HEXOKINASE OF THE ERYTHROCYTE

by Shelby Kashket

A Thesis

Submitted to the Faculty of Graduate Studies
and Research in partial fulfilment of the
requirements for the degree of Doctor
of Philosophy

McGill University
Montreal, Canada.

April, 1956.

PREFACE

The study described in this thesis represents part of a larger programme of research on the preservation of blood. Though the benefits resulting from the transfusion of blood were recognized a very long time ago, it has been only within the last two decades that serious attempts have been made to preserve human blood. With the outbreak of the Second World War, the demand for blood both in civilian hospitals and on the battle field was increased tremendously. At that time the practice was to collect the blood into isotonic sodium citrate and to store it at 5°C. However, specimens usually underwent hemolysis after four or five days so that it became urgent to devise methods whereby the storage period of the blood might be prolonged. Research on blood preservation was carried out in many countries. In Canada the Wartime Research Council, Medical Division, requested Prof. J. B. Collip, then head of the Biochemistry Department at McGill University, to undertake research on the problem. The investigation was assigned to Dr. O. F. Denstedt and it has been continued under his direction, on an expanding scale, since that time. After the end of World War II it was incorporated into the research programme supported by the Defence Research Board of the Department of National Defence.

During the initial phases of the research carried on in the McGill laboratories, attention was focused on the evaluation of the physical stability of the red blood cells during storage, and on the effects of storage, under various conditions, on the viability of the cells, as measured by their capacity to survive in the circulation after transfusion. As a result of this work several preservative media were developed in these laboratories, one of which is still a standard medium used by one of the commercial companies. However, it was appreciated as far back as 1942 that any real progress in the preservation of blood would come about only through an understanding of the energy metabolism of the red blood cell, and of the influence of various conditions of storage on the enzymes and metabolic processes of that cell. Rochlin, Andreae, Woodford, Pappius and Fishman, in the McGill group carried out extensive studies on the glycolytic activity and electrolyte balance of red blood cells during storage. Pappius and Fishman obtained evidence that the progressive depletion of the coenzymes DPN and ATP is closely linked with the metabolic changes and loss of viability during storage. Pappius held the view that the progressive fall in the concentration of DPN is probably the major initiating factor, while Fishman inclined to the view that the failure of the cell to replenish ATP is the dominant cause in the degeneration of the red blood cell. During the past few years an effort has been made to

resolve this problem. Dr. A. Malkin has studied the question as to whether the mature erythrocyte can synthesize DPN, while the writer undertook a study of the activity of the hexokinase system which requires ATP as a coenzyme. The failure of the latter enzyme system would explain the observed, progressive inability of preserved red blood cells to utilize glucose. The present study has permitted, also, an inquiry into the distribution of hexokinase in the membrane and cytoplasm of the cell. It was felt that such a study might shed much light on the mechanism by which glucose is transported into the red blood cell.

During the past ten years, much has been added to our knowledge of the metabolism of the erythrocyte. However, actual progress as to the improvement of the methods of blood preservation was disappointing, and it was only recently that two new developments were reported. One is the storage of glycerol-equilibrated red cells at very low temperatures, and the other is the remarkable observation that the addition of purine nucleosides, such as adenosine and inosine, to the blood, retards the rate of metabolic deterioration and improves the viability of the preserved red blood cells. The writer has participated in the studies on the latter development, and an elaboration of some of these findings represents a major contribution of this thesis.

ACKNOWLEDGEMENTS

I am most grateful to Dr. O.F. Denstedt for his continued interest and direction of this work, and also for his stimulation, understanding and patience shown to me throughout the course of this study.

I wish also to express my thanks to Dr. David Rubinstein, with whom the work on the preservation of blood was done.

To Mrs. Sheila M. Gosselin for her excellent technical assistance, and to Dr. Jean-Marie Loiselle and Mr. Paul Ottolenghi for their help at various stages of this study, my sincere thanks.

The stimulating discussions, criticism and fraternal spirit of the entire group were of inestimable value.

To Miss Phyllis J. Webb, for the typing of this thesis, and to Dr. Esau Hosein, for the proofreading of the manuscript, my thanks.

This study was supported by a grant from the Defence Research Board of Canada (Grant number 9350-01, Project number 50-93050-01).

And finally, I wish to express my gratitude to my parents.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AMP, ADP and ATP: adenosine mono-, di- and triphosphate;

CD and ACD: citrate-dextrose medium, and acid citrate-dextrose medium. Suffixes, +Ad. or +In., indicate the addition of adenosine or inosine, respectively, to the medium;

DPN, TPN: di- and triphosphopyridine nucleotide;

DPG: 2,3-diphosphoglycerate;

G-6-P, F-6-P: glucose-6-phosphate and fructose-6-phosphate;

Hb: hemoglobin;

M : molar ("moles" not abbreviated);

P_i, P_l, P_m, P_s: inorganic phosphate and labile, moderately stable and stable phosphate esters, respectively;

SFH: stroma-free hemolyzate;

YH: yeast hexokinase.

LIST OF FIGURES

Page

LIST OF TABLES

I N T R O D U C T I O N

Hexokinase - An Enzyme of Glycolysis

Early concepts.

In the November 30th issue of the Munchener medizinischer Wochenschrift of 1897 (1) an article appeared by Hans Buchner relating his brother Eduard's success in preparing a cell-free extract of yeast which was capable of fermenting[1] the sugar that had been added to it as a preservative. This relatively simple observation was to open up to the twentieth century a vast new horizon of biological concepts.

Several years earlier, Pfeiffer (see (2) for references) had succeeded in demonstrating the phenomenon of bacterolysis, and Behring had shown that the serum of animals, immunized with diphtheria toxoid, could be used for the protection of other animals against diphtheria. It was against such a background of knowledge that Eduard Buchner set to work to prepare cell-free extracts of the cholera and typhus bacilli. The brothers hoped that with such extracts they might be able to produce immunity directly by the injection

---

1. The reader is referred to the general textbooks of biochemistry (viz. 19) for the definition and discussion of the elementary aspects of fermentation, glycolysis, etc.

of the material into the animals. Eduard Buchner began by grinding yeast with infusorial earth and powdered quartz and then filtering the resulting, pasty concoction under high pressure. By this means, he was able to obtain a cell-free extract of the yeast, but when he tried to preserve this material with sugar, he noticed that the extract, like whole yeast, was capable of fermenting the added sugar. The Buchners succeeded, eventually, in preparing similar extracts from the cholera and typhus bacilli, as they originally had set out to do, but the legacy these workers left to biochemistry was the knowledge that cell-free extracts are capable of performing chemical transformations previously believed to be carried on only by living cells.

This observation did much to resolve one very important problem of the day. For years many authorities had insisted that yeast was nothing more than a simple organic material which happened to possess several properties of an "animal" nature. Thus Liebig believed that certain non-living, nitrogenous substances in yeast set up "unstabilizing vibrations" which are imparted to the molecules undergoing fermentation, and which lead to their breakdown and utilization. Traube advanced the theory that fermentation is brought about by the action of certain chemical substances in yeast, and that these substances are able to exert their influence on the fermentable molecules as a result of their specific chemical affinities. In the meantime, a considerable degree of success had been achieved in demonstrating the reproductive

and certain other characteristics of the yeast cell which marked it as a living organism, and it was not long before Pasteur put forth the idea that fermentation is linked indissolubly, with the life of the cell, i.e., only the intact cell can ferment sugars. Needless to say, this view was in direct opposition to the earlier theories of Liebig. The discovery of the Buchners finally resolved the problem. What Pasteur once thought to be impossible, had now been shown to take place. It was clear now that certain unknown substances are present in the cell which exert a catalytic action on fermentable molecules, and to these Kuhne had given the name, "enzyme". The question then arose whether zymase, the active yeast extract, was made up of one, or more than one of these enzymes.

