 5-day-old blood and stored at the temperatures as indicated. The activity of the SFH was assayed at intervals during storage.

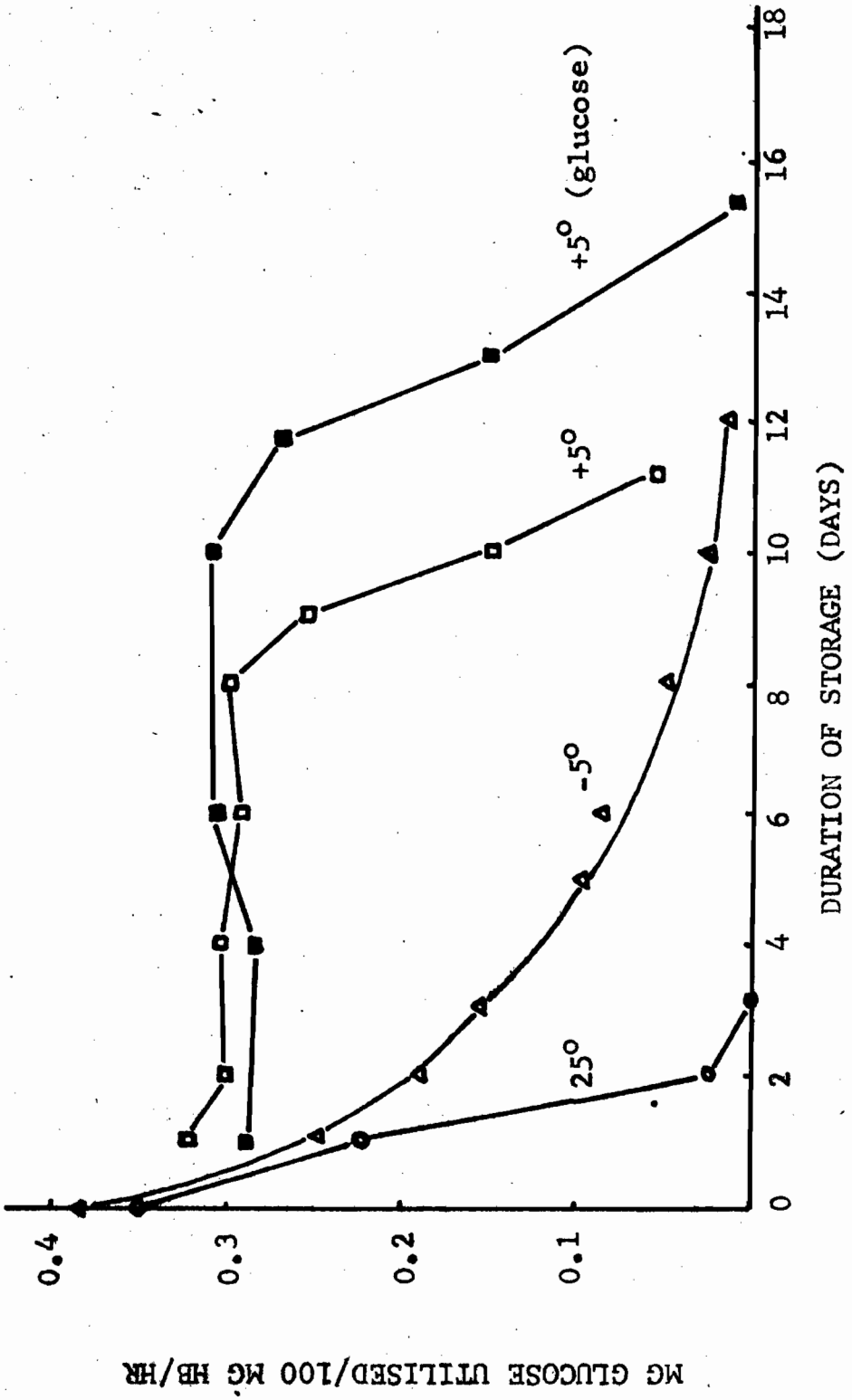

MG GLUCOSE UTILISED/100 MG HB/HR
0.4
0.3
0.2
0.1
0
2
4
6
8
10
12
14
16
18
DURATION OF STORAGE (DAYS)
+5° (glucose)
+5°
-5°
25°

