THE ASSAY OF HEXOKINASE ACTIVITY OF THE ERYTHROCYTE

by

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PREFACE

The work described in this thesis is part of a larger program of research on the preservation of blood. The research has been in continuous progress since the outbreak of World War II in 1939. At that time, the National Research Council, anticipating the extensive need for blood in the treatment of military and civilian casualties, requested Professor J.B. Collip, Head of the Department of Biochemistry at McGill University, and Chairman of the Committee on Medical Research of the National Research Council, to undertake research on the improvement of methods for the preservation of blood. The project was assigned to Dr. O.F. Denstedt, under whose direction it has been continued to the present time. This program has included investigations on several physical and chemical aspects of blood preservation, as well as metabolic studies on the erythrocyte. Since the end of World War II, the program has been supported by the Defence Research Board of Canada.

Initially, the research carried out in this laboratory was concerned with the effects of storage on the viability of erythrocytes, as determined by their post-transfusion survival. This work led to the development of improved preservation media. In order to obtain a better understanding of the changes that occur in blood storage, extensive
studies on the glycolytic activity and electrolyte balance of erythrocytes were carried out. This work was extended to include a comparison of the enzyme activity in reticulocytes and mature cells, and investigations on the pentose phosphate metabolic pathway. The utilisation of purine nucleosides and their effect on preserved blood were investigated by several workers in the group.

Kashket undertook a study of the hexokinase system in erythrocytes; his findings led him to believe that the failure of this system during blood preservation is responsible for the progressive inability of the cells to utilise glucose. However, Prevost, also in this laboratory, developed an improved hexokinase assay method and obtained results which did not confirm those of Kashket. Prevost found that the hexokinase activity is not diminished over a storage period of 30 days.

Thus, the purpose of the work described herein was to further investigate the hexokinase activity of stored erythrocytes, in the hope of settling the question of the stability of the enzyme during storage. In the initial part of this work, the writer used the glucose utilisation assay procedure developed by Prevost. However, this method proved unsatisfactory due to the nature of the measurements involved, and the results obtained were inconsistent. It was therefore decided to apply a more direct and precise method for the assay of hexokinase
activity, that is, the spectrophotometric measurement of TPNH in the hexokinase-glucose-6-phosphate dehydrogenase system. The development of this assay method, and its use in the investigations on hexokinase activity constitute the latter part of this work.
ACKNOWLEDGEMENTS

I am most grateful to Dr. D.F. Denstedt, my research director, for his patient guidance and encouragement during this study. The helpful suggestions of Dr. David Rubinstein are also acknowledged with thanks.

I am deeply indebted to both Miss Marlene Emblem and Dr. Arlene Maximchuk for proofreading this material in its various stages of preparation. Mrs. Roberta Jack has been extremely conscientious in the typing of this thesis.

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<table>
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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP, ADP and ATP</td>
<td>adenosine mono-, di- and triphosphate</td>
</tr>
<tr>
<td>CD, ACD, CPD</td>
<td>citrate-dextrose medium, acid citrate-dextrose medium, citrate-phosphate-dextrose medium</td>
</tr>
<tr>
<td>2,3-diPG</td>
<td>2,3-diphosphoglycerate</td>
</tr>
<tr>
<td>DPN, DPNH</td>
<td>diphosphopyridine nucleotide, reduced DPN</td>
</tr>
<tr>
<td>F-6-P, F-1,6-diP</td>
<td>fructose-6-phosphate, fructose-1,6-diphosphate</td>
</tr>
<tr>
<td>G-6-P, G-6-P DH</td>
<td>glucose-6-phosphate, G-6-P dehydrogenase</td>
</tr>
<tr>
<td>Hb</td>
<td>hemoglobin</td>
</tr>
<tr>
<td>HMP</td>
<td>hexose monophosphate</td>
</tr>
<tr>
<td>M, mM</td>
<td>molar, millimolar concentration</td>
</tr>
<tr>
<td>2-PG, 3-PG</td>
<td>2- and 3-phosphoglycerate</td>
</tr>
<tr>
<td>6-PGl, 6-PGl DH</td>
<td>6-phosphogluconic acid, 6-PGl dehydrogenase</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>R-1-P, R-5-P, R-1,5-diP</td>
<td>ribose-1-phosphate, ribose-5-phosphate, ribose-1,5,-diphosphate</td>
</tr>
<tr>
<td>Ru-5-P</td>
<td>ribulose-5-phosphate</td>
</tr>
<tr>
<td>SFH</td>
<td>stroma-free hemolysate</td>
</tr>
<tr>
<td>SH</td>
<td>sulphhydryl</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TPN, TPNH</td>
<td>triphosphopyridine nucleotide, reduced TPN</td>
</tr>
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</table>
INTRODUCTION

Erythrocyte Metabolism

The metabolism of the mature mammalian erythrocyte can be considered anomalous in relation to most types of somatic cells, including those from which the erythrocyte is derived. Erythropoiesis takes place mainly in the bone marrow, and is presumed to occur in the following stages: stem blast cell → erythroblast → normoblast → reticulocyte → erythrocyte. The cells up to and including the normoblast stage have a well defined nucleus, whereas the reticulocyte contains only stainable granules believed to be ribonucleoprotein. The mammalian erythrocyte lacks both the nucleus and stainable granules. Rubinstein et al. (1) found that reticulocytes show considerable respiratory activity, which is inhibited by fluoride and malonate and stimulated by succinate, and showed that they possess both tricarboxylic acid and cytochrome systems. A comparison of the enzyme activity of blood samples containing a large proportion of reticulocytes to that of normal blood specimens showed that the activity of succinic dehydrogenase, aconitase and cytochrome oxidase is lost during maturation, while that of fumarase, hexokinase and pyrophosphatase is very much reduced. In the process of maturation, the respiratory capacity of the cell is lost and the metabolic activity greatly reduced. Thus,
the energy requirement of the mature erythrocyte is derived largely through the glycolysis of glucose to lactate, and, to a lesser extent, through the oxidation of glucose by way of the hexose monophosphate shunt and pentose phosphate metabolic pathway. These pathways are illustrated in Figure 1.

Chapman et al. (2) determined the optimum conditions for the conversion of glucose to lactate in both intact erythrocytes and the hemolysates from human blood. In the optimum range of hydrogen ion concentration, namely between pH 7.8 and 8.6, the cells produce 2 moles of lactate per mole of glucose utilised. In addition, there is a definite requirement for inorganic phosphate, while the rate of glycolysis is independent of the external Mg++ ion concentration. In the reconstructed hemolysate system, containing added ATP, DPN, Mg++ and inorganic phosphate and at the optimum pH 8.1, glucose is converted to lactate also in the molar ratio of 1:2. The rate of glycolysis in the hemolysate is comparable with that in the intact cells. Measurement of the glycolytic enzyme activity in the hemolysate system showed that the enzymes concerned with ATP-utilising reactions have lower activity than those concerned with ATP-generating reactions, hexokinase having the lowest activity of all.

The literature concerning the properties of hexokinase is reviewed in detail in the following chapter. Rapoport et al. (3)
FIGURE 1

The Glycolytic and Pentose Phosphate Metabolic Pathways of the Erythrocyte.

Glycolytic Pathway:

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Enzyme</th>
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<tbody>
<tr>
<td>1</td>
<td>Phosphorylase</td>
</tr>
<tr>
<td>2</td>
<td>Phosphoglucomutase</td>
</tr>
<tr>
<td>3</td>
<td>1-phosphogluokinase</td>
</tr>
<tr>
<td>4</td>
<td>Glucose-1,6-diphosphatase</td>
</tr>
<tr>
<td>5,7</td>
<td>Hexokinase</td>
</tr>
<tr>
<td>6</td>
<td>Phosphoglucoisomerase</td>
</tr>
<tr>
<td>8</td>
<td>Phosphofructokinase</td>
</tr>
<tr>
<td>9</td>
<td>Aldolase</td>
</tr>
<tr>
<td>10</td>
<td>Triosephosphate dehydrogenase</td>
</tr>
<tr>
<td>11</td>
<td>Phosphoglyceric kinase</td>
</tr>
<tr>
<td>12</td>
<td>Diphosphoglyceric mutase</td>
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<td>13</td>
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<td>15</td>
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</tr>
<tr>
<td>16</td>
<td>Pyruvic kinase</td>
</tr>
<tr>
<td>17</td>
<td>Lactic dehydrogenase</td>
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</table>

Pentose Phosphate Pathway:

<table>
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<th>Enzyme</th>
</tr>
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<tbody>
<tr>
<td>18</td>
<td>Nucleoside Phosphorylase</td>
</tr>
<tr>
<td>19</td>
<td>Phosphoribomutase</td>
</tr>
<tr>
<td>20</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>21</td>
<td>Gluconolactonase</td>
</tr>
<tr>
<td>22,23</td>
<td>6-phosphogluconic dehydrogenase</td>
</tr>
<tr>
<td>24</td>
<td>Pentose phosphate isomerase</td>
</tr>
<tr>
<td>25</td>
<td>Ketopentose phosphate isomerase</td>
</tr>
<tr>
<td>26, 28</td>
<td>Transketolase</td>
</tr>
<tr>
<td>27</td>
<td>Transaldolase</td>
</tr>
</tbody>
</table>
Pentose metabolic pathway represented by solid lines and glycolytic pathway by broken lines.
studied the characteristics of anaerobic glycolysis and of hexokinase activity in erythrocytes. The activity of hexokinase was found to be low compared to that of other glycolytic enzymes; hexokinase activity and glycolytic capacity show similar pH optima; there is a parallelism of the two processes on maturation of erythrocytes, and in different species. These observations suggest that the reaction catalysed by hexokinase is rate-limiting in the glycolytic sequence.

A correlation can be made between the activity of the glycolytic enzymes and the steady-state concentration of the glycolytic intermediates. With the aid of ion-exchange chromatography, Bartlett (4) was able to separate and determine quantitatively several of the phosphorylated intermediates and the nucleotide cofactors of the glycolytic system. The intermediate that occurs in the greatest amount is 2,3-diphosphoglycerate (2,3-diPG), which is present in concentrations up to 5 μmoles per ml of red blood cells. Other intermediates that accumulate are glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), fructose-1,6-diphosphate (F-1,6-diP), 2-phosphoglycerate (2-PG) and 3-phosphoglycerate (3-PG). ATP, ADP, and AMP were found in concentrations of 1.0, 0.2 and 0.02 μmoles per ml cells, respectively. The relatively low activity of phosphofructokinase, aldolase and enolase, as reported by Chapman et al. (2), accounts for the accumulation of their substrates, F-6-P, F-1,6-diP and 2-PG, respectively.
The presence, in the human erythrocyte, of all the enzymes of the hexose monophosphate shunt and the pentose phosphate pathway was demonstrated by Brownstone (5). Murphy (6) found that, in air-CO₂ mixture at pH 7.5, the proportion of glucose metabolised via the hexose monophosphate (HMP) shunt is such that 25% of the potential energy derived from glucose metabolism must be in the form of TPNH, and 75% in the form of ATP. Investigations by deLoecker and Prankerd (7) on the rate of production of C₁⁴O₂ from glucose-1-C₁⁴ by human red cells indicate that 8.5% of the glucose utilisation occurs by way of the shunt pathway. Addition of methylene blue increases the proportion to 70%. Herman et al. (8) found that when TPN is added to a suspension of intact red cells, glucose utilisation through the HMP shunt is increased, while it is only mildly stimulated by TPNH, and is unaffected by DPN and DPNH.

The glucose-6-phosphate dehydrogenase (G-6-P DH) activity of erythrocytes has been extensively studied. Marks et al. (9) and Ramot et al. (10) found evidence for a stroma-bound activator of G-6-P DH. The presence of firmly bound TPN stabilises the enzyme against inactivation by dilution (9) and heat (9,11). The proportion of glucose metabolism proceeding by way of the shunt appears to be limited by the rate of reoxidation of the TPNH which is produced in the G-6-P dehydrogenase and 6-phosphogluconic dehydrogenase (6-PG1DH) reactions. TPNH oxidase, or
methemoglobin reductase, has been purified from human erythrocytes (12,13), and found to be active with oxygen, methemoglobin or cytochrome C as the terminal electron acceptor (12). Methylene blue accelerates the rate of reoxidation of TPNH by TPNH oxidase by serving as an electron carrier (13). This action is probably responsible for the stimulatory effect of methylene blue on the oxidation of glucose via the shunt pathway.