In 1905 Harden and Young (3) showed that the addition of a preparation of boiled yeast juice, or "Kochsaft", to yeast greatly increased the ability of the yeast to ferment sugar. This observation suggested that certain thermostable and dialyzable substances are present in the fermenting juices, and that without these fermentation is impossible. The dialyzable cofactors were later identified as consisting of inorganic phosphate ions and a certain organic phosphate fraction which was subsequently characterized as diphosphopyridine nucleotide (DPN) [see (4) for list of references]. The full significance of these findings was realized only later when Meyerhof, Embden, Harden, Young, Robison and many others, illustrated the essential role played by phosphorylated intermediates in fermentation and glycolysis.

The discovery of hexokinase

In 1926, Meyerhof (5) obtained an active glycolyzing extract from skeletal muscle, the "glycolytic enzyme" or "myozymaze", thereby demonstrating with muscle that which Eduard Buchner had succeeded in showing some thirty years earlier with yeast, namely that glycolysis or fermentation can proceed in the absence of the intact cell structure. It was shown also that glycolysis in muscle, like fermentation in yeast, requires inorganic phosphate ions and certain other heat stable factors found in the "Kochsaft" of fresh muscle. These factors were later shown to be cozymase (DPN) (vid. 4) and adenosine triphosphate. In 1927, Meyerhof (6) became aware of the presence in yeast, of a substance capable of accelerating glycolysis in muscle extracts. Earlier work had shown that the glycogen of muscle is broken down rapidly during muscular activity. However, with the extracts of rabbit muscle, Meyerhof found that free hexoses could be converted to lactic acid only at a slower rate than endogenous glycogen. If he now added the activator prepared from baker's yeast, he found that the muscle extracts metabolized hexoses twice as rapidly as glycogen. Meyerhof named the activator "hexokinase". By adding an excess of the yeast hexokinase preparation to a muscle extract, and restricting the amount of glucose or inorganic phosphate added, he showed that the formation of lactic acid or the esterification of phosphate proceeded until the supply of substrate was almost used up.

Meanwhile, Embden and Zimmerman (7) had succeeded in extracting a hexose monophosphate from fresh muscle and they found that this compound was almost identical with the monophosphate which Robison (8) had extracted from yeast. Since the Embden ester was glycolyzed by muscle extracts at about the same rate at which glucose was utilized by the yeast hexokinase - muscle extract preparation, it was concluded that the monophosphate (glucose-6-phosphate) was the first product formed in the glycolysis of glucose. It was also suspected that glucose-6-phosphate was the first ester formed in the breakdown of glycogen.

In a later publication Meyerhof (9) reported that hexokinase is present in the cell-free preparations from erythrocytes. While carrying out his investigations on a wide variety of erythrocytes, including the nucleated red cells from the goose and the non-nucleated cells from the rabbit and Man, Meyerhof became aware of the peculiar lability of hexokinase in certain species. He found that while the addition of yeast hexokinase was necessary to the erythrocyte preparations from some species, in order to restore the glycolytic activity to that of the intact erythrocyte, this was not so with others. Meyerhof noted that an analogous situation exists in certain muscle preparations. For instance, frog muscle extracts require the addition of yeast enzyme in order to glycolyze glucose, whereas freshly prepared rabbit muscle extracts can utilize glucose without

the addition of any activators. The cell-free preparations from erythrocytes, like those from muscle, were found also to require the cofactors ATP and Mg^{++} ions. Meyerhof noted especially the extreme lability of the naturally-occurring coenzymes. In this respect, it would appear that Meyerhof's experiments would have been foredoomed to failure because of his neglect of coenzyme I. However, it must be realized that the preparations of ATP available at the time were impure and probably contained sufficient DPN to permit glycolysis. Also, hemolysis of the erythrocytes and subsequent removal of the particulate matter by centrifugation were always carried out at 0-1°C; these conditions were favorable to the preservation of endogenous DPN. In the light of these circumstances, it can be understood how Meyerhof could obtain the above results.

Von Euler and Adler (10) rediscovered hexokinase three years after Meyerhof's studies were reported but, since the former workers were not aware of these earlier publications, they named the enzyme "heterophosphatese". About this time Warburg and Christian (11) found an enzyme, "zwischenferment", in yeast maceration juice which catalyzed the oxidation of glucose-6-phosphate in the presence of triphosphopyridine nucleotide (TPN). When von Euler and Adler studied zwischenferment they found that glucose and fructose could be oxidized, too, provided ATP was present. Without this nucleotide only glucose-6-phosphate could be utilized;

with it free sugars could be oxidized since heterophosphatese was shown to be present and could mediate the transfer of phosphate from ATP to hexose. Evidently the primary product of the transphosphorylation catalyzed by heterophosphatese was glucose-6-phosphate. This was the same intermediate which Embden (7) had postulated, several years earlier, as being the product first formed during the glycolysis of glucose or the breakdown of glycogen. Subsequently the identity of heterophosphatese with hexokinase was realized and the latter name came into preferred use. However, the term heterophosphatese has not been rejected entirely and it is still to be found in occasional publications.

Adenylate kinase (myokinase).

Von Euler and Adler (10) made a mistake, however, in postulating that the phosphorylation of hexose from ATP proceeded as follows:

ATP + 2 Hexose → 2 Hexosephosphate + AMP (I)

About ten years later, Colowick and Kalckar (12) demonstrated that the phosphate donor in the hexokinase system is ATP and not ADP. As a result, these authors suggested that the reaction catalyzed by the enzyme from baker's yeast occurs as follows:

ATP + Hexose → ADP + Hexose monophosphate (II)

and not as von Euler and Adler had proposed. Glucose and fructose were equally efficient in accepting the terminal labile phosphate group from ATP, and the esterification of

the hexose molecule was found to take place specifically at the carbon 6 of the sugar molecule. About the same time a paper by Kalckar (13) appeared describing an enzyme, "myokinase", which catalyzed the reversible dismutation of ADP as follows:

$$2\ ADP \rightleftarrows ATP + AMP \qquad (III)$$

Thus it became evident why the earlier workers had been led to believe that ATP could bring about the phosphorylation of two equivalents of glucose. The assumption underlying this was the notion that ADP could be utilized in the hexokinase system. With the work of Colowick and Kalckar it became evident that this was not so. What actually occurred was an initial phosphorylation according to reaction II, followed by a dismutation of ADP as in III, and finally, a phosphorylation of a second molecule of glucose by the newly-generated molecule of ATP. Thus it was found that those enzyme preparations which contained myokinase could utilize adenine nucleotides more efficiently than those without the enzyme.

It must be mentioned, parenthetically, that the tendency, in recent years, has been to refer to myokinase as "adenylate kinase". The latter name seems to be more truly descriptive of the enzyme (cf. 14), and therefore it will be used in the following text.

Thus far, studies has been restricted mainly to the hexokinase of yeast. In 1945, however, Price et al. (15) reported that insulin can influence the activity of hexokinase

of various tissues of the rat while having no effect on the hexokinase of yeast. This unusual demonstration led workers in many laboratories to study the effect of various hormones on the enzymes involved in carbohydrate metabolism, and thereby stimulated a great deal of research on the properties of hexokinase.