FIGURE 19

The rate of loss was greatly reduced when the SFH preparation was kept at -5°C. The decline in glucose utilization appeared to follow an exponential course at this temperature. At +5°C the activity of SFH was found to be well maintained until about the 8th day of storage. Thereafter the activity rapidly decreased. When glucose (0.1%) was added to the SFH, maintenance of the activity of the SFH was extended by 3 or 4 days. The protective effect of glucose on the enzymes had previously been observed with intact red cells during incubation. The inferior preservation of the SFH at -5°C compared to that at +5°C possibly may be attributable to damage caused to some of the enzymes in the SFH during the freezing and thawing process.

The experiment was repeated with a sample of ACD-preserved blood on the 1st and 50th days of storage. SFH was prepared from KCl washed and unwashed red cells and the preparations were kept at 5°C. The results are illustrated in Figure 20. It is evident that the behaviour of the SFH prepared from fresh and from out-dated blood with regard to the rate of loss of activity was similar. However, in both the blood samples the SFH prepared from unwashed cells maintained the activity to utilize glucose for longer than did the washed cell preparation. The superior preservation may be attributed to the presence of glucose in the SFH prepared from the unwashed specimen.

The decrease in glucose utilization of SFH during storage has been attributed to the decrease in activity of the enzyme G-6-P DH in the preparation (116). If this be true, on prolonging storage of SFH, the reduction in the activity of

FIGURE 20

Influence of Duration of Storage on the Glucose Utilization of SFH Prepared from Washed and Unwashed Erythrocytes.

Legend

A SFH from washed cells from fresh blood.

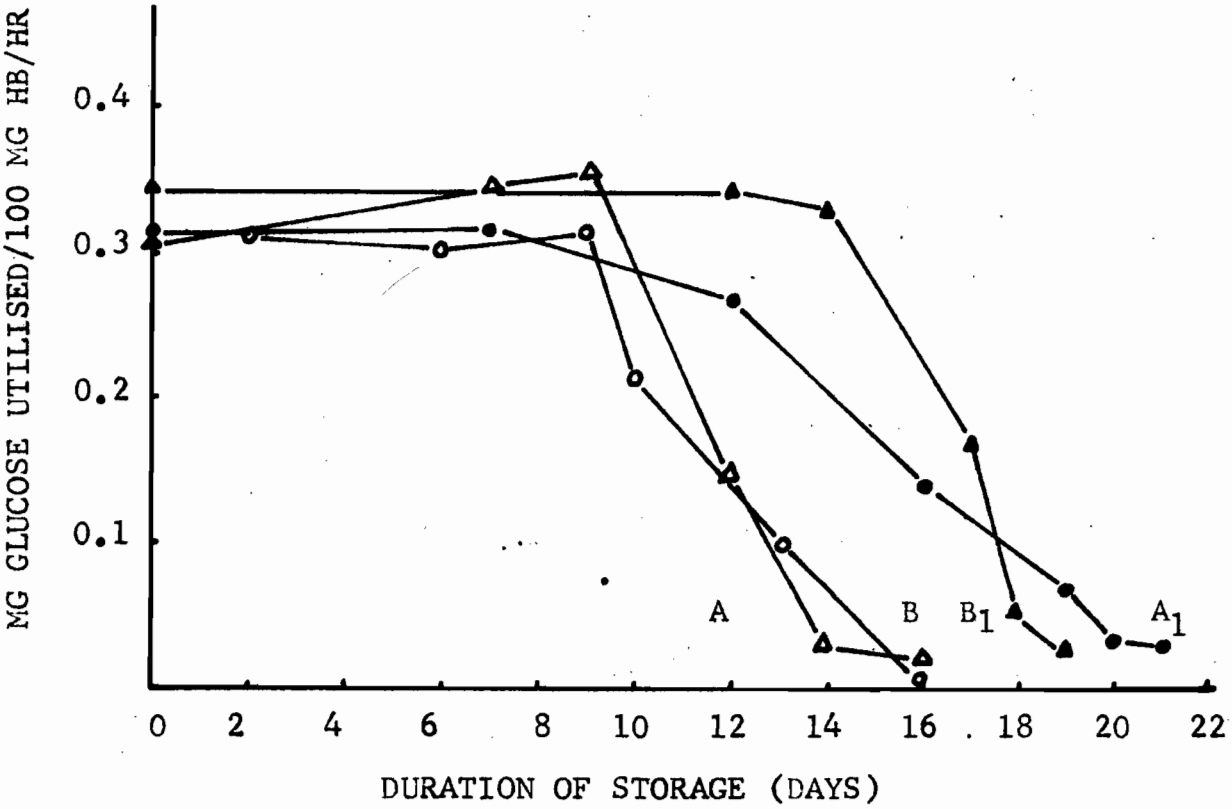
A_1 SFH from unwashed cells from fresh blood.