The rejuvenating action of added nucleosides on aged erythrocytes is attributable, in part, to the utilisation of the ribose moiety of the nucleoside via the pentose phosphate pathway. Rubinstein and Denstedt (14) demonstrated that adenosine added to intact human erythrocytes or hemolysates first undergoes deamination to inosine, which is then phosphorolytically cleaved yielding hypoxanthine and ribose-1-phosphate (R-1-P). When intact erythrocytes or hemolysates are incubated with a purine nucleoside, there is utilisation of the ribose and inorganic phosphate and accumulation of the deaminated purine base. There is evidence that the phosphorolysis of the nucleoside is not the rate-limiting step in the metabolic sequence. Gabrio et al. (15) isolated and purified the enzyme purine nucleoside phosphorylase from erythrocytes, and demonstrated that only inosine and guanosine are split readily by the enzyme. During the incubation of fresh human red cells with inosine, Vanderheiden (16) found that the concentration of ribose-1,5-diphosphate (R-1,5-diP) increased greatly, compared to the
amount present in red cells incubated without the nucleoside. He suggests that R-1,5-diP is a co-enzyme in the interconversion of R-1-P and R-5-P, catalysed by the enzyme phosphoribomutase, the occurrence of which was demonstrated in human blood by Guarino and Sable (17).

The viability of the mature erythrocyte is dependent, largely, on the cell content of ATP. However, on maturation from the reticulocyte, the erythrocyte loses its ability to synthesize ATP and other nucleotides de novo. Lowy et al. (18) showed, with rabbit red cells, that the specific radioactivity of the ATP, synthesized in vivo from labeled glycine, decreased exponentially and had a half-life of 13 days. Erythrocytes, in vitro, are unable to incorporate labeled glycine and other small-molecule precursors into ATP, whereas they are able to incorporate labeled formate and 5-amino-4-imidazole carboxamide riboside. Since the plasma levels of the latter two compounds are negligible, Lowy concluded that there is no turnover of ATP in mature erythrocytes, and that the observed decline in radioactivity of the cells in circulation is attributable to dilution of the older cells with newly formed unlabeled cells.

Yoshikawa and Nakao (19) found that hemolysates of fresh human erythrocytes are capable of incorporating adenine-8-C\textsuperscript{14} into ATP, in the presence of inosine, MgCl\textsubscript{2}, DPN, inorganic phosphate, nicotinamide and ATP. Whereas the presence of a catalytic amount of ATP is essential for
ATP synthesis, inosine could be replaced by R-5-P, and DPN by phosphoenolpyruvate. Further investigation by Lowy et al. on rabbit (20) and human erythrocytes (21) confirmed the formation of nucleoside triphosphates from the respective purine and nucleoside components. In human erythrocytes, as in rabbit red cells, there is no synthesis of purine nucleotides from purine ring precursors. In addition, these preparations are unable to convert IMP to adenine nucleotides.

The level of ATP in the erythrocyte is influenced also by the activity of adenylate kinase, which catalyses the reaction: $2\text{ADP} + \text{AMP} \rightarrow \text{ATP}$. Kashket (22) demonstrated the presence of this enzyme in the washed stroma of human red blood cells, as well as in the stroma-free hemolysate. Tatibana et al. (23) found that most of the adenylate kinase activity is in the cytoplasm. The reaction was found to reach a state of equilibrium in less than 20 seconds at $37^\circ\text{C}$. The enzyme activity is relatively constant over a range of hydrogen ion concentrations, from pH 7 to 9. Recently, Cerletti and DeRitis (24) were unable to demonstrate adenylate kinase activity in the erythrocyte membrane, nor a specific affinity of the enzyme for the membrane.

ATP also may be metabolised in the red cell by the action of ATPase, which hydrolyses it to ADP and inorganic phosphate. Laris et al. (25) compared the properties of stromal ATPase and the permeability of erythrocytes to sugars.
The enzyme activity in human and rabbit preparations is inhibited by phloretin, phloridzin and mercuric chloride. These workers suggest that ATPase and the mechanism of sugar transport in the stroma have features in common, but that the action of ATPase is not essential for the permeability of sugars. Investigations by Tatibana et al. (26) showed that 2-azaadenine triphosphate can serve as a substitute for ATP, both as substrate for stromal ATPase and as mediator of potassium transport and sphere-to-bowl shape transformation in aged erythrocytes. It was postulated that the active transport of cations and the maintenance of the shape of the erythrocyte are both determined by a common structural element which involves ATPase activity. Recently, Hashimoto and Yoshikawa (27) demonstrated that the characteristics of erythrocyte membrane ATPase, which is activated by Mg^{++} ions and dependent on the concentrations of Na^+ and K^+, are similar to the properties of the ATP-dependent binding of rubidium ions by erythrocyte ghosts. Thus, it would appear that the activity of ATPase is closely associated with the active transport of cations and the permeability of the erythrocyte membrane to sugars.

Closely related to the metabolism of the erythrocyte is the mechanism of entry of inorganic phosphate (P_1) into the cell. Prankerd and Altman (28) postulated that P_1 is involved in the triosephosphate dehydrogenase reaction in the stroma, since incubation of erythrocytes with P_1^{32}
resulted in a faster rate of labeling of intracellular 2,3-diPG and ATP than of intracellular P\textsubscript{i}. Furthermore, while phosphate exchange across the membrane is inhibited by iodoacetate and sodium fluoride, it is stimulated by adenosine and guanosine. It was suggested that P\textsubscript{i} also enters the cell by means of phosphorolysis of purine nucleosides. When blood was incubated with P\textsubscript{i}\textsuperscript{32}, Bartlett (29) found that the rate of labeling of intracellular phosphate esters was as follows, in decreasing order: adenylyl > hexose phosphate = P\textsubscript{i} > phosphoglycerate. These results indicate that labeled phosphate enters the cell via triosephosphate dehydrogenase, producing 1,3-diPG labeled in the 1 position. Immediate transfer of the labeled group to ATP via 3-PG kinase, then to hexose via hexokinase, would give the observed sequence of labeling. However, DeVerdier (30) has discovered recently that there is a rapid exchange of phosphate between P\textsubscript{i} and the terminal group of ATP, in hemolysates of human blood. The implication of these results is that it is risky to draw conclusions about the glucose metabolism of the red cell based on studies of labeled phosphate incorporation into different intermediates of the erythrocyte.

Another membrane phenomenon associated with the metabolism of the erythrocyte is hemolysis. The osmotic fragility of the cell, or the degree of susceptibility to hemolysis by osmotic agents, is partially dependent on the energy state of the cell.
It is well known that the failure of glycolysis in erythrocytes, either by depletion of glucose or induced by an inhibitory agent, results in increased osmotic fragility of the cells. The osmotic fragility of erythrocytes has been shown to increase with aging of the cells (31); one method of obtaining fractions of different mean cell age is based on differential osmotic hemolysis. This change in osmotic fragility may be related to the metabolic changes, to the changes in the erythrocyte membrane, or to the disc-to-sphere shape changes that occur on aging, or, perhaps, to any combination of these.

Nonmetabolic studies on the protective effect of various substances have shown that nonelectrolytes, to which the membrane is either permeable or nonpermeable, have less protective effect against osmotic hemolysis of erythrocytes than do osmotically equivalent solutions of electrolytes (32). With respect to hemolysis by freezing and thawing, the protective effect of compounds such as sugars, polymers and glycols is related to the concentration of potential hydrogen-bonding groups provided by the compounds (33).

Hexokinase

The literature on hexokinase, including that on the discovery and early investigations of the enzyme, has been reviewed by Kashket (22). The first crystalline preparations of hexokinase were obtained from yeast by Berger (34) in 1946 and by Kunitz and McDonald (35) in 1946, who calculated the
molecular weight to be 96,000. The enzyme has been prepared also in various degrees of purity from other sources. The main sources of the enzyme commercially available at the present time are yeast and muscle. Recently, Agren et al. (36) have developed a column chromatographic procedure for the further purification of the commercial preparations. Although the enzyme has not yet been purified from erythrocytes, Hennessy et al. (37) have prepared a hemoglobin-free protein fraction from erythrocytes which contains hexokinase and other glycolytic enzymes.

Kashket (22) found that the hexokinase of rabbit erythrocytes is characterised by the following Michaelis constants: $K_m(\text{glucose}) \ 2.8 \times 10^{-4} \text{M}$, $K_m(\text{Mg, ATP}) \ 1.5 \times 10^{-3} \text{M}$. At pH 7.8, the rate of phosphorylation of various sugars, in decreasing order, is D-glucose > D-mannose > D-fructose > D-galactose. Sols and Crane (38) concluded that hexose, to be a substrate for the enzyme, must be in the pyranoid form, and that the hydroxyl groups in positions 1, 3, 4, and 6 are involved in the formation of an active glucose-hexokinase complex. Studying the hexokinase activity of the adipose tissue of rats, DiPietro (39) found that the enzyme shows a specific requirement for ATP, is activated by both magnesium and manganese ions, and that it can phosphorylate both glucose and fructose, with $K_m(\text{glucose})$ of $7 \times 10^{-5} \text{M}$ and $K_m(\text{fructose})$ of $3 \times 10^{-3} \text{M}$.

Recent studies with purified yeast hexokinase have
indicated that a stable phospho-enzyme intermediate probably is not formed during the reaction. Hass et al. (40) isolated the enzyme after incubation with glucose and an excess of isotopically labeled ATP under optimal conditions, and they estimated the relative radioactivity of the hexokinase to be 1/500th that of the ATP. However, closer examination of the enzyme revealed the presence of a bound phosphoryl acceptor, presumably glucose-6-phosphate, since it is acid-stable and easily dissociated from hexokinase. Furthermore, the enzyme acts as a phosphoryl acceptor only after further exposure to glucose. Similarly, Trayser and Colowick (41) found no evidence for a phospho-enzyme intermediate when crystalline, phosphate-free hexokinase is incubated with ATP$^{32}$ and Mg$^{++}$ in the presence or absence of glucose. In addition, these authors were able to demonstrate the continuous formation of ADP when hexokinase is incubated with ATP and Mg$^{++}$ ions. They suggested that the enzyme has weak ATPase activity, which is not diminished on further purification of the hexokinase.

Kinetic studies on yeast hexokinase led Fromm and Zewe (42) to conclude that the substrates glucose and ATP add in a random fashion to the enzyme, and that the formation either of an enzyme-phosphate or an enzyme-glucose intermediate is unlikely.

Kashket (22) found that the hexokinase activity of the human erythrocyte exhibits two distinct pH optima, namely, a large peak at pH 7.8 and a lesser one at pH 6.0. However,
Prevost (43) used stroma-free hemolysates of human red blood cells, and was able to determine only one peak, at pH 7.8. Hinterberger et al. (44) found the pH optimum of hexokinase in rabbit erythrocytes to be pH 8.1.

The inhibition of brain hexokinase by G-6-P was first reported by Weil-Malherbe and Bone (45) and confirmed by Crane and Sols (46), who showed that the inhibition is of the noncompetitive type, and concluded that the enzyme must contain at least three binding sites, including one for G-6-P. Kashket (22) demonstrated the inhibition of the hexokinase of rabbit erythrocytes by G-6-P, and also the inhibition of both human and rabbit erythrocyte hexokinase by AMP and ADP. Prevost (43) also demonstrated the inhibition of human erythrocyte hexokinase by G-6-P.

Most investigations concerning the effect of sulphydryl-binding reagents indicate that the presence of free sulphydryl groups on hexokinase are essential for its activity. Sols and Crane (47) found that the inhibition of hexokinase activity by p-chloromercuribenzoate could be reversed by removal of the reagent with added cysteine. Disulfiram (tetraethylthiuram disulphide) is a potent inhibitor of both yeast and brain hexokinase preparations (48). The inhibition can be reversed by the addition of cysteamine and prevented by the addition of the reduced form of glutathione. With yeast hexokinase, Barnard and Ramel (49) showed that the titration of the enzyme with various SH-inhibitors in aqueous systems produced inactivation only at temperatures higher than 30°C. These
authors determined that hexokinase contains 4 free sulphydryl (SH) groups per molecule (M.W.47,000), one or more of which is required for activity. These groups become available for titration only at temperatures higher than 30°C. However, Fasella and Hammes (50) were able to titrate 6 SH groups per 100,000 g of yeast hexokinase with p-mercuribenzoate, in the presence of glucose. Only after the titration is complete does a spontaneous denaturation of the enzyme occur. A similar loss of activity takes place gradually during the titration in the presence of G-6-P. On the basis of these results, it was concluded that sulphydryl groups are not involved in the catalytic process, but help stabilise the active enzyme configuration. Recently, Eldjarn and Bremer (51) demonstrated inhibition of glucose utilisation of human erythrocytes by high concentrations of cystamine and cysteamine. Since these substances have no effect on the utilisation of adenosine or inosine, these workers conclude that the inhibition by the disulphides occurs at the hexokinase level in the system.