Substrate specificity

In 1947, Colowick et al. (16) reported that the hexokinase of beef brain brought about the phosphorylation of glucose on carbon 6. This finding confirmed the earlier results of Colowick and Kalckar (12), who found that phosphorylation of glucose by yeast hexokinase took place only in the 6 position. Later, Gottschalk (17), while studying the relative behavior of glucose, fructose and mannose with respect to yeast hexokinase, concluded that both the α- and β-mutarotatory forms of glucose and mannose could be phosphorylated by the enzyme, provided the sugars were of the pyranose structure. Fructose, on the other hand, proved to be inactive, enzymatically, in the pyranose configuration, and could be phosphorylated only when present as the β-D-fructofuranose. Furthermore, Gottschalk observed that configurational changes at C-4 of D-glucopyranose, as occur in D-galactose, or substitution on C-1 by large groups such as fructose, as in the case of sucrose, render the resulting saccharide unsuitable as a phosphate acceptor for hexokinase. Also, a configurational change in the C-1 and C-2 positions, or a change from the pyranose-1,5 to the furanose -1,4 ring structure can be tolerated by hexokinase, as may be

seen by comparing the behavior of glucose and fructose in the phosphorylations. Finally, by assuming that the group undergoing phosphorylation is involved in a reaction with the enzyme, Gottschalk concluded that the various substrate molecules become attached to hexokinase through the hydroxyl groups at carbons 3 or 4, and 6. Slein et al. (18) reported a similar specificity of hexokinase for gluco- and manno-pyranose and fructofuranose. More recently, Sols and Crane (20,21) analyzed the behavior of brain hexokinase towards thirty-five different compounds representing substitution or modification at each carbon atom of the glucose molecule. Sixteen of these compounds, including 5- and 7-carbon sugars, and several non-reducing compounds were found capable of being phosphorylated, although the suitability of these substances as phosphate acceptors was found to vary greatly from one compound to another. As a result of these studies, the authors were able to confirm that the glucopyranose and fructofuranose forms are essential for the phosphorylation process. They reasoned, by analogy, that the pyranoid forms of the other aldohexoses and heptuloses must be the substrate structures actually participating in the enzymatic reaction. Evidently the removal of enzyme-binding groups from either side of the sugar molecule, or the introduction of groups that cause steric interference, seriously affects the enzyme-substrate affinity and the maximal velocity of phosphorylation. These workers suggested, furthermore, that each hydroxyl group on the sugar molecule has a specific, quantitative influence on the affinity

of the enzyme for the sugar, and thereby affects the actual numerical value of the Michaelis constant as it is obtained experimentally. Finally, as a result of their experiments, Sols and Crane concluded that the formation of the active glucose-hexokinase complex must involve the hydroxyl groups at carbons 1, 3, 4 and 6.

Recently, Gamble and Najjar (22) obtained evidence for the reversibility of the hexokinase reaction. They did this by measuring the exchange reaction, catalyzed by purified yeast hexokinase, either with C^{14}-labeled glucose or G-6-P. By this means, though they found no net production of glucose, they were able to measure the reverse reaction and found it to proceed at 1/50th the rate of the forward reaction. It is interesting that the reverse reaction has been considered previously to be thermodynamically improbable.

Glucokinase and fructokinase.

In 1948, Meyerhof and Wilson (23), while studying the effects of varying concentrations of sugar on the rate of hexokinase activity in homogenates prepared from rat brain, found that the rate of phosphorylation of fructose is about half that of glucose. A similar difference in effect was noted with respect to the action of inhibitors, such as naphthaquinone sulfonate and caprylic alcohol, on the phosphorylation of the two sugars. These observations suggested the possible existence of two phosphorylating enzymes in rat brain, one being specific for glucose and the other for fructose.

A similar observation was made with regard to tumor tissue which was found also to utilize the two sugars at different rates. A few years earlier, Berger et al. (24) had succeeded in crystallizing hexokinase from baker's yeast. When these workers measure the rates of utilizatiom of hexoses by the purified enzyme, they found that the relative rates of phosphorylation of fructose, glucose and mannose could be represented by the ratios 1.4:1.0:0.3, respectively. However, glucose and fructose were found to inhibit each other, and Berger and co-workers concluded that only one enzyme is operative in the phosphorylation of hexoses by yeast (cf.25). Slein et al. (18) reported similar ratios for the relative rates of phosphorylation of the three sugars indicated above, by hexokinase from beef and sheep brain. The affinity of the enzymes for these sugars (represented quantitatively by the constant K_s), however, was found to decrease in the order mannose, glucose and fructose. Furthermore, the phosphorylation of fructose was inhibited by the two other sugars, although the reverse was not true, and glucose and mannose competed with each other in the phosphorylative process. It was evident that only one enzyme is involved in the phosphorylation of these sugars in brain, the same as in yeast.

Rabbit muscle, on the other hand, was found to contain two enzymes, one a glucokinase, specific for glucose, and the other a fructokinase, specific for fructose (18); with these enzymes there was no competition between the sugars for phosphorylation. In rat liver, only fructokinase

could be demonstrated. This enzyme requires magnesium ions for activation, and catalyzes the formation of fructose-1-phosphate from fructose and ATP. A mutase present in liver converts the C-1-ester to the C-6-ester, thus making the overall transformation analogous to that of glucose. Vestling et al. (26) also obtained evidence of the existence of a separate enzyme for the phosphorylation of fructose in rat liver homogenates. The fructokinase activity was observed to vary greatly in liver tissue from different species. Thus rabbit liver homogenate exhibited no fructokinase activity whatsoever. It is of significance that these workers (26) found that liver slices phosphorylate fructose only, and are completely inactive towards glucose. Mackler and Guest (27) concluded from their own studies, that fructose phosphorylation in the intact animal must proceed mainly by way of fructokinase since, with normal concentrations of glucose, the phosphorylation of fructose by hexokinase must be almost completely inhibited. Hers (28), too, demonstrated an enzyme in liver which was capable of phosphorylating fructose in the presence of ATP. However, since he found that the enzyme catalyzed the phosphorylation of sorbose and tagatose, also, he named the enzyme "ketokinase". Ketokinase was shown to be completely inactive towards the aldoses glucose, galactose and mannose, and strongly activated by potassium acetate or chloride, even though the latter substances were shown to inactivate liver glucokinase. Hers

also showed that the primary product of the phosphorylation of the ketoses was either fructose-1- or sorbose-1-phosphate.

Galactokinase.

An enzyme, distinct from hexokinase, and capable of phosphorylating galactose, has been shown to be present in many tissues. It had been known for a long time (see references in 29) that certain strains of yeast which normally cannot ferment galactose could be adapted to ferment this sugar by growing the cells in a galactose-containing medium. Kosterlitz (vid. 29) made the important observation that galactose-1-phosphate tends to accumulate in the liver of rats fed galactose, and that this intermediate is in turn converted to an acid-stable ester, presumably G-6-P. On the basis of these findings he postulated the existence of an enzyme which catalyzes the phosphorylation of galactose to galactose-1-phosphate by ATP. Cardini and Leloir(29) later proved him to be correct, and named the enzyme "galactokinase". These authors demonstrated also that galactokinase brings about the phosphorylation of both galactose and galactosamine, the two compounds, when present together, competing with each other for the enzyme. Earlier, Caputto et al. (30) had discovered a heat-stable coenzyme, uridine-diphosphate-glucose, and an enzyme, phosphogalactoisomerase, which catalyzes the conversion of galactose-1-phosphate to glucose-1-phosphate. These significant discoveries elucidated the mechanism by which gal-

actose metabolism is linked with that of glucose.

Hexokinase activity in different tissues.

In recent years, several papers have appeared dealing with the distribution of hexokinase in various animal and plant tissues. In 1951, Long (31) published the results of a systematic study on the occurrence of hexokinase in the tissues of the rat. Prior to the appearance of this paper, relatively few values for the activity of hexokinase in different tissues had been reported. Even then, most of these values were obtained with crude homogenates which had been prepared in an attempt to discover whether the hexokinase activity of animal tissues could be influenced by the addition, in vitro, of hormonal preparations (vid. 16,26,31). However, an inherent source of error in the estimation of the enzyme content of these preparations, as later shown by Crane and Sols (32), lay in the mode of preparation of the extracts. These authors demonstrated that almost all of the hexokinase activity of the tissues is associated with the sedimentable particles, probably the mitochondria. However, the degree of binding of the enzyme in the particulate matter was found to vary with the tissue and, furthermore, it seemed to vary with the mode of preparation of the tissue. Hence, it may be assumed

that a considerable portion of the hexokinase activity of the extracts which were used in the earlier studies, was lost, and as a result, the values reported for the hexokinase content of various tissues by many workers, must be accepted with reserve. It is interesting to note that the hexokinase activity of plants also appears to be associated with the mitochondria (33). In consideration of the above observations, Long (31) undertook a detailed study of the distribution of hexokinase in the tissues of the rat. Brain was found to exhibit the greatest enzyme activity, while heart, small intestine, spleen, kidney and liver exhibited progressively smaller enzyme activities, the approximate relative values being 27.1, 14.5, 11.7, 8.3, 7.9 and 1.4, respectively.