B SFH from washed cells from out-dated (50-day-old) blood.

B_1 SFH from unwashed cells from out-dated (50-day-old) blood.

Conditions: SFH samples were stored at $4^{o}C$.

FIGURE 20


MG GLUCOSE UTILISED/100 MG HB/HR
0.4
0.3
0.2
0.1
A
B
B1
A1
0
2
4
6
8
10
12
14
16
18
20
22
DURATION OF STORAGE (DAYS)

G-6-P DH may be expected to reach the stage where the hexokinase catalyzed reaction no longer is the rate limiting step in the reaction sequence. In order to test the validity of this explanation, the ability of the SFH to utilize glucose and G-6-P as the substrate was estimated during the preservation of SFH at $5^{o}C$. The effect of addition of glutathione, glucose or TPN to the SFH on the activity of the enzyme also was studied.

It is evident from Table XVIII that the decrease in the utilization of glucose during storage of the SFH was indeed accompanied by a reduction in the activity of the enzyme G-6-P DH. Again, the protective effect of glucose and glutathione on the enzyme activity was observed. The presence of TPN in the SFH, however, did not produce any beneficial effect in the maintenance of the enzyme activity. Chung and Langdon (193) have reported that TPN, the coenzyme of G-6-P DH was found to activate the enzyme as well as to protect it against inactivation. The prevention against loss of the activity was not observed in this experiment.

There is no experimental evidence that hexokinase activity is maintained in stored SFH. Nevertheless, careful examination of the results in Table XVIII shows that the activity of hexokinase as well as G-6-P DH must also undergo alteration. For instance the decrease in the G-6-P DH activity from 4.8 to 1.5 corresponds to a reduction of the activity to less than 70% of the original value. However, with glucose as substrate, the corresponding reduction in overall activity

TABLE XVIII

Influence of Glucose and Glutathione on Hexokinase and G-6-P Dehydrogenase Activity of SFH during Storage at 5°C.

Duration of Storage (days)	REAGENT ADDED									
	Control		Glucose (0.5mM)		Glutathione (GSH (0.1%)		Glucose (0.5mM) + GSH (0.1%)		TPN (2.4mM)	
	HK Act.*	G-6-P DH Act.	HK Act.	G-6-P DH Act.	HK Act.	G-6-P DH Act.	HK Act.	G-6-P DH Act.	HK Act.	G-6-P DH Act.
0	0.37	5.1	0.33	5.0	0.35	5.2	0.34	4.8	-	-
1	0.35	5.1	-	-	0.37	5.2	-	-	0.40	7.1
3	-	-	0.35	4.8	0.35	5.1	0.35	4.7	0.39	7.3
5	0.35	5.2	0.36	4.7	-	-	0.36	4.5	0.30	7.1
8	0.31	4.8	0.34	4.9	0.35	5.2	0.33	4.6	0.21	6.8
9	0.06	1.5	0.18	2.8	0.24	4.3	0.30	4.3	0.19	3.0
10	0.01	0.8	0.14	2.2	-	-	0.31	4.1	0.11	0.3
11	0.01	0.9	0.13	1.1	0.20	3.4	0.25	3.3	-	-
13	0	0.7	-	-	0.18	3.0	-	-	0	0