Metabolism in Aged Erythrocytes

The metabolic changes that occur during aging of erythrocytes have been studied by numerous investigators. The red cell ages normally in the circulation, and has a mean life span of about 120 days. Using Ashby's technique (52) of differential agglutination to estimate the number of donor cells in the recipient's circulation, and in vivo isotopic
labeling of erythrocytes, it has been possible to distinguish groups of cells of different ages. Some of the alterations in enzyme activity which occur on aging in vivo have also been observed in blood preserved in vitro. However, the characteristics of the storage defect, described by Gabrio and Finch (53), result from the confinement of an aging population of erythrocytes in an isolated medium, which cannot be replenished with essential substrates nor cleansed of waste products. The storage defect in the cells is partially reversible on reintroduction of the preserved blood sample into the circulation.

When provided with a substantial amount of glucose, as is present in the commonly used preservatives, citrate-dextrose (CD) and acid-citrate-dextrose (ACD), preserved red blood cells continue to metabolise this substrate with a resulting accumulation of lactic acid, and a concomitant rise in hydrogen ion concentration. As long as the glycolytic system continues to operate, pyruvate and other intermediate metabolites accumulate. However, with the gradual depletion of substrate and the endogenous energy reserve, ATP, as well as inhibition of certain glycolytic enzymes by their accumulated products and by the increasing hydrogen ion concentration, the steady state equilibrium of glycolysis is disrupted, and the energy-producing mechanism gradually fails. It has been observed that if glucose is added to preserved citrated blood after the endogenous glucose supply
has been depleted, there is a greatly diminished utilization of this substrate (54).

Kashket et al. (55) investigated the effect of the hydrogen ion concentration on the glycolytic changes in blood preserved both in CD and ACD media. At the beginning of storage, the pH of blood in CD medium is 7.4. On the other hand, addition of the relatively acid ACD medium to blood results in an immediate drop in the pH from 7.4 to 7.1. The accumulation of lactate in blood specimens preserved in either medium causes a progressive increase in the hydrogen ion concentration, such that, by the end of the fourth week of storage, the pH of CD-preserved blood is 7.0, while that of ACD-preserved blood is 6.5. In any case, as the pH decreases to 7.0, the activity of hexokinase is depressed, and glycerate-2,3-diphosphatase approaches its maximum activity. These changes in enzyme activity result in a diminished utilization of glucose and increased hydrolysis of 2,3-diPG, with an accompanying increase in the concentration of inorganic phosphate. On account of the differences between the pH of blood specimens preserved in CD and ACD, the changes in glucose utilization and in the concentration of 2,3-diPG and inorganic phosphate proceed from the beginning of storage in ACD medium, but only after the second week of storage in CD medium. Conversely, the concentration of ATP in the erythrocytes of blood stored in ACD decreases only after the second week of storage at 4°C., whereas there is a progressive loss of ATP from the
beginning of storage in CD medium (22). Bartlett and Bar- 
et (56) found relatively little change in the concentration of ADP throughout the storage of blood samples in ACD at 4°C. 

While Kashket (22) found that the activity of hexokinase decreased to 40% of the original level by the 30th day of storage, later work by Prevost (43) indicated that this enzyme remains stable. Lühr and Waller (57) observed no differences in the hexokinase activity of fractions containing erythrocytes of various ages, as prepared by differential osmotic hemolysis of fresh blood.

There is a sharp initial decrease in both the activity of phosphofructokinase (58) and the concentration of its product, fructose-1,6-diphosphate (56), on the storage of blood in ACD at 5°C. However, the rate of the phosphofructokinase reaction does not decrease below that of the succeeding reactions in the Embden-Meyerhof sequence until after the second week of storage.

The activity of triosephosphate dehydrogenase was shown to be decreased in erythrocytes which have aged both in vivo and in vitro (59). Prankerd (60) attributes the decline in activity of this and other stroma-bound enzymes to denaturation of stromal proteins during aging of the erythrocyte. The decreased activity of both phosphofructokinase and triosephosphate dehydrogenase would give rise to a progressive decrease in the concentration of DPN. This would result, in turn, in retardation of the lactic dehydro-
genase activity and an accumulation of pyruvate in the preserved blood sample. Prevost (43) found that pyruvate is distributed unequally between the intracellular and extracellular components of stored blood, the plasma concentration exceeding that in the red cells. The concentration of pyruvate in the cells does not increase appreciably until after the second week of storage in either CD or ACD medium.

There is considerable evidence that the activity of glucose-6-phosphate dehydrogenase (G-6-P DH) decreases with increasing cell age, both in vivo and in vitro. The studies with erythrocytes aged in vivo include those of Marks et al. (61), who obtained fractions of erythrocytes of differing mean cell ages, by means of differential osmotic hemolysis and centrifugation. The fractions richer in the old red blood cells contained less G-6-P DH activity than those richer in younger cells. Similar results were obtained by Lohr and coworkers (59,57), who observed that the G-6-P DH activity of the red cells is reduced to 40% of its original activity by the 40th day of aging in vivo. The enzyme, 6-phosphogluconic dehydrogenase (6-PG1 DH) also is found to be decreased in erythrocytes which have aged in vivo (61).

Palek (62) measured the ability of erythrocytes to produce pentose from glucose during storage of blood at room temperature. A marked decrease was observed both in
the amount of glucose utilised and the amount of pentose
produced, in the first week of storage. Furthermore, the
amount of glucose converted to pentose and the ability of
methylene blue to stimulate the pentose production were
diminished also in the first week. The results were in-
terpreted as evidence that the decrease in activity of
G-6-P DH is greater than that of hexokinase during the
aging of erythrocytes on the storage of blood at room
temperature.

Prankerd (60) has suggested that although the glucose-
6-phosphate dehydrogenase activity falls off, the overall
activity of the HMP shunt is increased during storage.
This increase could be due to an enhanced rate of reoxidation
of TPNH, which, in turn, would result from the increased
production of methemoglobin in aged erythrocytes. It has
been shown that the percentage of total hemoglobin in the
form of methemoglobin increases from 1% to 8% after 80 days
of aging in vivo (57).

Brownstone (5) demonstrated that the activity of
transaldolase and transketolase remains constant in the
stroma-free hemolysates of blood stored at 4°C., and that
there is no impairment of the ability to metabolise ribose-5-
phosphate via the pentose phosphate metabolic pathway. Marks
et al. (61) also found no difference in the activity of purine
nucleoside phosphorylase in erythrocytes of differing age
groups.
In 1955, Gabrio et al. (63) discovered that the addition of adenosine to stored blood effects a restoration of metabolic activity towards normal. Shortly thereafter, Rubinstein et al. (64) found that inosine is utilised at the same rate as adenosine and produces the same effect. The same authors showed that when samples of blood are incubated with adenosine, it is rapidly deaminated to inosine, which then is metabolised to hypoxanthine and ribose-1-phosphate.

Adenosine, when added to preserved blood specimens, induces in the erythrocytes a re-esterification of inorganic phosphate, an increase in the concentration of ATP, hexose-phosphates and 2,3-diPG (63), along with a stimulation of glucose utilisation and, even more so, of lactate production (64). In addition, the deamination of adenosine produces a rise in the pH of the blood sample (65), which is partially responsible for the stimulation of glucose utilisation, since hexokinase is more active at the higher pH. The optimum concentration of adenosine, with respect to the restoration of organic phosphate esters, was found to be 2.5 μmoles/ml red blood cells (63). Adenosine causes an improvement in the post-transfusion survival of stored erythrocytes (66), indicating that the restoration of metabolic activity represents a true restoration of viability of the cells. The addition of both adenosine and inorganic phosphate results in a further increase in the concentration of 2,3-diPG in the red cells, although
there is no alteration in the concentration of ATP, glucose, lactate or pyruvate with respect to the samples containing only added adenosine (64).

Further work by Gabrio et al. (67) established that the relative order of effectiveness of purine nucleosides, according to their restorative effect on phosphorylated esters in preserved erythrocytes is as follows: inosine > adenosine > guanosine > xanthosine. Also, inosine administered intravenously has a much less hypotensive action than adenosine, although all purine nucleosides cause an increase in the urinary uric acid concentration of the recipient (68). Whereas the addition of inosine to preserved blood does not retard the in vitro storage changes in the plasma constituents or in the osmotic fragility of the erythrocytes, it does enhance the post-transfusion survival of the cells (68). It was also found that, in blood stored at 4°C., the period of maintenance of organic phosphate esters is directly proportional to the concentration of inosine added (69). However, in blood stored in ACD containing inosine, there is an impairment in glucose utilisation and a stimulation in the utilisation of the ribose moiety of the nucleoside, as compared to blood stored in ACD-adenosine. This effect is due to the lower pH of the inosine-containing sample (65). When inosine is added to preserved blood of various ages, its capacity to replenish 2,3-diPG is not altered, but its ability to cause resynthesis of ATP diminishes as storage progresses (69,70).
Nakao et al. (71) found that the ATP concentration in 8-week-old blood stored in ACD can be restored only when adenine is added along with inosine or another purine nucleoside. Since adenine-C\textsuperscript{14} is incorporated into the re-synthesized ATP in this case, it was postulated that the purine nucleoside serves as a source of pentose phosphate for the synthesis of ATP from adenine. These investigators later confirmed this, showing that R-5-P can serve as a substitute for inosine in the synthesis of ATP in fresh blood (19). ACD medium containing inosine and adenine is more effective than ACD-inosine with respect to the ability to cause a sphere-to-bowl shape transformation in aged erythrocytes, and the maintenance of post-transfusion survival of the preserved blood sample. However, the effectiveness of the two media is reversed, in relation to their ability to maintain the concentration of 2,3-diPG and hexose mono- and diphosphates (19).

Simon et al. (72) studied the effect of added adenine alone on the preservation of blood in ACD. The addition of adenine enhances the viability and the glycolytic capacity of the erythrocytes and favours maintenance of a slightly increased intracellular ATP level throughout storage. Whereas guanine effects a slight improvement in the viability of the preserved red cells, hypoxanthine and the pyrimidine bases have no beneficial effect on the erythrocytes (73).

Prevost (43) demonstrated that when 3',5'-cyclic AMP
is added to 3-week-old blood preserved in ACD, its effects are similar to those obtained with inosine. There is an increase in pH, stimulation of ribose utilisation, resynthesis of 2,3-diPG and a decrease in inorganic phosphate, although the latter two effects are not as lasting as when produced with inosine.

Early work by Gabrio et al. (74) supported the view that the surrounding medium of erythrocytes preserved in ACD at 4°C had no deleterious effect on the red cells. The osmotic fragility, the partition of phosphate across the cell membrane, and the post-transfusion survival of the erythrocytes were unaffected by the presence of citrate, plasma, leucocytes, reticulocytes or hemolysate in their medium. On the other hand, it has been shown recently that ACD itself interferes with the glycolytic metabolism of the erythrocytes. DeVerdier and Killander (75) compared the metabolism of glucose-1-C¹⁴ in blood to which either heparin or ACD had been added. The concentration of both 2,3-diPG and lactate, as well as the relative amount of isotope appearing in 2,3-diPG, are decreased by the addition of ACD to the blood. Washing and resuspension of the ACD-stored cells in neutral buffer does not restore the rate of production of 2,3-diPG from radioactive glucose. These authors suggest that ACD interferes with the portion of the glycolytic sequence which involves diPG, possibly as a result of chelation of metal ions by citrate. This conclusion,
however, is open to doubt, since Garby (76) has confirmed the findings of the earlier workers. He was able to find no penetration of citrate-C\textsuperscript{14} into erythrocytes from the plasma, either when fresh heparinized blood is incubated at room temperature, or when citrate-C\textsuperscript{14}-ACD blood is stored at 4°C.