Purification of hexokinase.

In 1945, two groups of workers - Berger et al. (24) and Kunitz and McDonald (34) - succeeded in crystallizing hexokinase from baker's yeast. Berger and his coworkers used an alcohol fractionation procedure which led to a thirty-fold increase in the specific activity of the preparation. This product gave a turnover number of 13,000 moles/10^5 grams protein/minute at 30°C and pH 7.5, with glucose as the substrate. The preparation was not amenable to further purification by ammonium sulfate precipitation. Furthermore, the crystalline enzyme ex-

hibited a definite dependence on magnesium ions and a temperature coefficient (Q_{10}) of 1.9, between 0 and 30°C. The dissolved enzyme was unstable on dilution, but the instability was diminished in the presence of other proteins, especially insulin. Kunitz and McDonald (34) obtained the crystalline enzyme from baker's yeast which had been autolyzed in the presence of toluene. The method involved precipitation first with ammonium sulfate, and then with alcohol. These workers found the purified enzyme to be a protein of the albumin type with a molecular weight of 96,600 and an isoelectric point of 4.8. They found also that the enzyme was most stable at pH 5, and also in the presence of sugars, the protective effect of the sugars varying in the following descending order: glucose, mannose, sucrose and fructose. Finally, physico-chemical studies revealed that the diffusion constant of the crystalline hexokinase was 2.9×10^{-7} cm.^{2} sec.$^{-1}$ at 1°C. in acetate buffer, pH 5.5, and the sedimentation constant was 3.1×10^{-3} cm.sec.$^{-1}$ dyne^{-1} gm. at 1°C and pH 5.5.

Crane and Sols (32) succeeded in purifying the hexokinase from calf brain. By the combined use of fractional centrifugation and treatment with lipase and desoxycholate they obtained a particulate preparation with a 45- to 50-fold increase in the specific activity

of the enzyme. In their preliminary studies they had found that both lipase and desoxycholate cause irreversible damage to the hexokinase, but later they were able to control the action of these reagents so as to achieve a high degree of purification with a minimum degree of damage to the enzyme. The purified preparation was free from interfering enzymes such as ATP-ase, adenylate kinase, phosphomonoesterase and phosphofructokinase, and was quite stable in the cold. The Michaelis constants for the various substrates of hexokinase were as follows: mannose, 6×10^{-6}M, glucose, 1×10^{-5}M, 2-desoxyglucose, 2.4×10^{-5}M, glucosamine, 1.1×10^{-4}M (cf. 35), and ATP, 1.3×10^{-4}M.

Inhibitors of hexokinase.

In the course of their studies on the hexokinase of brain, Crane and Sols (32) found that the enzyme is inhibited by G-6-P and ADP. Actually, Colowick and Kalckar (12) were the first to suggest the possibility of such an inhibition. However, following the discovery of adenylate kinase by Kalckar (13), Colowick and Kalckar found that AMP inhibits only the latter enzyme, and that yeast hexokinase is not inhibited by either AMP or G-6-P. The failure of glucose-1-phosphate and G-6-P to inhibit yeast hexokinase was confirmed later by Slein et al. (18). A report by Wajzer (36) that yeast hexokinase is inhibited by G-6-P was refuted subsequently by Crane and Sols (21)

who claimed that the inhibition was an apparent one resulting from the methods employed. Weil-Malherbe and Bone (37) found, however, that brain hexokinase is inhibited by hexose monophosphate, and that the inhibition is non-competitive (cf. 38) with respect to glucose and ATP. Evidently, only G-6-P is responsible for the inhibition and neither fructose-6-phosphate nor hexose diphosphate was found to be effective in this capacity. When glucose and hexose monophosphate were added to the rat brain preparation, in the presence of ATP, the phosphorylation of glucose was found to lag behind that of the hexose phosphate until a considerable portion of the phosphate ester had been removed from the medium. Thereafter, the rate of phosphorylation of glucose was found to increase gradually. Crane and Sols (32) observed a similar inhibition of their purified preparation of brain hexokinase by hexose-6-phosphate. They found that the rate of glucose utilization by the enzyme diminished rapidly with time, apparently as a result of the accumulation of G-6-P. Furthermore, the addition of G-6-P augmented the inhibition, while the addition of purified phosphofructokinase, in large amounts, relieved the inhibition and permitted the reaction to revert to zero-order kinetics. On the basis of these observations, Crane and Sols developed an equation which enabled them to calculate the true activity of brain hexokinase from the observed activity. In a later

paper these authors (21) reported a study of the type and degree of inhibition produced by six out of a total of twenty-five analogues of G-6-P. They came to the conclusion that only those hexose phosphates with the proper configuration of the hydroxyl groups at carbons 2 and 4, a pyranose ring structure and with the phosphate groups in the 6-position, can inhibit brain hexokinase. Furthermore, since the inhibition produced by G-6-P proved to be of the non-competitive type, Crane and Sols concluded that the enzyme must possess at least three distinct sites for the attachment of substrates and inhibitor. Thus, one site would be reserved for the attachment of the sugar molecule, another for ATP and a third for the attachment of G-6-P. It follows then that, after the phosphorylation of a sugar molecule, the phosphate ester is released from the site of its formation and combines, subsequently, with another site on the enzyme. It is from this latter site that the hexose phosphate can interfere with the phosphorylation of other sugar molecules. These authors postulated, furthermore, that the metabolism of hexoses in animal tissues can be characterized by a certain "steady state". In this "steady state" the rate of phosphorylation of sugars would be limited, at all times, by the conditions existing within the hexokinase-G-6-P-phosphofructokinase

system. The alteration of any of these factors in a tissue, or during the course of an in vitro experiment, would tend also to modify the rate of phosphorylation of sugars. A similar concept has played an important part in the study of the metabolic derangements involved in diabetes mellitus. It is interesting to note also, as Crane and Sols pointed out, that of the physiologically important hexoses, only glucose gives rise to an inhibitory phosphate ester.

Lardy et al. (44) found that l-glyceraldehyde depressed the hexokinase activity of beef brain. To elucidate the mechanism of this inhibition, they showed that sorbose-1-phosphate is formed by aldolase from l-glyceraldehyde and triose phosphate, and that sorbose-1-phosphate inhibits hexokinase and not phospho-hexokinase.

ADP was found also to have an inhibitory effect on brain hexokinase (18), but not on the hexokinase from yeast (12,37). Sols and Crane (39) demonstrated the competitive nature of the inhibition and found that the affinity of brain hexokinase for ADP was about the same as that for ATP. It is possible, therefore, that the intracellular ratio of ATP:ADP, like the G-6-P system mentioned earlier, may afford a means of controlling the activity of hexokinase and, indeed, of the metabolism of the cell.

Sulfhydryl reagents and war gases.

Studies on the effect of reagents such as p-chloromercuribenzoate, o-iodosobenzoate and alloxan, which combine with andmask sulfhydryl groups[2], indicate that the activity of brain hexokinase is dependent upon the integrity of certain sulfhydryl groups on the enzyme molecule (39). The addition of cysteine to the system reverses the inhibition produced by p-chloromercuribenzoate, but not that produced by o-iodosobenzoate. Metal-binding agents such as versene were found to protect hexokinase against inactivation by dilution of the medium, while insulin and serum albumin proved to be less effective in this respect. Saltman (33) observed that inhibitors such as the above-mentioned compounds caused only a slight inhibition of the hexokinase from higher plants. He interpreted this as indicating that these enzymes, much the same as the hexokinase from yeast, are not markedly dependent upon functional -SH groups for activity. However, the data reported by Saltman show that the inhibition is actually of considerable magnitude, and differs only in degree from that reported for brain hexokinase (32,39). Thus, while 5×10^{-4} M $CuSO_4$ produced complete inhibition of the

---

2. The reader is referred to the excellent review by Fraenkel-Conrat (40) for a more complete coverage of the chemical reactions of enzyme proteins.

purified brain hexokinase, 1 x 10^{-3}M $CuSO_4$ caused only 19% inhibition of the plant enzyme. Moreover, it is apparent from the reports of Dixon and coworkers that sulfhydryl groups on the hexokinase from yeast are essential for the activity of the enzyme.