* "Act." = activity

(0.31 → 0.06) was over 80%. Analysis from other results in Table XIII similarily shows that the rate of decrease of G-6-P DH and the succeeding reactions was less than that of the overall decrease in activity. Thus a decrease in hexokinase activity must have occurred to account for the large decrease in glucose utilization. The results are consistent with Kashket's observation that the activity of hexokinase in hemolysate and SFH preparation diminishes fairly rapidly during storage of the preparation for several days at $5^{o}C$ (43).

DISCUSSION

The lack of a standard assay for the estimation of hexokinase in the red blood cell has presented an obstacle to the elucidation of problems concerning the utilization of glucose by red cells during preservation. The question as to whether hexokinase remains stable in red cells during storage has arisen mainly out of the differences in the method of assay used and the conditions under which the enzyme was studied. In view of the difficulties involved in the isolation and purification of hexokinase from the erythrocytes, it has not as yet been possible to isolate the enzyme in pure form and to study it independently of other glycolytic enzymes.

The assay of hexokinase in intact red cells offers the advantage that the activity of the enzyme may be studied in the intracellular environment in which they are preserved. The methods involve either the measurement of the rate of disappearance of glucose or the production of G-6-P. On the other hand, this indirect approach may not be specific for the assay of the enzyme activity. For instance, the accumulation of glycolytic intermediates may influence the rate of the hexokinase reaction considerably thus making the result liable to misinterpretation by failing to take into account the activity of other enzymes. The use of methylene blue (MeB) in the hexokinase assay system to enhance the utilization of glucose through the hexose-monophosphate shunt in the red cells also

is open to criticism since the dye stimulates both TPN and DPN redox systems non-specifically. The measurement of the hexokinase activity from the rate of oxygen uptake of the system may be influenced by various factors. It has been reported that MeB may stimulate hexokinase (115). Furthermore, the rate of oxygen consumption in the presence of MeB may be influenced by reactions involving the coenzymes TPN or DPN as well as by the hexokinase reaction.

The assay of hexokinase in the SFH makes possible a more thorough study of the enzyme since the composition of the assay system and the concentration of the various reactants may be adjusted at will. In the assay method used in the present investigation the hexokinase activity can be studied under a more strictly controlled condition by using G-6-P as the substrate to determine the behaviour of other enzymes involved in the assay. This method is therefore more reliable for estimating the actual change in the activity of the enzyme itself while the influence of other factors on the activity of the enzyme can be accurately analysed. With the intact cell, one can demonstrate the significance of the concentration of ATP in the hexokinase activity. The proof of the stability of the apo-enzyme of hexokinase during the preservation of red cells, on the other hand, depends on the determination of the enzyme activity in the hemolysate. It should be mentioned, however, that the hexokinase in the SFH was assayed under the optimum conditions for the enzyme,

hence the estimated value may not necessarily represent the activity of the enzyme system under the intracellular conditions.

The view that the stability of hexokinase is the sensitive point of the multi-enzyme glycolytic system of the erythrocyte storage is based on the observation that the activity of the hexokinase system declines during storage of blood. Kashket (43) claimed that the hexokinase of the red cell is inactivated irreversibly during preservation of blood at $5^{o}C$ such that after 4 weeks of storage, 40% of the activity was lost. It has been observed also by various workers that addition of the intermediates of the shunt preserved blood specimens induced the synthesis of ATP thus the glycolytic pathways involved in the ATP-generating reactions are believed to remain intact during storage of blood. The glycolytic failure therefore must be attributed to the inactivation of hexokinase or some early steps in glycolysis. It was postulated also that since the hexokinase reaction is the rate-limiting step in the glycolysis in the erythrocyte, a small diminution in the activity of this enzyme may lead to a decrease in the ATP reserve, and reduce the glycolytic activity of the cell. However, no conclusive evidence is available to support the hypothesis that the instability of hexokinase is the primary cause of the metabolic failure in the red cells during blood preservation. On the other hand, the author has provided evidence in favor of the stability of the apo-enzymes of hexokinase, G-6-P DH and 6-PGA DH in red cells during preservation in ACD or CD medium at $5^{o}C$ for a period of 50 days. (Figure 4 and Table IV.)