Kritzman et al. (77) compared the rate of amino acid uptake by fresh and stored red blood cells. Of the nine amino acids tested, only DL-methionine was found to be taken up by fresh erythrocytes faster than by preserved cells.

Both the age of the erythrocyte and the storage environment are known to influence the ionic gradients across the cell membrane. Borum (78) studied the differences in ionic composition and uptake between specimens of younger and older erythrocytes, obtained by differential centrifugal sedimentation of the red cells in fresh blood. It was found that the older human erythrocytes contain less potassium, chloride and bicarbonate ions than the younger cells, but the two age groups of cells contain comparable amounts of sodium and magnesium ions. Furthermore, a reticulocyte-rich specimen of rabbit cells shows greater uptake of K\textsuperscript{42} and lower uptake of Na\textsuperscript{22} than do mature erythrocytes. It would seem that, on maturation of the reticulocytes and aging of the erythrocytes, there is an alteration in the permeability properties of the cell membrane. Blostein et al. (79) showed that the metabolic control of the cation
transport and gradients which normally is operative at 37°C. is virtually lost at 4°C. At the lower temperature, cation transport across the membrane is influenced by the concentration of inorganic phosphate and the pH, but is not affected by the slow endogenous metabolic activity nor the ATP concentration. Hence the cells cannot maintain the normal cation gradients, and thus tend to lose potassium ions and take up sodium ions. When the temperature is raised to 37°C., the extent to which the cation concentrations are restored to normal does depend on the metabolic activity and ATP content of the preserved erythrocytes.

The osmotic properties of red blood cells are related also to the age of the cells. Young erythrocytes are more resistant than older cells to osmotic hemolysis by hypotonic saline solutions. Marks and Johnson (31) demonstrated this effect on specimens of cells whose age was determined after in vivo labeling with Fe^{59}. Thus, fractions of erythrocytes of differing mean age can be obtained by graded osmotic hemolysis. Jaffe et al. (80) and workers in this laboratory have found that the ability of added adenosine or inosine to enhance the osmotic resistance of erythrocytes is more pronounced in the younger than in the older cells.

The increased osmotic fragility of older erythrocytes is probably due to the loss of intracellular water and other constituents during aging (78). Associated with this, is
the increase in density of the older cells, such that centrifugation of a blood sample produces a sedimentation distribution of the erythrocytes according to age. By means of Fe$^{59}$ labeling technique, Borun et al. (81) proved that the top, middle and bottom layers of centrifuged erythrocytes have youngest, intermediate and oldest mean ages of cells, respectively. Prankerd (82) suggests that the increase in density with age is attributable also to a decrease in lipid content of the cell membrane.

Examination of the structure of erythrocyte membranes with the aid of the electron microscope shows that the membranes of the old cells are thinner, smoother and have a less granular surface than those of young cells (83). Approximately one third of a normal cell population exhibits the membrane characteristics of old cells. However, it has been found that there is no significant alteration in the proportion of structurally old red cell membranes throughout the storage of a blood sample (84). This evidence would suggest that there is a difference between the physiological aging of erythrocytes in vivo and the aging in vitro which is accompanied by the biochemical changes typical of the storage defect.

Recent investigations on the physical properties of hemoglobin indicate that hemoglobin itself changes during the aging of red blood cells. Rosa et al. (85) electrophoretically separated and isolated two distinct fractions
of hemoglobin, and demonstrated that the proportion of the
two fractions varies with the age of the erythrocytes, that
is, the age of the hemoglobin. The hemoglobin was labeled
in vivo by injecting valine-$^{14}$C or arginine-$^{14}$C into the
animal. In another experiment, the same authors (86) sub-
jected isolated hemoglobin, which had been previously labeled
in vivo with Fe$^{59}$, to ion-exchange chromatography. Three
fractions were obtained, which could be related to the
hemoglobin from the young, intermediate and old erythrocytes
in the specimen.

Sandler et al. (87) recently have endeavoured to
reverse the chemical changes that occur during storage of
blood in the cold, by the treatment of specimens preserved
in ACD with a mixture of anion and cation exchange resins.
This treatment results in restoration of the hydrogen ion
concentration of the blood to normal, and a decrease in the
plasma concentrations of K$^+$, Mg$^{++}$, Ca$^{++}$, pyruvate, citrate
and hemoglobin.

Investigations on different methods of blood preserva-
tion include those of Danstedt et al. (88), who found that
red blood cells preserved in citrate-phosphate-dextrose (CPD)
medium exhibit a slight increase in post-transfusion survival
compared to cells stored in ACD. Recent studies by Gibson
et al. (89) showed that the changes in pH, plasma hemoglobin,
potassium concentration gradient, intracellular inorganic
phosphate and organic phosphate concentrations are either
delayed or are less drastic on storage in CPD than in ACD.

There has been much investigation on the technique of preservation of blood at extremely low temperatures. Such a procedure has the obvious advantage of greatly prolonging the storage period. However, the problem of preventing cell damage by ice formation has been a serious one. Rinfret (90) recommends the use of polyvinylpyrrolidone (PVP) as the protective additive, cooling in liquid nitrogen, storage temperature of -100°C or less, and warming in water at 45°C. Under these conditions, 90% of the red blood cells survived the freezing, storage and thawing procedure.
A. General Methods

The chemicals and reagents used were obtained from commercial sources. Glucose-6-phosphate dehydrogenase was purchased from Sigma Chemical Company; the crystalline enzyme preparation was reconstituted to a stock solution which was kept at 5°C and then diluted as required for use. Solutions of ATP, ADP, DPN and TPN were prepared in 0.154 M KCl, and the pH adjusted approximately to 7.5. All pH measurements were done with a "Radiometer Copenhagen pH22" instrument.

Blood from student donors was collected into sterile bottles containing ACD solution, and stored immediately at 5°C. The ACD medium contained 1.32% sodium citrate, 0.44% citric acid, and 1.47% dextrose. The ratio of blood to preservative solution was 4:1 (volume/volume). Occasionally, bottles of unusable blood were obtained through the courtesy of the Montreal Depot of the Red Cross blood transfusion service.

Stroma-free hemolysates (SFH) of the saline-washed red cells were prepared as follows:

A sample of blood, removed aseptically from the storage bottle, was centrifuged in the cold, and the plasma and white cell layer were removed and discarded. The remaining red blood cells were made up to the original blood volume with
cold, isotonic KCl, resuspended and centrifuged. The washing and resuspension were repeated three times. The centrifugation was done with an International Refrigerated Centrifuge, Model PR1, with head #269, at 3000 r.p.m. for 15 minutes at 3-5°C. After the final washing and resuspension, the sample was hemolyzed by rapid freezing in ethanol-dry ice (-78°C) and thawing, repeating the treatment three times. The hemolysed sample was centrifuged in an International high-speed refrigerated centrifuge, Model HR1, at 10,000g, for 30 minutes at 5°C. The clear stroma-free hemolysate was removed, taking care not to disturb the lower cloudy zone and the sedimented pellet. Unless stated otherwise, the SFH samples were used for assay within 12 to 18 hours after preparation, and meanwhile were kept frozen at -15°C.

B. Analyses

1. Glucose.

Glucose was estimated by a glucose oxidase method, using the "Glucostat" reagents obtained from Worthington Biochemical Corporation, Freehold, New Jersey. To 2 ml of a protein-free filtrate, prepared according to the Nelson-Somogyi method (91), were added 2 ml of the glucostat reagent. After exactly 10 minutes, the reaction was stopped by the addition of a drop of 4 N HCl. The stable yellow colour was read in a Coleman Junior spectrophotometer at 400 mp. Standards containing 5 to 25 ug glucose/ml filtrate were
run along with the unknowns.

2. Lactic acid; differential phosphate analysis.

Lactate was estimated by the method of Barker and Summerson (92) as modified by Lepage (93).

Inorganic phosphate was determined by the method of Fiske and Subbarow (94). The organic phosphate esters were determined by a fractional hydrolysis procedure, as described by Pappius et al. (95).

For the analysis of whole blood, the lactate and phosphate were determined on a protein-free filtrate prepared by the addition of 9.0 ml 10% trichloroacetic acid (TCA) to 1.0 ml blood. In the lactate determinations, the TCA-filtrate was further treated with Ca(OH)$_2$-CuSO$_4$. For the analysis of lactate in samples of the hexokinase assay medium, Ca(OH)$_2$-CuSO$_4$ alone was used as the protein precipitant.

3. Hemoglobin.

Hemoglobin was estimated by Brownstone's (5) modification of the method of King (96).

C. Hexokinase Assay

1. Hexokinase assay procedure A.

In the initial part of the work, the hexokinase assay method used was that developed by Prevost (43), in which the rate of glucose utilisation by SFH preparations was measured during incubation at 37°C. The composition of the assay
medium was as follows:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration (Molar)</th>
<th>Volume Added (ml)</th>
<th>Final Concentration (milli Molar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycylglycine buffer (pH 7.7)</td>
<td>0.40</td>
<td>0.40</td>
<td>53.2</td>
</tr>
<tr>
<td>PO₄ buffer (pH 7.7)</td>
<td>0.20</td>
<td>0.20</td>
<td>13.3</td>
</tr>
<tr>
<td>ADP</td>
<td>0.054</td>
<td>0.20</td>
<td>3.6</td>
</tr>
<tr>
<td>ATP</td>
<td>0.075</td>
<td>0.20</td>
<td>5.0</td>
</tr>
<tr>
<td>DPN</td>
<td>0.03</td>
<td>0.20</td>
<td>2.0</td>
</tr>
<tr>
<td>NaOH</td>
<td>0.10</td>
<td>0.35</td>
<td>}</td>
</tr>
<tr>
<td>KCl</td>
<td>0.154</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFH</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.075</td>
<td>0.20</td>
<td>5.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.029</td>
<td>0.25</td>
<td>2.4</td>
</tr>
</tbody>
</table>

For this assay the SFH was diluted, when necessary, so that the hemoglobin concentration in the assay medium was 25 to 30 mg Hb/ml. A pH test first was carried out with a medium containing all the ingredients except NaOH and KCl, and the volume of NaOH required to bring the pH of the medium to 7.75 ± 0.05 was determined by titration. In the preparation of the actual assay medium, the ingredients were added, with the exception of glucose, in the order listed, and the medium was incubated at 37°C for 10 minutes. At zero time, glucose was added; aliquots were removed for analysis of glucose, lactate and hemoglobin; the pH of the medium was checked; the preparation was placed in a water bath at 37°C for 180 minutes. At intervals of 45 and 90 minutes, aliquots of the medium were removed for glucose and lactate determinations, respectively; the final pH was checked at the end of the incubation. The hexokinase activity was determined from the slope of a glucose concentration vs. time plot, and expressed as mg glucose utilised/100mg Hb/hour.
2. Hexokinase assay procedure B.

The development of the optimal conditions for this hexokinase assay procedure will be discussed later. The method consists of the spectrophotometric measurement of the rate of reduction of TPN by the SFH on incubation with glucose, ATP and TPN. The reactions in this assay are catalysed by the enzymes hexokinase, glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase. The assay medium consisted of the following:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration (Molar)</th>
<th>Volume Added (ml)</th>
<th>Final Concentration (milli Molar)</th>
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<tbody>
<tr>
<td>Glycylglycine buffer (pH 7.7)</td>
<td>0.40</td>
<td>0.40</td>
<td>53.2</td>
</tr>
<tr>
<td>PO₄ buffer (pH 7.7)</td>
<td>0.20</td>
<td>0.20</td>
<td>13.3</td>
</tr>
<tr>
<td>ATP</td>
<td>0.075</td>
<td>0.40</td>
<td>10.0</td>
</tr>
<tr>
<td>TPN</td>
<td>0.003</td>
<td>0.30</td>
<td>0.3</td>
</tr>
<tr>
<td>KOH</td>
<td>0.154</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>0.154</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFH</td>
<td>-</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.075</td>
<td>0.40</td>
<td>10.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.029</td>
<td>0.25</td>
<td>2.4</td>
</tr>
</tbody>
</table>

In each case, the SFH was diluted with isotonic KCl to obtain a concentration of 30 mg Hb/ml, and a final concentration in the assay medium of 1.2 mg Hb/ml. The amount of KOH required to bring the pH of the medium to 7.75 ± 0.05 was determined in a test medium, as with assay method A. The medium was made up to 2.75 ml with KCl, in quartz cuvettes with a light path of 1.0 cm, and preincubated at 30°C without glucose. Using a Beckman DU spectrophotometer, readings of the optical density at 340 mp were taken during this period, to ascertain that no reaction was taking place in the absence
of glucose. At zero time, 0.25 ml of glucose was added to the sample cuvette, and 0.25 ml of KCl to the blank. Readings of the optical density were taken at 5-minute intervals until the rate of increase in optical density became constant, usually within 60 to 70 minutes. Between readings, the cuvettes were suspended in a water bath at 30°C. At the end of the assay period, the samples were removed, 1.0 ml of the medium used for hemoglobin determination, and the final pH of the medium checked. Taking the value of 6.22x10³ as the molar extinction coefficient of TPNH at 340 mµ, and a ratio of 2/1 as the molar ratio of TPNH formation/glucose utilisation, the activity was calculated from the rate of the change in optical density, and expressed as mg glucose utilised/100 mg Hb/hour.
EXPERIMENTAL RESULTS

A. Hexokinase Activity Determined by Assay Method A.

1. Influence of added ADP on hexokinase activity of preserved erythrocytes:

One of the aims of this phase of the study was to determine which of contradictory findings, those of Kashket or those of Prevost, are correct, with respect to the stability of hexokinase activity during the preservation of blood. Kashket obtained evidence that the hexokinase of the red cells is labile and tends to decrease in activity during storage of blood (22). Prevost, on the other hand, found no decrease in the activity of the enzyme in blood preserved for 30 days (43). The main difference in the assay methods used by the two workers was that Prevost added ADP to the assay medium.