Dixon and Needham (41), in their work on chemical warfare agents during World War II, tried to correlate the physiological action of these substances with the effects they could be shown to have on specific enzyme systems. They found that all vesicants (blistering agents), regardless of their chemical constitution, were capable of inhibiting yeast hexokinase in vitro, whereas all nonvesicants, even though some of them were chemically very similar to certain of the vesicants, failed to inhibit the enzyme. Furthermore, a direct relationship was found to exist between the intensity of the vesicant action of mustard gas and the degree of inhibition of the hexokinase activity of rat skin. Thus it was found that after short periods of exposure of the skin to the vesicant, a reduced, though persistent, hexokinase activity could still be demonstrated. However, when blistering had commenced, glycolysis was no longer operative in the skin, and the hexokinase activity had been reduced to zero. On the other hand, the capacity of the tissue to utilize hexose diphosphate remained unimpaired throughout. Lewisite, another vesicant, also caused an inhibition

of hexokinase and other -SH enzymes, but in all cases, BAL (British antilewisite, dithiopropanol), a powerful antidote against Lewisite, was found to protect the enzymes against the action of the gas. Monothiols such as cysteine exerted a similar protective action against Lewisite. Dixon and Needham observed, further, that lachrymators (irritant agents which provoke a discharge of tears) inhibit -SH enzymes such as hexokinase, alcohol dehydrogenase and triose phosphate dehydrogenase. The accumulated evidence indicated that the in vivo effect of these chemical warfar agents is involved, if not primarily, at least intimately with the inactivation of the -SH enzymes, among them hexokinase, of the tissues.

Dixon and Needham (41) and also Bailey (42) were able to measure the combination, in certain small proportions, of mustard gas with yeast hexokinase. However, these proportions were not significantly different from those observed in the binding of the vesicant by other proteins such as ovalbumin, fibrinogen and other serum proteins. These authors concluded therefore, that the sensitivity of hexokinase to mustard gas was not due to any special reactivity of the enzyme molecule as a whole with the gas, but rather, that it depended upon the affinity of certain groups, such as the -SH groups which are essential for the enzyme activity of the molecule, for mustard gas. Griffiths (43) found that alloxan and

ninhydrin inhibit the hexokinase of rat muscle, and that cysteine can relieve this inhibition. However, while 5 x 10^{-3}M cysteine was found to counteract, completely, the inhibitory effect of 2.86 x 10^{-3}M alloxan, a concentration of cysteine as high as 1 x 10^{-2}M could only partially counteract the inhibition produced by 2.5 x 10^{-3}M ninhydrin. Griffiths also made a study of the stoichiometry of the inhibition, and of the reactivation of the enzyme by cysteine, and concluded that the destruction of the thiol groups of hexokinase by alloxan and ninhydrin could not alone account for the inhibitory effect of these agents. He suggested, therefore, that other reactive groups on the enzyme, such as the amino and hydroxyl groups, may be involved in the reaction of hexokinase with alloxan and ninhydrin (cf. 40). By analogy, it may be assumed that similar considerations apply also to the mode of action of the war gases on hexokinase.

Insulin, hormones and hexokinase.

In 1945, Price et al. (15) reported that extracts prepared from the tissues of rats previously injected with anterior pituitary extracts exhibited considerably less than normal hexokinase activity. Addition, <u>in vitro</u>, of the pituitary extracts to the enzyme preparations from nontreated animals likewise

brought about an inhibition of hexokinase. Furthermore, extracts from the tissues of rats made diabetic with alloxan also were found to show less than normal enzyme activity, but most important of all, insulin was shown to increase the hexokinase activity in all of the above cases, even though the hormone, when added to the tissues of normal, control animals, produced no effect. These findings, and their confirmation by Colowick et al. (16), led to a great deal of speculation concerning the mode of action of insulin and its effect on hexokinase. However, other workers were not successful in repeating these experiments. On the contrary, Brok-Kahn and Mirsky (45) found that the hexokinase activity of rats made diabetic with alloxan remained normal. Furthermore, the addition of insulin did not affect the extracts from either diabetic or normal muscle. These authors concluded, therefore, that the carbohydrate disorders of diabetes must be attributable to some factor other than the inhibition of hexokinase.

More recently, Chaikoff (46) studied the metabolism of glucose-C^{14} and fructose-C^{14} by rat liver slices. He found that, whereas the oxidation of glucose-C^{14} by liver slices from diabetic animals was reduced below normal, the oxidation of fructose-C^{14} by these slices remained unaffected. The addition of insulin brought about a complete restoration of the ability

of the tissue to oxidize the C^{14}-labeled glucose. Chaikoff then showed that the conversion of fructose -C^{14} to glucose-C^{14} in diabetic liver was not impaired, and also that liver homogenates from diabetic animals could convert G-6-P to F-6-P with the same ease as homogenates from normal livers. In this respect, the Coris (47) had shown earlier, that, accompanying the increased utilization of glucose by animal tissues in the presence of insulin, there is a simultaneous increase in the level of the hexose monophosphates of these tissues. However, the relative rate of metabolism of the phosphate esters was found to be unchanged. Chaikoff concluded, as a result of his experiments, that hexokinase is blocked in the diabetic state, and that insulin acts directly on hexokinase to activate it. Broh-Kahn and Mirsky (45) reported that insulin does not affect glucose-6-phosphatase.

In recent years a great deal of work on the problem of diabetes has been carried out on the isolated diaphragm of the rat. Krahl and Cori (48) and Park (49) reported that insulin increases the uptake of glucose by the diaphragms of normal and diabetic rats, and also by the diaphragms of rats injected with extracts of the anterior pituitary and adrenal cortex (50). Villee and coworkers (51), using C^{14}-labeled glucose, found that insulin increases the uptake of

glucose, the synthesis of glycogen and the oxidation of glucose to CO_2 by rat diaphragm. Beloff-Chain et al. (52) obtained similar results and found that insulin does not affect the synthesis of glycogen from glucose -1-phosphate. Mackler et al. (27) observed that only the glucose and not the fructose utilization of rat diaphragm is affected by a fall in pH. Subsequently, these workers found that the hypoglycemic action of insulin, in the intact animal, is antagonized by a condition of acidosis.

Stadie et al. (53,54) were able to demonstrate that insulin is taken up and tightly bound to rat diaphragm when the tissue is temporarily suspended in an insulin-containing medium. The bound insulin cannot be removed from the diaphragm by washing with a phosphate buffer, and yet it can exert an influence on the glucose metabolism of the muscle. Lee and Williams (55) infused I^{131}-labeled insulin into rat liver and found that the hormone passes into the hepatic cells quite readily and then combines, irreversibly, with the various intracellular components.

Goldstein et al. (56) studied the effect of insulin on the distribution of sugars within the eviscerated-nephrectomized dog. They found that insulin brings about a shift of administered glucose, galactose, d-xylose and l-arabinose from the extracellular fluid

into the intracellular spaces of the tissues. The authors interpreted this as indicating that insulin "facilitates" the transfer of hexoses across the cellular membranes. The distribution of d-arabinose, d-sorbitol, l-sorbose, d-mannose and d-fructose in the fluids of the body, however, was found not to be affected by insulin. Wick and Drury (57) confirmed these results and found that glucose and galactose compete with each other in the transfer reaction which is "facilitated" by insulin. In many ways [the non-facilitated transfer of mannose is an exception (see p. 12)] this mechanism has been found to behave like hexokinase. Indeed, it has been suggested that hexokinase participates in the transport of glucose across cell membranes (see "Discussion").