Also, it was demonstrated that during incubation of the red cells at 30°C, the glycolytic activity of erythrocytes decreases in spite of the stability of hexokinase. (Table V) Furthermore, various investigators have shown that the glycolytic activity of preserved red cells may be restored when the cells are introduced into the circulation. This state is possible only if the enzymes of the glycolytic system remain intact during the preservation of blood. Hexokinase must therefore remain stable and at least potentially active, in the preserved red cells. This evidence disproves the hypothesis that the diminution of the activity of the enzyme itself, either by denaturation or progressive inactivation, is responsible for the decline of metabolic activity during storage of the red cells.

Any reduction in the ATP or ADP content during storage of blood would result in impairment in the metabolism of the red cells. Prankerd (194) has shown that when the ATP content falls below a critical level of about 0.3 µmole per ml cells, the phosphorylation of glucose cannot take place. This level of ATP occurs in ACD preserved cells at 5°C about the third week of storage (Figure 3). The time corresponds closely with the time of onset of the rapid loss of viability of the red cells under these conditions (Figure 5). A diminution in the ADP concentration in the cell would result in a diminished activity of triose phosphate dehydrogenase which is necessary for the regeneration of the high energy compounds

1,3-DPG and ATP. It has been observed recently that the activity of the ATPase system, which hydrolyses ATP to AMP controls the ADP content and thus regulates the glycolytic activities of the red cell membrane (195).

Another factor which participates in the control of the utilization of glucose is the concentration of G-6-P. The accumulation of this product inhibits the activity of hexokinase and thus its removal increases the hexokinase reaction. This was shown by the addition of G-6-P DH to the assay system in our study (Table XVI). The level of G-6-P normally depends on the activity of phosphofructokinase which also is found to undergo diminished activity in red cells during storage in the cold. Rose et al. (115) have demonstrated that the glucose utilization by intact red blood cells is inversely proportional to the concentration of intracellular G-6-P. Control of the G-6-P content by the action of other enzymes of the glycolytic or the pentose phosphate system may therefore, directly or indirectly, play an important role in the kinetics of glycolysis in the steady state.

The increase in hydrogen ion concentration, as a result of the accumulation of lactic, pyruvic and phosphoric acids in preserved erythrocytes, causes a diminution in the hexokinase activity and hence in the glycolytic activity. However, since a decrease in the hydrogen ion concentration from pH 7.4 to 6.8 resulted only in a decrease of about 7% in the activity of hexokinase (Figure 10). The significance of this factor

thus is questionable with respect to the relative large reduction in the glycolytic activity during the storage period during which the above mentioned change in pH occurred.

The reduction in DPN content of blood during cold storage apparently is not a primary factor since DPN is relatively more stable than other coenzymes (e.g. ATP) during the storage of blood in the cold. Rapoport (133) has observed that the activity of triosephosphate dehydrogenase decreases markedly before the onset of significant reduction in DPN content during aging of the cells *in vivo*.