Accordingly, experiments were carried out, in which the activity of SFH samples, prepared from washed erythrocytes, was assayed with or without added ADP, at intervals during storage of blood specimens at 5°C. In each case, the assay medium was prepared as described for assay method A, (see p.33). When ADP was omitted from the medium, it was replaced by an equal volume of 0.154 M KCl.

The results of experiments a and b, in which blood was preserved in ACD medium, are given in Tables I and II.
and Figure 2. Considering the values obtained in experiment a with the complete assay medium (including added ADP), it is evident that there was no significant change in the hexokinase activity, while the results from experiment b indicate a tendency towards a decrease in the activity after the 9th day of storage. Although the values obtained from the assays without ADP are consistently but only slightly lower than those with ADP, there is no change in the pattern of activity throughout the storage period in either experiment.

TABLE I
Hexokinase Activity of SFH during Blood Storage at 5°C (Experiment a)

<table>
<thead>
<tr>
<th>Duration of Storage (days)</th>
<th>Hexokinase Activity (mg glucose utilised/100 mg Hb/hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With added ADP</td>
</tr>
<tr>
<td>7</td>
<td>0.43</td>
</tr>
<tr>
<td>12</td>
<td>0.40</td>
</tr>
<tr>
<td>15</td>
<td>0.43</td>
</tr>
<tr>
<td>21</td>
<td>0.44</td>
</tr>
<tr>
<td>28</td>
<td>0.43</td>
</tr>
<tr>
<td>34</td>
<td>0.43</td>
</tr>
<tr>
<td>38</td>
<td>0.43</td>
</tr>
<tr>
<td>40</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Conditions:

The hexokinase assay with added ADP is identical to assay method A, described on p. 33. In the assay without added ADP, the ADP was replaced with an equal volume of 0.154 M KCl.
TABLE II

Hexokinase Activity of SFH during Blood Storage at 5°C (Experiment b)

<table>
<thead>
<tr>
<th>Duration of Storage (days)</th>
<th>Hexokinase Activity (mg glucose utilised/100 mg Hb/hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SFH from washed cells + ADP</td>
</tr>
<tr>
<td>0</td>
<td>0.47</td>
</tr>
<tr>
<td>4</td>
<td>0.51</td>
</tr>
<tr>
<td>9</td>
<td>0.55</td>
</tr>
<tr>
<td>14</td>
<td>0.53</td>
</tr>
<tr>
<td>23</td>
<td>0.41</td>
</tr>
<tr>
<td>31</td>
<td>0.39</td>
</tr>
<tr>
<td>37</td>
<td>0.34</td>
</tr>
<tr>
<td>44</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Conditions:

The hexokinase assays with and without added ADP are described under Table I. The SFH from unwashed cells was prepared as indicated on p. 42.

2. Hexokinase activity of SFH preparations from washed and unwashed preserved erythrocytes:

During previous experiments, the writer had observed that washing of the red cells from older specimens of preserved blood, with isotonic saline, resulted in hemolysis of a considerable proportion of the red cells. It is known that erythrocytes become increasingly susceptible to osmotic
Hexokinase Activity during Blood Storage at 5°C: Effect of Added ADP.

I Experiment a
II Experiment b

Legend:

- Assay with ADP
- Assay without ADP

Conditions: As described under Tables I and II.
hemolysis with increase in age (31). Thus, it is reasonable to suppose that the cells which remain after washing the older preserved specimens with isotonic KCl represent the younger portion of the cells from the original, unwashed sample.

The procedure used in the preparation of SFH from washed cells is described on page 31. For the unwashed preparation, whole blood was centrifuged, and the plasma and white cell layer removed. The remaining red cells were resuspended in isotonic KCl, hemolysed by freezing and thawing, and the SFH prepared as previously described.

In experiment b, the blood was stored at 5°C. These results are given in Table II and Figure 3. The succeeding experiments, c, d, and e, were performed on samples of blood stored at 15°C. The results are indicated in Table III and Figure 4. From the rate of depletion of glucose in all four experiments, as shown in Figure 5, it is evident that the increment of 10°C in the storage temperature results in an acceleration of the changes during storage by a factor of approximately two. There was no marked difference between the hexokinase activity of stroma-free hemolysates prepared from washed or unwashed erythrocytes at intervals during storage, other than a tendency of the SFH from the unwashed cells to show a slightly higher activity during the initial 14 days at 5°C, or 7 to 20 days at 15°C. Furthermore, as already noted in the case of experiment b at 5°C, there would appear to be a gradual decrease in the hexokinase
FIGURE 3

Hexokinase Activity in SFH Samples Prepared from Washed and Unwashed Erythrocytes of Blood Stored at 5°C.

Legend:

•••• SFH from washed cells
•••• SFH from unwashed cells

Conditions: As described under Table II.
FIGURE 3

MG GLUCOSE UTILISED/100 MG HB./HR.

DURATION OF STORAGE (DAYS)
activity of both types of samples prepared from blood stored at 15°C.

TABLE III

Hexokinase Activity of SFH during Blood Storage at 15°C.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Duration of Storage</th>
<th>Hexokinase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>days</td>
<td>mg glucose utilised /100 mg Hb/hr</td>
</tr>
<tr>
<td>c</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.56 ± 0.01*</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.41 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>d</td>
<td>0</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.45 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>e</td>
<td>0</td>
<td>0.50 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.48 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>0.41 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.30 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>0.26 ± 0.01</td>
</tr>
</tbody>
</table>

Conditions:

SFH was prepared from washed or unwashed cells as described on p. 42. Hexokinase assay method A is described on p. 33.

* The first figure expresses the mean of duplicate assays, and the second figure represents the deviation.
FIGURE 4

Hexokinase Activity during Blood Storage at 15°C

I  Experiment a
II Experiment d
III Experiment e

Legend:

•—•  SFH from washed cells
○—○  SFH from unwashed cells

Conditions:  As described under Table III
FIGURE 4

I

MG GLUCOSE UTILISED / 100 MG HB./HR.

DURATION OF STORAGE (DAYS)

II

III
FIGURE 5

Utilisation of Glucose in Blood Specimens Preserved at Different Temperatures.

Legend:

Blood stored at 5°C: •—• Experiment b
Blood stored at 15°C: o—o Experiment c
Δ—Δ Experiment d
α—α Experiment e
FIGURE 5

MG GLUCOSE / ML BLOOD

DURATION OF STORAGE (DAYS)
3. Evaluation of the hexokinase assay method A.

During the assay of a given sample of SFH, aliquots of the medium were removed for the estimation of glucose at 0, 45, 90, 135 and 180 minutes, and of lactate at 0, 90 and 180 minutes. The results presented in Table IV show values for the ratio of the total amount of glucose utilised to the total amount of lactate produced, in μmoles/100 mg Hb, over the full assay period of 180 minutes, unless otherwise indicated.

TABLE IV

Relative Proportion of Glucose Converted to Lactate during Hexokinase Assay Procedure A.

<table>
<thead>
<tr>
<th>Duration of Storage</th>
<th>Ratio: Glucose utilised (μmoles/100 mg Hb)</th>
<th>Lactate produced (μmoles/100 mg Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment c</td>
<td>Experiment d</td>
</tr>
<tr>
<td>days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>0.95</td>
</tr>
<tr>
<td>2</td>
<td>1.40</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>1.10*</td>
<td>0.97</td>
</tr>
<tr>
<td>13</td>
<td>0.56</td>
<td>0.78</td>
</tr>
<tr>
<td>20</td>
<td>0.86</td>
<td>1.61</td>
</tr>
<tr>
<td>27</td>
<td>∞**</td>
<td>3.18</td>
</tr>
</tbody>
</table>

Conditions:

SFH preparations from washed erythrocytes

Hexokinase assay method A (see p.33).

* These values were calculated for the initial 90-minute incubation period of the assay. All other values represent the full 180-minute incubation period.

** In this case, there was no net production of lactate.
Except in the assay of the SFH prepared on day 13 of experiment 2, the ratios were all greater than 0.5, and increased further with increasing age of the blood sample. It was evident during the assay, that the glucose is not completely metabolised to lactate, but that there is an accumulation of glycolytic intermediates between these two stages, which occurs to an even greater extent when the rate of glucose utilisation is low, as in older blood samples. This observation raises serious doubts as to whether the hexokinase reaction is rate-limiting, with respect to the rate of glucose utilisation as measured by this assay method.

A further objection to the use of this method for the assay of hexokinase activity is that the rate of glucose utilisation is seldom constant throughout the assay period. Thus, a considerable error is introduced into the calculation of the slope of the glucose concentration vs time plot.

A SFH sample prepared from 15-day-old blood was assayed in media containing, respectively, 2.4, 3.6 and 4.8 mM glucose. The glucose concentration curves obtained are shown in Figure 6. In the assay in which the initial glucose concentration was 2.4 mM, the slope of each segment of the curve is given in the following table, (V). The greatest deviation from the mean is 0.07, or 16%. It is apparent also that the rate of glucose utilisation did not become constant when the initial glucose concentration was increased 1.5 or 2.0 times. Although the duplicate determinations
shown in Table III generally show a deviation of 10% or less from the mean, a possible error of up to 25% could occur in the estimation of the value of the hexokinase activity.

TABLE V

Rates of Glucose Utilisation Throughout the Assay Period.

<table>
<thead>
<tr>
<th>Interval (min.)</th>
<th>Slope (mg glucose/100mg Hb/hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 45</td>
<td>-0.39</td>
</tr>
<tr>
<td>45 - 90</td>
<td>-0.42</td>
</tr>
<tr>
<td>90 - 135</td>
<td>-0.49</td>
</tr>
<tr>
<td>135 - 180</td>
<td>-0.39</td>
</tr>
<tr>
<td>Mean</td>
<td>-0.42</td>
</tr>
</tbody>
</table>

On the basis of these criticisms, we endeavoured to devise a more precise method for the determination of hexokinase activity in samples of SFH.
Glucose Utilisation during Incubation: Assay Method A

Legend:

- Assay i
- Assay ii
- Assay iii

Conditions:

Hexokinase assay method A, using SFH prepared from blood stored in ACD at 5°C for 15 days. The initial concentration of glucose in each assay medium is as follows:

i. 2.4 mM

ii. 3.6 mM

iii. 4.8 mM
FIGURE 6

MG GLUCOSE /100 MG HB.

DURATION OF INCUBATION (MIN.)
B. Hexokinase Activity Determined by Assay Method B.