It was noted above that insulin increases the utilization of glucose by isolated rat diaphragm. However, the effect of insulin on this tissue, as also on the intact animal, is influenced by hormones from the anterior pituitary and adrenal cortex. Thus, it has been found (58) that diaphragms from hypophysectomized animals utilize glucose at a faster rate than those from normal animals. When anterior pituitary extracts are administered to the hypophysectomized animals, the activity of the diaphragms, <u>in vitro</u>, is decreased, but this occurs only if the animals have not been adrenalectomized. Administration of pituitary extracts to

hypophysectomized - adrenalectomized rats produces no change in the glucose utilization of the isolated diaphragms. However, injection of cortical extracts along with the pituitary extracts leads to a diminution in the glucose utilization of the diaphragms. Hypophysectomy or adrenalectomy of alloxan-diabetic rats restores the activity of the isolated diaphragms to normal (48,49). From these results it was concluded that the function of the adrenal steroids is to facilitate the inhibition of glucose utilization by certain pituitary factors.

Serum of alloxan-diabetic rats was found to inhibit the uptake of glucose by isolated diaphragms of normal rats, and insulin was shown to reverse this inhibition (49). Bornstein (59) found that the inhibitory substances are present in the serum β-lipoproteins. The β-lipoprotein fraction from the serum of normal or diabetic-hypophysectomized rats was found to be non-inhibitory to the isolated diaphragms of normal rats. However, if the normal or diabetic-hypophysectomized rats were pretreated with growth hormone and cortisone, an inhibitory substance could be shown to be present in the serum. Bornstein also noted that the inhibitory β-lipoproteins are easily inactivated by freezing, or even by standing in an ice-bath. This observation may be helpful in explaining the inconsistencies frequently

reported in experiments with the inhibitory factors of serum and also of other tissues. Finally, Bornstein concluded that insulin-reversible inhibition of the isolated diaphragms by β-lipoproteins must be localized at the hexokinase step.

The growth hormone is frequently considered to be identical with the hyperglycemic factor of the anterior pituitary. Krahl and Park (60) observed that during the first few days of injection of pituitary extracts or crystalline growth hormone into hypophysectomized rats, the glucose utilization of the isolated diaphragms actually was increased; only after this initial period was the rate of glucose utilization diminished. Park (49) was unable to separate the growth hormone from the diabetogenic or hyperglycemic factor. He suggested that the growth hormone may actually be the hypoglycemic factor, and that, through the mediation of the adrenal cortical hormones, this substance is converted, subsequently, into the inhibitory or hyperglycemic factor. According to Stadie et al. (53,54) the hyperglycemic factor appears to compete with insulin for the same binding sites in the diaphragm.

In 1923 Murlin et al, (61) discovered a hyperglycemic factor in the extracts of pancreatic tissue and named the factor "glucagon". Sutherland (62) suggested that the factor probably is elaborated in the

α-cells of the pancreas. He found also that this hyperglycemic-glycogenolytic (H-G) factor, as well as adrenalin, can protect the α-phosphorylase activity of rat liver slices. A similar effect of adrenalin, but not of the H-G factor, was observed on the α-phosphorylase of isolated rat diaphragms. McDermott et al. (63) found that adrenalin also causes a discharge of the anterior pituitary and adrenal cortex. These observations may in some measure, explain the general hyperglycemic effect of adrenalin, in vivo.

Recently, Grossman et al. (64) reported that hydrocortisone and cortisone decrease the utilization of glucose by isolated rat diaphragms. Corticosterone, on the other hand, depresses only the oxidation of glucose without affecting its uptake and conversion to glycogen. Desoxycorticosterone does not affect the oxidation, but inhibits the conversion of glucose to glycogen. Weber et al (65) injected cortisone into rats and found an increased glycogen content and also an increased glucose -6-phosphatase activity in the liver homogenates of these rats. Luthy and Verzar (66) showed that adrenalectomy leads to a diminution in the ATP-ase activity of the intestinal mucosa. Administration of corticosteroids restores this activity without affecting the activity of hexokinase. These observations serve to indicate the many ways in which the cortical hormones may act. The

postulated effect of these hormones on the pituitary factors, and thereby on hexokinase, has been referred to already.

E X P E R I M E N T A L

1) MATERIALS:

A. Substrates.

The substrates and reagents used throughout were commercial preparations. The adenine nucleotides, glucose-6-phosphate, adenosine, inosine and the sodium salt of heparin were obtained from Nutritional Biochemicals Corporation, and the dipotassium salt of ATP and TPN "90", from Sigma Chemical Company. Glucose-6-phosphate was supplied as the barium salt and was converted to the sodium or potassium salt by precipitation of the barium with sodium or potassium sulfate. The solutions of the phosphorylated intermediates were neutralized with $KHCO_3$.

Solutions of ATP and ADP were subjected to paper electrophoresis[3] and it was found that fresh preparations of the nucleotides were essentially pure. On standing in the refrigerator, however, the nucleotides, whether in the powdered form, or in solution, became contaminated with ADP and AMP.

B. Enzymes.

(i) Lipase (steapsin) and pepsin (porcine origin) were obtained from Nutritional Biochemicals

---

3. These assays were carried out with the assistance of Dr. J.-M. Loiselle of our laboratory.

Corporation and Armour Laboratories, respectively. Solutions containing 5 mg./10 ml. in isotonic KCl were prepared from the commercial materials without further purification.

(ii) Glucose-6-phosphate dehydrogenase was kindly supplied by Mr. P. Ottolenghi of our laboratory, who prepared the enzyme from brewer's yeast by a modification of the method of Kornberg (67).

(iii) Yeast hexokinase was obtained from Nutritional Biochemicals Corporation. The preparation was specified to contain 28,000 Kunitz-McDonald units (34) per gram, at 30°C. A diluted stock solution containing 10 mg. of enzyme per ml. was used in the experiments.

(iv) Rabbit hexokinase. Blood was collected from the ear of adult rabbits, with heparin as the anticoagulant. The blood specimen was diluted with three or four volumes of cold, isotonic KCl, mixed, and centrifuged at low speed in a refrigerated centrifuge. [In the earlier experiments, isotonic sodium chloride was used both for washing the erythrocytes and in the incubation media. Later, the sodium salts of the various reagents and substrates were replaced by the corresponding potassium salts and, consequently, the routine washing of the erythrocytes and the stroma was carried out with isotonic potassium chloride (0.154M)].

After centrifugation, the clear supernatant

and the buffy coat of white blood cells[4] were withdrawn and discarded. The procedure was repeated four times in all, and the resulting "washed erythrocytes" were made up to the original volume of the blood with isotonic KCl.

The erythrocyte suspension was hemolyzed by freezing in a mixture of acetone and dry ice, and thawing. This procedure was repeated three times to ensure complete hemolysis of the erythrocytes. To obtain a stroma-free hemolyzate (SFH) preparation, the whole hemolyzate was centrifuged at about 2000 x g for 5 minutes, at 3°C. The sparkling clear supernatant was then removed from the sedimented stroma (membrane) material, and diluted with a quantity of isotonic KCl proportional to the volume of packed stroma. When desired, the stroma ("unwashed stroma") was washed by suspending it in isotonic KCl and centrifuging. The washing procedure was repeated from four to eight times. However, regardless of the number of washings, the final product was always pink in color. The amount of pigment present in the material, however, was too small to be measured by the usual hemoglobin method (see below). Finally, the "washed stroma" or the "unwashed stroma" was diluted with KCl to the original

---

4. The reader is referred to the paper by Bartlett and Marlow (68) for a comparison of the rates of glucose utilization by white blood cells and erythrocytes. From the values reported by these workers, it may be assumed that, in the present experiments, glucose utilization is due almost entirely to the erythrocytes.

volume of the whole hemolyzate.