It was found in the present study that high concentrations of the inorganic phosphate ion inhibit hexokinase in the SFH (Figure 18). The results are consistent with the finding by Chapman *et al.* that high concentrations of inorganic phosphate inhibit glycolysis both in intact cells and in the hemolysate (55). The inorganic phosphate content of the red cell increases rapidly with progressive hydrolysis of organic phosphates during storage. For instance, in blood specimens preserved in the ACD medium at $5^{o}C$, the inorganic phosphate concentration rises to about 8 μ mol/ml of blood, corresponding to about 6 times the initial concentration in the fresh blood. This level of phosphate ion would produce an inhibition of greater than 10% in the hexokinase in the SFH (Figure 18). Since the rate of escape of inorganic phosphate ion is extremely small in the red cell at $5^{o}C$, the increase in the intracellular inorganic phosphate concentration must produce an increasing inhibitory effect on the hexokinase during storage of the blood. This

may account for the circumstance that even when nucleotides are added to preserved blood, the glycolytic activity of the cell is not significantly improved in spite of the induced increase in ATP concentration.

The mechanism of the inhibition of hexokinase by high inorganic phosphate level is not known. It was found that the adenine nucleotides (ATP, ADP and AMP) also inhibit the enzyme (Table X). Crane and Sols(94) have provided evidence indicating that brain hexokinase possesses, in addition to two binding sites (A,B) for glucose and ATP respectively, a third site (C) specific for the binding of G-6-P or its phosphorylated analogue. The site C presumably is located in between A and B and serves as the receptor for the reaction product. It is plausible therefore, that the inhibition of adenine nucleotides or inorganic phosphate is brought about by the inactivation of this receptor site either by the ionic effect or by competitive inhibition. It is possible also that high concentration of these components simply precipitate the Mg^{++} as an inactive complex and thus make unavailable the metallic ion cofactor required by hexokinase.

The observed effects of inhibitors on the hexokinase activity stress the importance of SH-groups in the catalytic functioning of the enzyme. The observation that inhibitors of active transport, such as ouabain, exert no inhibitory influence on hexokinase appears to indicate that the enzyme may not be the mediator for the transport of glucose through the cell membrane of the red cell, since this is where the ATPase system is thought to play a role.

The finding that glucose and glutathione exert protective action on hexokinase, in both the intact cells and the SFH, is of special interest. Presumably the enzyme is more stable in the form of a complex with the substrate glucose. This explanation is consistent with the observation by Pappius (89), that if the red cells run out of glucose, even temporarily, subsequent addition of the sugar fails to restore the capacity of the cell to utilize the substrate. Glucose may serve also as the source of energy for maintaining the integrity of the enzyme. Glutathione stabilizes hexokinase possibly by protecting the essential free thiol (SH) group against oxidation or other alteration.

The finding from our studies, that the red cell hexokinase is stable during preservation of blood at $5^{o}C$, while the enzyme in SFH stored at this temperature, was found to be unstable may imply that the enzyme exists in a protected state in the intact cell. Such protection may be afforded by the endogenous glucose, glutathione or other reducing substances. The former two compounds have been found in an undiminished concentration even in out-dated blood provided sufficient glucose is present in the extracellular medium.

The enzymes responsible for the utilization of glucose are, for the most part, soluble ones which are enclosed by the intracellular membranes. However, the importance of other enzyme systems in the cell membrane should not be overlooked. The incorporation of radioactive compounds into glutathione,

for instance, is an interesting aspect of the metabolism of red cell stroma. The occurrence of DPNase and ATPase in the membrane also may play a role, either directly or indirectly, in the utilization of glucose. It follows that the co-operation of the enzymes of the membrane and the soluble components of the cell possibly may be an important factor in the regulation of the glycolytic system. It is therefore not surprising that one or more factors which activate or stabilize the enzyme hexokinase, may be present in the erythrocyte. The existence of this factor is evidenced by the observed difference in activity with washed and unwashed red cells (Figure 14). Also the finding that when extracts of the washings were reintroduced into the assay system of the washed cells, a partial restoration of hexokinase activity comparable to unwashed cells was achieved (Table XIV).

Although the apo-hexokinase remains fully active during storage of blood at $5^{o}C$ for more than 7 weeks, some alteration in the physical state of the enzyme is indicated from the results of our study. It was found that there is a small but consistent difference in hexokinase activity between the young and old cells of fresh blood (Table XII). In addition, it appears that the hexokinase in stored blood is more susceptible to thermal denaturation than that in fresh samples (Figure 12 a).

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