1. Rationale.

There is ample evidence that human erythrocytes are capable of oxidizing glucose by way of the hexose monophosphate shunt (5,7,8). The reactions involved are the following:

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

\[
\text{G-6-P} + \text{TPN}^+ \xrightarrow{\text{G-6-P DH}} \text{6-Phosphogluconic acid (6-PGL)} + \text{TPNH} + \text{H}^+
\]

\[
\text{6-PGL} + \text{TPN}^+ \xrightarrow{\text{6-PGL DH}} \text{Ribulose-5-phosphate(Ru-5-P)} + \text{TPNH} + \text{H}^+ + \text{CO}_2
\]

Thus, for every mole of glucose utilised, 2 moles of TPN are reduced to TPNH. When a stroma-free hemolysate preparation is incubated with optimal amounts of glucose, ATP, Mg\(^{++}\) and TPN, the rate of production of TPNH should be directly related to the rate of the hexokinase reaction, if the latter is less rapid than the succeeding dehydrogenase reactions.

2. Preliminary experiments.

It was essential to ensure that the SFH preparations contained no glucose-6-phosphatase activity, which would hinder the assay of hexokinase by this method. To this end, a sample of dialysed SFH was incubated at 37\(^\circ\)C with G-6-P in the presence of iodoacetate, which was added to inhibit the triosephosphate dehydrogenase and thus prevent extensive
activity of the Embden-Meyerhof pathway. The amounts of glucose, inorganic phosphate and hexose monophosphate (represented by the stable phosphate fraction) were estimated before and after incubation of the preparation, and the percentage increase or decrease in each was calculated on the time basis of one hour. The results are given in Table VI.

**TABLE VI**
Assay of Glucose-6-phosphatase Activity in SFH

<table>
<thead>
<tr>
<th>Final Hb Concentration (mg/ml)</th>
<th>G-6-P Conc. (mM)</th>
<th>pH</th>
<th>Hexose-monophosphate</th>
<th>Inorganic phosphate</th>
<th>Glucose</th>
<th>Duration of Incubation (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.60</td>
<td>2.0</td>
<td>7.5</td>
<td>(percentage change/hour)</td>
<td>no change</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td>no change</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>3.0</td>
<td>7.0</td>
<td>no change</td>
<td>no change</td>
<td>no change</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>6.9</td>
<td>18</td>
<td>no change</td>
<td>no change</td>
<td></td>
</tr>
<tr>
<td>26.9</td>
<td>2.5</td>
<td>7.0</td>
<td>9</td>
<td>no change</td>
<td>0.7</td>
<td>90</td>
</tr>
<tr>
<td>26.7</td>
<td>2.5</td>
<td>7.5</td>
<td>22</td>
<td>no change</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>

**Conditions:**
SFH and G-6-P were added to give the final concentrations shown. The final concentrations of iodoacetate and glycyglycine buffer (pH 7.7) were 2.0 mM and 53.0 mM, respectively. 0.1 M NaOH was added to bring the medium to the indicated pH. 0.154 M KCl was added to bring the final volume to 6.0 ml.
When estimated at the beginning of the incubation, the stable phosphate fraction, obtained by subtracting the values for 100 minute-hydrolysable phosphate from the total acid-soluble phosphate, was shown to represent the total amount of G-6-P added. Although there was some utilisation of the G-6-P, which could occur via the hexose monophosphate shunt, there was no significant production of either free glucose or inorganic phosphate. We concluded, therefore, that the SFH contains no significant glucose-6-phosphatase activity.

In order to establish the 1:2 stoichiometric relationship between the glucose utilisation and the TPNH production, and determine the extent, if any, of reoxidation of TPNH, the following experiment was carried out. Assay media were prepared as described for assay method B on page 35, except that glucose was replaced by G-6-P in final concentrations of 0.019, 0.029 and 0.038 mM. Readings of the optical density were taken for at least 60 minutes after they reached a steady value. In each of the three samples, there was no decrease in optical density during this period, implying that no reoxidation of TPNH occurred. The theoretical amount of G-6-P utilised, as calculated from the final level of TPNH produced, was compared with the initial concentration of G-6-P in each sample. The results, given in Table VII, indicate that the production of 2 moles of TPNH resulted from the metabolism of each mole of G-6-P.
TABLE VII

Production of TPNH from Glucose-6-phosphate by SFH.

<table>
<thead>
<tr>
<th>G-6-P added (μM/ml)</th>
<th>TPNH produced (μM/ml)</th>
<th>Theoretical amount of G-6-P utilised (μM/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.019</td>
<td>0.038</td>
<td>0.019</td>
</tr>
<tr>
<td>0.029</td>
<td>0.052</td>
<td>0.026</td>
</tr>
<tr>
<td>0.038</td>
<td>0.070</td>
<td>0.035</td>
</tr>
</tbody>
</table>

To verify the rate-limiting role of hexokinase in the assay system, the rate of utilisation of substrate was compared for equimolar amounts of glucose and glucose-6-phosphate as substrate, the initial concentration of each being 2.4mM. It was found that the linear rate of change of optical density was 0.34 OD units/hr. for glucose, and 3.56 for G-6-P, which values correspond to rates of utilisation of 1.99 μmol glucose/100mg Hb/hr. and 21.65 μmol G-6-P/100mg Hb/hr. Calculations based on these findings indicate that the hexokinase reaction is slower than the succeeding dehydrogenase-catalysed reactions by a factor of 10.9.

3. Optimal conditions for assay method B:

The optimal concentrations of glucose, ATP, Mg++ and TPN, as given in the table on p.35, were established in a series of experiments in which the hexokinase activity was
FIGURE 7

Influence of Enzyme Concentration on Hexokinase Activity:
Assay Method B.

**Conditions:** Hexokinase assay method B. The volume of SFH in the medium was varied to give the final concentrations of hemoglobin indicated.
measured at various concentrations of each ingredient of the medium.

The influence of the enzyme concentration, as represented by the hemoglobin concentration of the medium, on the measured hexokinase activity is illustrated in Figure 7. It is apparent that a straight line best represents the trend of the experimental points in the range of 1.0 to 1.4 mg Hb/ml. The concentration of hemoglobin chosen for routine assay was $1.2 \pm 0.1$ mg Hb/ml.

The influence of the hydrogen ion concentration on the hexokinase activity, as measured by this assay procedure, is indicated in Figure 8. The final pH of the medium was adjusted by adding HCl or KOH in amounts predetermined from independent trials. The remainder of the medium was prepared as previously described (see p. 35). It is evident that the enzyme activity is maximum over a fairly broad range - pH 7.2 to 8.0 - and that the activity falls off sharply at pH values less than 7.0.

The temperature at which the assay medium was incubated was arbitrarily chosen as $30^\circ$C. The effect of different incubation temperatures on hexokinase activity is evident in Figure 9, which shows the data obtained from experiments on SFH samples from two bloods of different ages. From Figure 9, it is apparent that the rate of reaction began to fall off at $40^\circ$C, presumably owing to denaturation of enzymes, which would be expected to occur at that temperature.
FIGURE 8

Influence of Hydrogen Ion Concentration on Hexokinase Activity: Assay Method B.

Legend:

- SFH (i)
- SFH (ii)

Conditions: As described in the text, on page 61, using either SFH (i), prepared from 12-day-old blood or SFH (ii), prepared from fresh blood.
FIGURE 8

MG GLUCOSE UTILISED / 100 MG HB / HR.

pH
Influence of Temperature on Hexokinase Activity:
Assay Method B.

I. Hexokinase activity vs Temperature (°C).
II. \( \log_{10}(\text{activity} \times 10) \) vs \( 1/T_{\text{abs.}} \times 10^3 \).

Legend:
- SFH (i)
- SFH (ii)

Conditions: SFH (i) was prepared from 7-day-old blood, SFH (ii) from 36-day-old blood. Hexokinase assays were carried out at the indicated temperatures.
FIGURE 9

I

MG GLUCOSE UTILISED/100 MG HB./HR.

TEMPERATURE (°C)

II

LOG₁₀ (ACTIVITY x 10)

1 / Tₐｂｓ. x 10³
Figure 9,II represents the plot of the $\log_{10}$ activity ($x_{10}$) against the reciprocal of the absolute temperature, $T(x_{10}^3)$. Since the reaction followed zero-order kinetics, that is, the measured initial rate of reaction is linear in every case, the activation energy, $\mu$, can be calculated according to the following equation,

$$\mu = 4.6 \left( \frac{\log_{10} k_2 - \log_{10} k_1}{1/T_2 - 1/T_1} \right)$$

where $k_1$ and $k_2$ represent the rates of reaction (or activity) at temperatures $T_1$ and $T_2$, respectively. From the slope of the lines obtained in Figure 9,II, the calculated values of $\mu$ are 11,200 cal./mole for experiment a and 10,500 cal./mole for experiment b. Kashket (22) found the activation energy for the phosphorylation of glucose by stroma-free hemolysates to be 11,500 cal./mole. From the mean value of $\mu$ for experiments a and b, it was calculated that the mean Q$_{10}$ for the system is 1.8 between 25° and 35°C, and 1.7 between 30° and 40°C.

4. Hexokinase activity of blood during storage at 5°C:

In each of two storage experiments, a and b, the stroma-free hemolysate was prepared from red cells which had been treated in three different ways, and the hexokinase activity of each type of SFH determined by assay method B. The three types of treatment of the cells for the preparation of the SFH were as follows:

(i) Red cells washed with isotonic KCl, as described on page 31.
(ii) Unwashed red cells, as described on page 42.

(iii) Red cells obtained by centrifugation, after 1 volume of blood had been diluted with 4 volumes of hypotonic KCl, and permitted to remain at room temperature for 20 minutes. The unhemolysed red cells in the sample were then washed three times with isotonic KCl, and the SFH prepared as in (i) above.

As stated for similar experiments using assay A, preparation (ii) from unwashed cells would represent the cell contents of the whole erythrocyte population, whereas the washing procedure used in preparation (i) might remove the more osmotically fragile older cells, the proportion of which would tend to increase with the duration of storage. Similarly, an even younger population of cells would be expected to survive exposure to the hypotonic saline used in preparation (iii). Thus, if differences should be observed in the behaviour of the hexokinase activity of the various stroma-free hemolysates prepared at intervals during the storage period, the differences might be related to the general age characteristics of the cell populations represented in the SFH.

The results of experiments a and b are presented in Tables VIII and IX and Figure 10. The concentration of hypotonic KCl used in preparation (iii) was 0.077M (50% of isotonic) on days 4 and 7 in experiment a, and 0.093M (60% of isotonic) in
### TABLE VIII

Hexokinase Activity during Blood Storage at 5°C:
Assay Method B.
(Experiment a)

<table>
<thead>
<tr>
<th>Duration of Storage (days)</th>
<th>Hexokinase activity of SFH prepared from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(i) Cells washed in isotonic solution</td>
</tr>
<tr>
<td></td>
<td>(mg glucose utilised/100 mg Hb/hr.)</td>
</tr>
<tr>
<td>1</td>
<td>0.26</td>
</tr>
<tr>
<td>4</td>
<td>0.29</td>
</tr>
<tr>
<td>7</td>
<td>0.29</td>
</tr>
<tr>
<td>12</td>
<td>0.34</td>
</tr>
<tr>
<td>15</td>
<td>0.34</td>
</tr>
<tr>
<td>18</td>
<td>0.36</td>
</tr>
<tr>
<td>21</td>
<td>0.35</td>
</tr>
<tr>
<td>26</td>
<td>0.32</td>
</tr>
<tr>
<td>29</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>0.37</td>
</tr>
<tr>
<td>*</td>
<td>0.11</td>
</tr>
<tr>
<td>36</td>
<td>0.28</td>
</tr>
<tr>
<td>40</td>
<td>0.29</td>
</tr>
<tr>
<td>48</td>
<td>0.29</td>
</tr>
</tbody>
</table>

**Conditions:**

SFH preparations described in text on p. 66.

* A general power failure occurred on day 31 (see text p. 72)
TABLE IX

Hexokinase Activity during Blood Storage at 5°C: Assay Method B. (Experiment b)

<table>
<thead>
<tr>
<th>Duration of Storage (days)</th>
<th>Hexokinase activity of SFH prepared from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(i) Cells washed in isotonic solution</td>
</tr>
<tr>
<td></td>
<td>(ii) Unwashed cells</td>
</tr>
<tr>
<td></td>
<td>(iii) Cells exposed to hypotonic saline</td>
</tr>
<tr>
<td></td>
<td>(mg glucose utilised/100 mg Hb/hr.)</td>
</tr>
<tr>
<td>1</td>
<td>0.36</td>
</tr>
<tr>
<td>5</td>
<td>0.31</td>
</tr>
<tr>
<td>9</td>
<td>0.32</td>
</tr>
<tr>
<td>14</td>
<td>0.24</td>
</tr>
<tr>
<td>17</td>
<td>0.32</td>
</tr>
<tr>
<td>24</td>
<td>0.32</td>
</tr>
<tr>
<td>30</td>
<td>0.38</td>
</tr>
<tr>
<td>32</td>
<td>0.35</td>
</tr>
<tr>
<td>36</td>
<td>0.35</td>
</tr>
<tr>
<td>42</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Conditions:

SFH preparations described in text, on p. 66.
FIGURE 10

Hexokinase Activity during Blood Storage at 5°C: Assay Method B.