(v) Human hexokinase. Human blood was collected by venipuncture, with sodium heparin as the anticoagulant. The washing of the erythrocytes was carried out as described in the preceding paragraphs for rabbit erythrocytes, except that isotonic KCl was used throughout. It soon became evident, however, that the earlier procedure for hemolyzing the red blood cells, namely, by alternately freezing and thawing, would not be suitable for hemolyzing the human erythrocytes. When hemolysis was carried out by the above procedure, it was found impossible to separate the stroma from the soluble cytoplasmic portion of the hemolyzate, even when centrifugal forces as high as 24,000 x g were used. Only a small portion of the stroma was sedimented under these conditions, and the supernatant fluid remained turbid. Separation of the stroma from the turbid material could not be achieved even by filtration through a sintered glass filter, under reduced pressure, and lowering of the pH of the hemolyzate did not improve the efficiency of sedimentation of the stroma, either. In view of the above observations the following procedure was adopted in preparing the various fractions from human erythrocytes: one volume of washed red blood cells was well mixed with two volumes of ice-cold distilled water and the resulting mixture was kept at 3°C. for a period

of about thirty minutes, with occasional stirring. At the end of this time 0.2 volumes of 1.54M KCl was added to the hemolyzate so as to return the mixture to isotonicity with respect to the KCl. The addition of the salt solution was always accompanied by the appearance of a white cloudiness in the hemolyzate. With gentle mixing the cloudiness was dissipated, but the resulting suspension was definitely more turbid than the original, hypotonic hemolyzate. Apparently, this phenomenon was due to the process frequently referred to as "reversible hemolysis" (cf. 69,70). Centrifugal separation of the stroma was achieved quite readily with forces of the order of 1200 x g. Although the SFH which was obtained was sparkling clear, as with the preparations from the rabbit erythrocytes, the stroma contained a considerable amount of dark red material, presumably intact or partially hemolyzed erythrocytes (cf. 69). The residue or "stroma" was made up to the original hemolyzate volume by the addition of isotonic KCl. Subsequently, it was found possible to obtain a homogeneous stroma preparation by washing the residue with distilled water. The stroma was uniformly pink in color at the end of about six washings. Although the "washed stroma" was easily precipitable by centrifugation at 1200 x g, it became swollen in water and, as a result, the volume of the washed stroma was greater than

that of the original hemolyzate.

2) METHODS.

A. Assay of hexokinase.

Samples of erythrocyte preparations were incubated at 37°C. with the following, for periods usually of one to two hours duration:-

0.1 ml. phosphate buffer, 0.2M, pH 8;
0.2 ml. glycylglycine buffer, 0.2M, pH 8;
0.1 ml. $MgCl_2$, 0.075M;
0.1 ml. ATP, 0.075M;
and 0.1 ml. glucose, 0.033M.

(The molarities indicate the concentrations of the solutions added). Various quantities of the erythrocyte preparations, usually 0.2-0.5 ml., were used, and the final volume of the incubated mixture was made up to 1.5 ml. with isotonic KCl - the above solutions were usually made up in isotonic KCl. When required, 0.1 ml. of 0.15 M KF was included in the above system. It was found frequently that preincubation of the samples at 37°C. for 5 minutes, in the absence of glucose and ATP, led to more consistent results.

After incubation, 10 ml. of distilled water and 2 ml. each of $Ba(OH)_2$ and $ZnSO_4$ were added to the samples to precipitate proteins and phosphorylated intermediates. The precipitates were centrifuged and glucose was determined on a 2 ml. aliquot of the supernatant. "Blanks" or "zero-time" controls were prepared

as above, but the precipitation of the protein was carried out without incubation of the samples.

(i) Hexose determinations were carried out according to the method of Nelson (71). Standard curves were prepared for glucose, fructose, mannose and galactose. Adenosine was found to interfere with the method, but inosine did not affect it.

In the experiments in which extremely small quantities of glucose were used, hexose was estimated by the method of Park and Johnson (72). This change in procedure was necessitated by the fact that the Nelson method can be used to measure only 20-100 μg. of glucose, whereas the Park-Johnson method will estimate as little as 1-9 μg. of the sugar.

(ii) pH measurements were made at room temperature with a Beckman pH meter.

Hemoglobin was determined by the method of Evelyn and Malloy (73). 0.02 ml. of the blood sample was added to 10 ml. of distilled water, containing a drop of concentrated ammonia, and the optical density of the solution was determined at 540 mμ.

B. Preservation of blood.

Blood was collected from adult humans, by venipuncture, into two types of preservative media. One of these was a neutral, isotonic solution of sodium citrate and the other was an acidified citrate-dextrose solution,

designated as "ACD". The proportions of blood to preservative medium were as follows:

(a) Five parts of blood to 1.5 parts of a CD solution which contained 2.1% trisodium citrate dihydrate and 1.7% anhydrous dextrose;

(b) Four parts of blood to one part of an ACD solution containing 1.37% trisodium citrate, 0.5% citric acid monohydrate and 2.5% dextrose.

For storage experiments with added adenosine or inosine, the nucleosides were added to the preservative media before autoclaving. This permitted solution of the nucleosides in the preservative medium, since at room temperature, the solubility of the nucleosides is very low. However, in later studies the nucleosides were added at various times during the storage period, and the nucleosides were autoclaved separately, in the dry state. The quantity of nucleoside added was such as to give a concentration of 400-800 mg.% in the final stored sample. The blood specimens were stored at 4°C. and were agitated frequently to resuspend the cells.

(i) Chemical analyses. At appropriate intervals, aliquots of the stored specimens were withdrawn, with aseptic precautions, and the following chemical analyses were carried out: glucose, by the method of Nelson (71); ribose, by the method of Meijbaum (74); pyruvate, by the method of Friedman and Haugen (75);

lactate, by the method of Barker and Summerson (76), and inorganic phosphate, by the method of Fiske and SubbaRow (77).

A fractional analysis of the organic phosphate esters was carried out as described by Pappius et al. (78). The blood samples were deproteinized with trichloroacetic acid (10% solution) and the filtrates hydrolyzed in IN HCl at 100°C. The inorganic phosphate liberated by hydrolysis was determined by the method of Fiske and SubbaRow (77). For convenience of discussion, the various phosphate fractions have been designated as follows:

P_i - inorganic phosphate initially present;

P_l - "labile" organic phosphate esters which are hydrolyzable within seven minutes in N HCl at 100°C. P_l was obtained, therefore, by subtracting P_i from the total inorganic phosphate present after hydrolysis. This fraction represents mainly the two terminal phosphate groups of ATP and the phosphate esters attached to carbon 1 of hexose and pentose sugars.

P_m Organic esters of moderate stability which undergo hydrolysis within the interval between the 7th and 100th minute, i.e., total inorganic phosphate after hydrolysis minus $[P_l + P_i]$. These esters include the phosphoric esters on the terminal carbon atom

of hexose and pentose sugars.

P_s - Stable phosphate esters which are not hydrolyzed in N HCl at the end of 100 minutes. This fraction consists mainly of the phosphate groups of 2,3,-diphosphoglycerate (DPG).

(iii) Electrophoresis of nucleosides.

The blood samples were deproteinized with cold perchloric acid (2%) and 0.05 ml. aliquots of the filtrates were subjected to paper ionophoresis using a Spinco electrophoresis apparatus of the Durrum type. The buffer used was a 0.05M citrate buffer, pH 2.5, and a constant voltage of 400 volts was applied across the paper strips. At the end of three hours the paper strips containing the nucleosides were dried and photographed, by a contact process, under ultraviolet light (240 mμ).

3). EXPERIMENTAL RESULTS.

A. Hexokinase of the rabbit erythrocyte.

(i) Preparatory studies.

Preliminary experiments showed that hexokinase is present in the rabbit red blood cell. This finding confirmed those of Meyerhof (9) who demonstrated that hexokinase is present in the erythrocytes of several mammalian species, including the rabbit red blood cell.

The results presented in Table I show that when glucose is incubated with intact rabbit erythrocytes or with hemolyzates prepared from the erythrocytes, glucose disappears from the medium, and ATP is necessary for this to occur. The activity of intact cells in the absence of added ATP can be explained by the fact that red blood cells contain a considerable amoumt of endogenous ATP (79). Indeed, in the later experiments, the addition of ATP to the erythrocytes frequently failed to bring about an increase in the rate of glucose phosphorylation.