I  Experiment a
II  Experiment b

Legend:

•—•  SFH from cells washed in isotonic solution.
○—○  SFH from unwashed cells
□—□  SFH from cells exposed to hypotonic saline.

Conditions:  As described on p. 66. In Fig I, the dashed line represents the occurrence of the power failure.
FIGURE 10

I

MG Glucose Utilised / 100 mg Hb/hr.

II

Duration of Storage (Days)

0 10 20 30 40 50

0 0.2 0.4 0.6

0 0.2 0.4 0.6

0 10 20 30 40 50

0 0.2 0.4 0.6
every other case. A power failure occurred on day 31 of experiment a, such that the temperature of the blood bank increased to room temperature for a period of approximately 24 hours. In considering the results of experiment a up to the 31st day, and of experiment b, there appears to be no significant difference in the hexokinase activity of the three types of stroma-free hemolysate, and the activity remained relatively constant over the 31- and 42- day periods of storage.

An attempt was made to duplicate the results obtained from experiment a by deliberately interrupting the cold storage with a period of exposure of the blood sample to room temperature. A bottle of fresh blood, specimen A, was stored at 5°C. On the 6th day of storage, 50 ml of the blood was transferred aseptically to another container. From the latter, specimen B, a sample of blood was removed immediately for preparation of the SFH for assay of the hexokinase activity. Specimen B was then permitted to remain at room temperature for 16 hours, after which time it was returned to the blood bank (5°C). This procedure was repeated on day 27: specimen C was removed from bottle A, and kept at room temperature for 24 hours.

Stroma-free hemolysates were prepared at intervals during the storage of each specimen from cells washed in isotonic solution, in the usual manner, and the hexokinase activity was determined by assay method B. The results, given in Table X and Figure 11, indicated that the hexokinase activity was not affected by a period of exposure to ambient temperatures, either early or later in the storage period.
### TABLE X

**Hexokinase Activity during Blood Storage at 5°C: Effect of Exposure of Blood to Room Temperature.**

<table>
<thead>
<tr>
<th>Duration of Storage (days)</th>
<th>Hexokinase activity of SFH prepared from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specimen A</td>
</tr>
<tr>
<td>(mg glucose utilised/100 mg Hb/hr.)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>4</td>
<td>0.32</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>0.30</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>0.33</td>
</tr>
<tr>
<td>27</td>
<td>0.33</td>
</tr>
<tr>
<td>28</td>
<td>-</td>
</tr>
<tr>
<td>34</td>
<td>-</td>
</tr>
</tbody>
</table>

**Conditions:**

Hexokinase assay method B, as described on p. 35.

- Indicates the day on which blood was removed from specimen A, kept at room temperature for 16 hours (specimen B) or 24 hours (specimen C) before being returned to 5°C.

* Assay performed on SFH prepared from blood at the beginning of period of exposure to room temperature.
FIGURE 11

Hexokinase Activity during Blood Storage at 5°C: Effect of Exposure of Blood to Room Temperature.

Legend:
- - - Specimen A
- - Specimen B
- - Specimen C
↓ Exposure of specimen to room temperature.

Conditions:
As described under Table X and in text, on p. 72.
Hexokinase assay method B was used.
FIGURE II

MG Glucose Utilised /100 mg HB./hr.

DURATION OF STORAGE (DAYS)
5. **Hexokinase activity in erythrocytes of different age groups:**

It has been established that the erythrocytes in a given blood sample may be fractionated according to age by means of a graded osmotic hemolysis procedure (31). The following procedure of differential hemolysis was used in our study:

Aliquots of a specimen of blood were diluted with 4 volumes of KCl, of various concentrations from 45% of isotonic to isotonic, as given in Table XI, and allowed to stand at room temperature for 20 minutes. Each sample was centrifuged, as in the initial step of the SFH preparation, described on p.31. The supernatant 'S' was removed, and the remaining red cells washed 3 or 4 times with isotonic KCl, and a SFH prepared. The supernatant 'S' contained the plasma, KCl and the lysate of erythrocytes which were susceptible to osmotic hemolysis at the particular concentration of KCl used. The SFH thus represented only the cells which were resistant to osmotic hemolysis at that concentration of KCl.

Attempts were made to perform a hexokinase assay on the supernatant 'S' samples. However, it was not possible to obtain a 'control' value in the assay, since the 'supernatant 'S' obtained from blood that had been stored for only one week or so contained some residual glucose. The SFH samples prepared from the osmotically resistant erythrocytes were assayed for hexokinase activity by assay method B.
TABLE XI

Hexokinase Activity of Erythrocyte Fractions of Different Osmotic Resistance.

<table>
<thead>
<tr>
<th>Blood Specimen</th>
<th>Duration of Storage (days)</th>
<th>Concentration of KCl (% of isotonic)</th>
<th>Hemolysis Ratio</th>
<th>Hexokinase activity of SFH from resistant cells (mg glucose utilised/100 mg Hb/hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>2</td>
<td>45</td>
<td>0.79*</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>0.45</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>0.08</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>0.00</td>
<td>0.29</td>
</tr>
<tr>
<td>b</td>
<td>8</td>
<td>50</td>
<td>0.38</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>0.05</td>
<td>0.38</td>
</tr>
<tr>
<td>c</td>
<td>33</td>
<td>50</td>
<td>0.95</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>0.68</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>d</td>
<td>50</td>
<td>60</td>
<td>0.92</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td>0.65</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>0.15</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Conditions:

Hexokinase activity determined by assay method B.

\[* Hemolysis ratio = \frac{Hb \text{ conc.}(mg/ml) \text{ of supernatant from given fraction}}{Hb \text{ conc.}(mg/ml) \text{ of supernatant from 0\% fraction}}*\]
If the samples contained sufficient hemoglobin, they were assayed at a hemoglobin concentration of 1.2 mg/ml. Otherwise, the hemoglobin concentration was that obtained by using the maximum volume of SFH permitted by the assay procedure. The results obtained with four blood specimens of different ages are given in Table XI. The concentration of KCl used in the hemolysing step is expressed as % of isotonic: 0% represents water alone, and 100% represents isotonic (0.154M) KCl. The hemolysis ratio for each fraction represents the ratio of the hemoglobin concentration of the supernatant 'S' of a given sample to the hemoglobin concentration of the supernatant 'S' of the 0% sample, and is a measure of the relative amount of hemolysis that has occurred. As the degree of hemolysis increases, the osmotic resistance of the surviving erythrocytes becomes greater and thus, an increase in the ratio represents a progressively younger population of cells. In Figure 12, the hexokinase activity of the SFH from various fractions is plotted against the hemolysis ratio for that fraction. Blood specimens a and b, which had been stored for 2 and 8 days, respectively, show no difference in the hexokinase activity of the various fractions. However, the samples prepared from 33- and 50-day-old bloods, specimens c and d, do show a positive relationship between the hexokinase activity and the hemolysis ratio, thus indicating that the fractions with a higher proportion of younger erythrocytes have the higher hexokinase activity.
FIGURE 12

Hexokinase Activity in Erythrocyte Fractions of Different Osmotic Resistance.

Legend:

Blood Specimen a
Blood Specimen b
Blood Specimen c
Blood Specimen d

Conditions:

Hexokinase activity estimated by assay method B.
Fractions were prepared from blood specimens which had been stored for the following periods:

<table>
<thead>
<tr>
<th>Blood Specimen</th>
<th>Storage Period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>2</td>
</tr>
<tr>
<td>b</td>
<td>8</td>
</tr>
<tr>
<td>c</td>
<td>33</td>
</tr>
<tr>
<td>d</td>
<td>50</td>
</tr>
</tbody>
</table>
FIGURE 12

Graph showing the relationship between MG glucose utilised/100 mg HB/hr and hemolysis ratio.
Whether these results represent a true difference in the fractions representing younger and older erythrocytes, or in the fractionation of fresh and old bloods, or whether the behaviour is merely an artifact resulting from uncontrollable variability in the procedure, is not known. More extensive studies under more strictly controlled conditions are warranted.

6. **Hexokinase activity in stored stroma-free hemolysates:**

Throughout the previous experiments, it became evident that the hexokinase activity diminished during storage of the stroma-free hemolysates. Several SFH preparations from bloods of various ages were assayed for hexokinase activity at intervals during storage at 5°C and -15°C (frozen storage), respectively. For each specimen, the activity measured on the day of preparation was taken as 100%, and the activity of the stored specimens was calculated as a percentage of the original. Figure 13 illustrates the accumulated results obtained with six specimens stored at -15°C (Fig. 13-I) and with two specimens stored at 5°C (Fig. 13-II). The SFH specimens prepared from blood samples less than 1 week old are represented as solid dots; those from blood samples more than 1 week old are represented as open circles.

It is evident that the age of the blood from which the SFH was prepared had no effect on the rate of loss of activity. In the SFH preparations stored at -15°C, the rate of loss of activity appears to follow an exponential-
FIGURE 13

Influence of Duration of Storage of SFH on Hexokinase Activity.

I  SFH samples stored at -15°C.
II SFH samples stored at 5°C.

Legend:
• SFH prepared from blood less than 1 week old.
○ SFH prepared from blood more than 1 week old.

Conditions:
Hexokinase activity estimated by assay method B.
FIGURE 13

DURATION OF STORAGE (DAYS)

PERCENTAGE OF ORIGINAL (DAYS 0) ACTIVITY
type decline, such that only 20% of the original activity remained at the 17th day. As may be expected, the activity decreased much more rapidly in the specimens stored at 5°C than in those at -15°C, there being no measurable activity after 7 days of storage at the higher temperature. A sample of SFH (II) was assayed on the 15th day in three different media; first, with glucose as the substrate, as in assay method B; secondly, with glucose as the substrate, in the presence of added yeast hexokinase; and thirdly, with glucose-6-phosphate as the substrate. Again, the activity is expressed as a percentage of the original 0-day activity.

**TABLE XII**

Hexokinase Activity in SFH Stored at 5°C for 15 Days (I).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Final Concentration</th>
<th>Final Concentration of Hexokinase</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>2.4</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.4</td>
<td>9.3</td>
<td>7.2</td>
</tr>
<tr>
<td>G-6-P</td>
<td>0.08</td>
<td>-</td>
<td>14.9</td>
</tr>
</tbody>
</table>

Although no definite conclusion could be drawn from the results obtained from single additions of an arbitrary amount of these substances, it appeared that a factor other than lability of the hexokinase enzyme is responsible for the apparent loss of hexokinase activity, as measured by
this assay method. Two other explanations were considered: the decrease might occur as a result of an accumulation of an inhibitory metabolite, or a loss of glucose-6-phosphate dehydrogenase activity. The following experiment was designed to examine these possibilities.

A portion of a SFH preparation, which had been stored at 5°C for 15 days, was dialysed against isotonic KCl for 17 hours at 5°C. The dialysed and nondialysed specimens were assayed for hexokinase activity with and without the addition of G-6-P DH (final concentration 7.5 units/ml) to the medium. The activity is represented as a percentage of the original activity.

### TABLE XIII

Hexokinase Activity in SFH Stored at 5°C for 15 Days (II).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Addition</th>
<th>Activity (x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nondialysed SFH</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>G-6-P DH</td>
<td>127</td>
</tr>
<tr>
<td>Dialysed SFH</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>G-6-P DH</td>
<td>98</td>
</tr>
</tbody>
</table>

Dialysis of the stroma-free hemolysate did not affect an increase in the activity. However, the addition of G-6-P DH resulted in a restoration of the activity to the original level in the dialysed specimen, and to a still higher level in the nondialysed sample.
7. **Effect of added G-6-P DH on the hexokinase activity as assayed by method B:**

In view of the pronounced effect of the addition of glucose-6-phosphate dehydrogenase on the "hexokinase" activity observed with stored samples of SFH, the effect of analogous additions on the activity of freshly-prepared stroma-free hemolysates was investigated.

SFH samples were prepared from erythrocytes on the first and sixth days of storage of a blood specimen, and were assayed with the addition of various amounts of G-6-P DH to the medium. The concentration of G-6-P DH is expressed in Kornberg units/ml, the unit being the amount of enzyme which causes the reduction of 1.0 μmole TPN/min. in the presence of G-6-P at pH 7.4 and 25°C. The results are given in Table XIV and Figure 14. Again, it is evident that G-6-P DH has a marked stimulatory effect on the rate of reduction of TPN in the assay system. A final concentration of 5.0 units/ml of the assay medium caused a 64% increase in the rate of reaction.
TABLE XIV

Effect of Added G-6-P DH on Rate of Reaction in Hexokinase Assay Method B.

<table>
<thead>
<tr>
<th>Duration of Storage</th>
<th>Concentration of G-6-P DH (units/ml)</th>
<th>Activity (mg glucose utilised/100 mg Hb/hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>1.67</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>3.33</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>6.67</td>
<td>0.69</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>6.54</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>8.17</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Conditions:
Hexokinase assay method B, as described on p. 35.
Influence of Added G-6-P DH on Hexokinase Activity.

Legend:

- - - SFH (i)

○ --- ○ SFH (ii)

Conditions:

Hexokinase activity was determined by assay method B, in the presence of G-6-P DH, added to the assay medium to give the final concentrations indicated.

SFH (i) was prepared from 1-day-old blood, and SFH (ii) from 6-day-old blood.
FIGURE 14

MG GLUCOSE UTILISED/100 MG HB./HR.

G-6-P DH CONCENTRATION (UNITS/ML)
DISCUSSION

There has been considerable confusion about the properties of hexokinase in fresh and stored erythrocytes. This has arisen mainly from the variety of procedures used for the assay of hexokinase activity. Since this enzyme has not yet been isolated in a pure form from erythrocytes, its activity has been studied only in the presence of many other enzyme systems, including those of the glycolytic pathway. Thus, it has been extremely difficult to obtain consistent values for the rate of the hexokinase-catalysed reaction, especially when preparations of hemolysed cells are used, because of the large variation in the amount of glycolytic cofactors, either endogenous to the preparation or added to the assay medium.

Investigators in our laboratory, using different hexokinase assay methods, have obtained conflicting evidence concerning the behaviour of hexokinase activity during the preservation of blood. It may be recalled that Kashket et al. (55) found evidence that hexokinase became irreversibly impaired during storage of blood at 5°C. Prevost (43), on the other hand, found no diminution in the hexokinase activity of erythrocytes throughout a storage period of 30 days, at 5°C. Both workers measured the enzyme activity by following the rate of utilisation of glucose by a hemolysed preparation of washed red cells.
Kashket measured glucose uptake by whole hemolysates in the presence of added ATP, but without added ADP or DPN. Prevost, having shown that ADP and DPN stimulate glucose utilisation, included these two compounds in the assay medium. In two storage experiments, the present author has confirmed the stimulatory effect of added ADP in the assay medium (see fig. 2). However, with Prevost's assay method, the results obtained were inconsistent with respect to the stability of hexokinase during storage.

A serious disadvantage of the type of hexokinase assay involving measurements of glucose utilisation is the incomplete conversion of glucose to lactate by the stroma-free hemolysates. This is evident in the results indicated in Table IV (p.50). Prevost (43) found that, in suspensions of intact erythrocytes, all the glucose utilised is metabolised to lactate, whereas the SFH preparations convert only 50% of the glucose to lactate. During the assay, therefore, there must be an accumulation of one or more of the glycolytic intermediates, and this condition doubtless has an effect on the rate of the hexokinase reaction.

Hexokinase assay procedures based on measurements of the rate of reduction of TPN during the oxidation of glucose via the hexose monophosphate shunt pathway, have been used by several workers, including DiPietro (39) and
Chapman et al. (2). The present author has modified a procedure of this type and designated it assay method B.

The range of hydrogen ion concentration for optimum hexokinase activity in stroma-free hemolysates, as determined by assay method B (see Fig. 8, p. 63) is very similar to that obtained by Prevost (43) using assay method A. The maximum activity is obtained at pH 7.8. Chapman et al. (2) and Rapoport et al. (3) both report a pH optimum of 8.1 for hexokinase activity of the erythrocyte.

The SFH preparations were found to metabolise glucose-6-phosphate with an accompanying production of 2 moles of TPNH per mole of G-6-P utilised (see p. 57), indicating that both glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase were active in the assay system. The net rate of these two reactions was found to be eleven times faster than the rate of operation of the system with the hexokinase reaction as an obligatory step in the sequence (see p. 58). It appears, therefore, that the hexokinase reaction is the rate-limiting step in the system.

Later experiments, however, showed that the addition of exogenous glucose-6-phosphate dehydrogenase greatly stimulates the rate of reduction of TPN (Table XIV and Figure 10). Moreover, the degree of stimulation, in the range of enzyme concentration used, is roughly proportional to the amount of G-6-P DH added. This may be explained on the basis of inhibition of the hexokinase reaction by its product, glucose-6-phosphate, as has been demonstrated by several
workers (45, 46, 22, 43). Despite the fact that G-6-P dehydrogenase can metabolise G-6-P as fast as it is formed, the presence of any G-6-P at the active centre of hexokinase would tend to cause inhibition of the reaction. An increase in the amount of dehydrogenase could cause a decrease in the steady-state concentration of G-6-P and permit the hexokinase reaction to proceed at a slightly faster rate. Although the hexokinase reaction is the slowest step in the assay system, and therefore is the rate-limiting reaction, it is continuously inhibited by glucose-6-phosphate. The inhibition can be relieved, to a certain extent, by increasing the amount of glucose-6-phosphate dehydrogenase available to remove the inhibitor.

It was observed that storage of stroma-free hemolysates, either in the frozen state or at 5°C, resulted in a progressive loss of hexokinase activity (see Fig. 13). This finding was unexpected, since Prevost (43) had observed no alteration in the hexokinase activity of SFH preparations that had been stored at -10°C for a week. However, since the addition of either yeast hexokinase or glucose-6-phosphate failed to effect a restoration of "activity", it became evident that the failure of the assay system was not restricted to the hexokinase reaction. Restoration of the activity to the original level on addition of glucose-6-phosphate dehydrogenase to the assay medium indicated that
the hexokinase, in fact, had remained fully active. Other investigators have found that purified preparations of G-6-P dehydrogenase are inactivated on dilution (9), and on storage at 4°C (97) in the absence of TPN. In the experiments mentioned above, the SFH samples had been diluted at the time of preparation, so that the hemoglobin concentration was approximately 1/5th that in whole blood. Although it is not known whether the amount of TPN present in the stored SFH preparations was sufficient to stabilise the enzyme, it is possible that inactivation of G-6-P dehydrogenase was responsible for the apparent loss of activity.

Bishop (98) recently has developed a further modification of the hexokinase assay procedure involving the reactions of the HMP shunt. This method consists in the measurement of the rate of oxygen uptake by a suspension of intact erythrocytes in the presence of added glucose and methylene blue. It was found that the oxygen uptake, with glucose as substrate, was decreased in the red cells from blood that had been stored in ACD in the cold, whereas the uptake of identical cell samples remained unchanged when inosine was used as the substrate. The enzyme sequence involved in the metabolism of glucose includes the hexokinase reaction, whereas the utilisation of inosine does not involve this enzyme. Hence, Bishop has suggested that failure of the hexokinase system, meaning the enzyme and its cofactors, is responsible for the observed decrease
in oxygen uptake on incubation of stored erythrocytes with glucose. It must be noted, however, that the rate of oxygen uptake in the presence of methylene blue may be affected more directly by reactions involving TPN or DPN than by the hexokinase reaction. Additional experiments indicated that the decrease in oxygen uptake was parallel to the decrease in the ATP concentration in the preserved cells, and, moreover, that the rate of oxygen uptake is returned to normal when the ATP level is restored, by incubating the stored blood specimens with adenine and inosine. It would appear, then, that the hexokinase apoenzyme suffers no damage during storage, and that the depressed rate of glucose phosphorylation is attributable to the decrease in intracellular concentration of ATP.

The stability of the hexokinase system in preserved erythrocytes was confirmed in the present study, using the hexokinase assay method B. It is evident from Figures 10 and 11 that there was no significant change in the hexokinase activity throughout the storage of blood specimens at 5°C for 31, 42 and 34 days, respectively. Furthermore, the results presented in Tables VIII and IX indicate that the hexokinase activity in the SFH samples which represent the entire population of erythrocytes did not differ from that in the samples which are presumed to represent red cells of younger age groups. Lohr and Waller (57) demonstrated that fractions of the whole red cell popula-
tion, obtained by osmotic hemolysis of fresh blood specimens, and corresponding to groups of erythrocytes of different mean ages, show no difference in the activity of hexokinase. The experiments in the present study, which are concerned with a similar osmotic fractionation, yielded conflicting results (see Fig. 12). The fractions that were obtained from a given specimen of relatively fresh blood (stored for either 2 or 8 days at 5°C) showed no significant difference in hexokinase activity. These results confirm those of Lohr and Waller, mentioned above. On the other hand, the results obtained on osmotic fractionation of old blood (stored for 33 or 50 days) indicated that there is a positive relationship between hexokinase activity and the hemolysis ratio of the fraction. We have no explanation for this observation.

It must be emphasised that the hexokinase activity of a stroma-free hemolysate, assayed at an optimum pH and in the presence of optimal concentrations of all the required cofactors, does not necessarily represent the level of activity present in the original cellular environment. Although it can be concluded that hexokinase retains the ability to phosphorylate glucose when optimal conditions are restored, it is obvious that the actual level of activity of this enzyme is depressed during storage of blood.

The increase in hydrogen ion concentration, caused by the accumulation of lactic acid in preserved erythrocytes,
no doubt results in a diminished activity of hexokinase (see p.16). The concentration of ATP in the cells also is critical, with respect to the hexokinase activity. Numerous investigators have shown that the improvement in the viability of the erythrocytes and the increase in their capacity to utilise glucose are parallel to the increase in intracellular ATP, produced by incubating preserved erythrocytes with nucleosides. Prankerd (99) demonstrated that the incubation of blood, stored at 4°C for 6 weeks, with added adenosine causes a resynthesis of ATP and a restoration of glucose utilisation. Recently, Nakao et al. (100) showed that similar effects are obtained by incubating 8-week-old blood with adenine and inosine. Thus, it is probable that the observed decrease in the intracellular concentration of ATP is largely responsible for the depression in glucose utilisation which occurs during the storage of blood in vitro.
SUMMARY

The hexokinase assay procedure A, based on measurements of the rate of glucose utilisation by stroma-free hemolysates of blood, as was used by former workers of this laboratory, has been shown to be unreliable. The writer has developed a modification of the hexokinase assay method involving the dehydrogenase reactions of the hexose monophosphate shunt. Studies concerned with the latter assay method (B) have indicated that the hexokinase reaction is continuously inhibited by its product, glucose-6-phosphate. This conclusion is based on the observation that added G-6-P dehydrogenase stimulates the rate of reduction of TPN, despite the fact that the hexokinase reaction is the slowest step in the sequence.

The results obtained from blood preservation experiments confirm the stability of the hexokinase of erythrocytes during storage at 5°C, for periods up to 6 weeks. Changes in the method of preparation of the stroma-free hemolysates, designed to vary the age characteristics of the erythrocytes represented in the SFH, did not result in a significant alteration in the hexokinase activity during blood storage.

Experiments involving osmotic fractionation of red cell specimens yielded inconsistent results. The hexokinase activity did not vary in the red cell fractions
obtained from fresh blood, but the activity of the fractions from aged blood increased with the osmotic resistance of the erythrocytes.

It is herein postulated that inactivation of the glucose-6-phosphate dehydrogenase of the stroma-free hemolysate is responsible for the apparent loss of hexokinase activity during storage of the SFH. At -15°C, the activity remaining at the 17th day of storage was 20% of the original; at 5°C, no activity could be measured after 7 days of storage. The activity was restored to the original level on addition of G-6-P dehydrogenase to the assay medium, whereas similar additions of either G-6-P or hexokinase had only a minimal effect.

Evidence from the literature suggests that the depressed rate of glucose utilisation by preserved erythrocytes results primarily from the decreased intracellular concentration of ATP.
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