TABLE I

	Δ µg. glucose/hour
Intact erythrocytes	67
Intact erythrocytes + ATP	90
Hemolyzate	0
Hemolyzate + ATP	147

It was interesting that the enzyme activity of the hemolyzate was higher than that of the intact cells. Whether the cell membrane or internal cell structure may act as a barrier to the free diffusion of substrate to the site of the enzyme, was not known. Meyerhof (9) found that the hexokinase activity of the stroma-free hemolyzate

of rabbit erythrocytes was only about half of that of the intact cells. However, Meyerhof maintained his reaction media under conditions which were different from those used in the present experiments, and this may explain the discrepancy in the results.

In order to ascertain whether the depletion of substrate or the accumulation of the products of the reaction interfered with the rate of glucose phosphorylation, an experiment was carried out in which aliquots of a red cell hemolyzate were incubated with glucose, ATP, Mg^{++} ions, inorganic phosphate, TRIS buffer (pH 8) and fluoride ions for various lengths of time. The rate of utilization of glucose was essentially constant with time, thus indicating that the reaction was of a zero -order. By the end of the experimental period (eighty minutes) about 30% of the glucose present had been utilized. This was estimated to be equivalent to a utilization of about 13% of the added ATP. In another experiment the amount of hemolyzate added was varied and it was found that the rate of phosphorylation of glucose was proportional to the amount of enzyme until at least 78% of the added glucose and, theoretically, 35% of the added ATP had been utilized. These results indicate that under the conditions of the experiments, hexokinase remained fully active, and that there was no depletion of substrates, or an accumulation of the products of the

reaction (i.e., ADP and G-6-P). The latter is evident from the fact that these products were subsequently shown to produce an inhibition of the enzyme.

(ii) Behavior of hexokinase toward ATP

The dependence of hexokinase on the concentration of ATP is illustrated in Fig. I (A and B). There was reason to suspect that an ATPase is present in the hemolyzate of rabbit erythrocytes, and that this might interfere with the determination of hexokinase in the erythrocyte preparations (vid. 80). When fluoride (1×10^{-2}M) was added to the hemolyzate, there resulted an increase in the rate of glucose phosphorylation as indicated in Fig. IA. However, the optimal concentration of ATP remained the same as in the absence of fluoride. Variation of the concentration of fluoride from 1×10^{-3}M to 5×10^{-2}M caused no significant change in the activity of the enzyme. With a stroma-free hemolyzate, on the other hand, addition of fluoride produced no change in the rate of glucose phosphorylation. Evidently, there is present in the stroma an ATPase which is inhibited by fluoride. Clarkson and Maizels (80) and Szekely et al. (81) came to a similar conclusion regarding the location of ATPase in the red blood cell. It may be concluded, furthermore, that fluoride does not inhibit hexokinase.

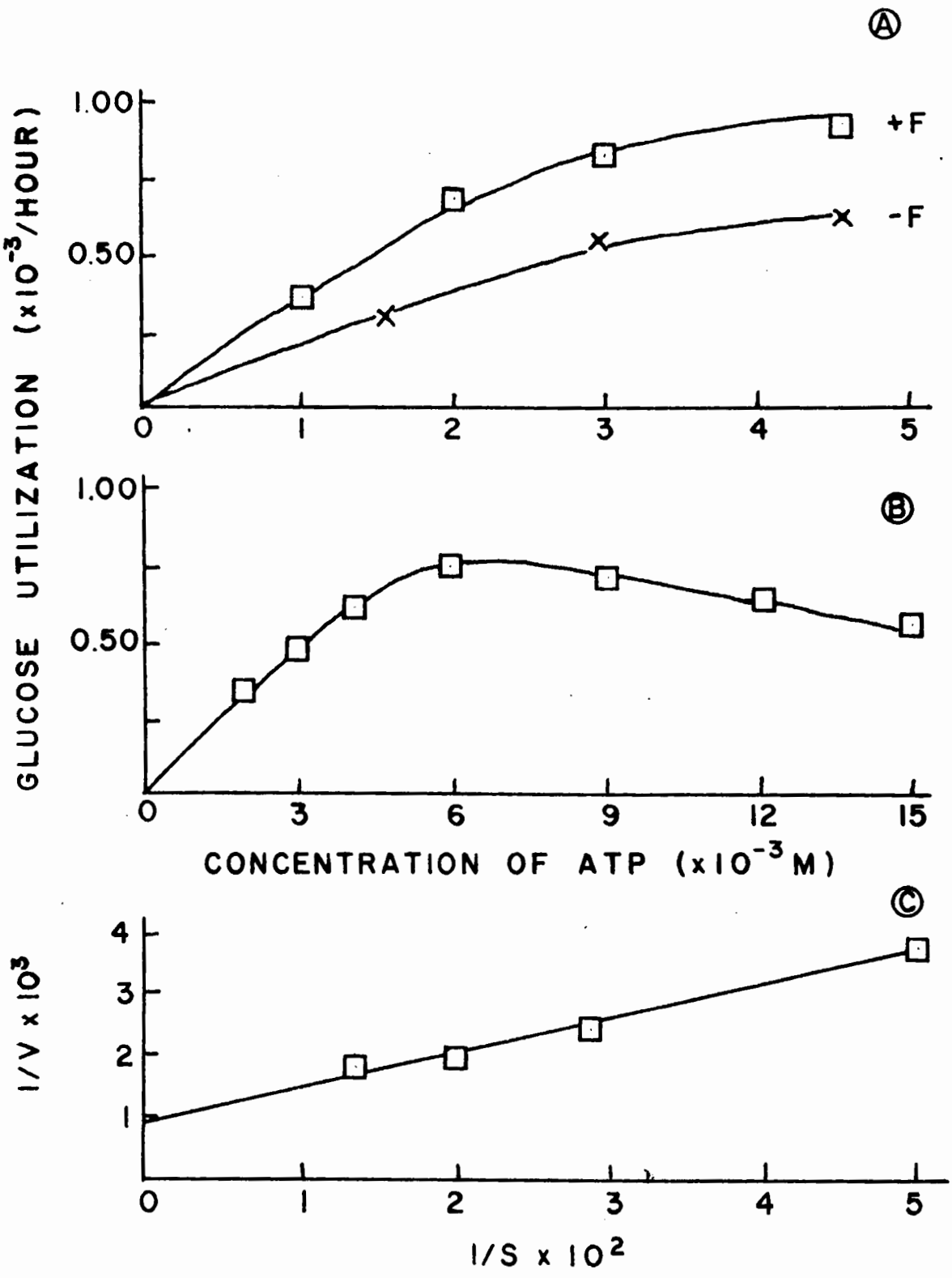
FIGURE I

The dependence of hexokinase of the rabbit erythrocyte on the concentration of ATP

Conditions:-

0.5 ml. SFH incubated with 1.33mM phosphate and 2.67mM glycylglycine buffer, pH 8; 5mM $MgCl_2$; 2.2mM glucose; ATP as indicated in the Figure; 0.154 M KCl to make the final volume up to 1.5 ml. Incubation temperature, 37°C. Glucose was measured by the method of Nelson (71).

FIGURE 1


Ⓐ
1.00
0.50
+F
-F
0
1
2
3
4
5
GLUCOSE UTILIZATION (x10⁻³/HOUR)
Ⓑ
1.00
0.50
0
3
6
9
12
15
CONCENTRATION OF ATP (x10⁻³ M)
Ⓒ
4
3
2
1
0
1
2
3
4
5
1/V x 10³
1/S x 10²

The effect of fluoride on the utlization of glucose by intact red blood cells was found to be different from that observed with the cell-free erythrocyte preparations. This is indicated in Table II. In this Table, glucose utilization by the intact red blood cells without fluoride or ATP is represented as 100%. It is evident that fluoride inhibited the phosphorylation of glucose by the intact cells. Presumably this was due to the inhibition of enolase by the fluoride ions (cf.19). The same result was obtained both in the presence and

TABLE II

Effect of fluoride on glucose utilization by rabbit erythrocytes.

ATP	Fluoride	Enzyme activity (%)
-	-	100
-	+	23
+	-	100
+	+	45

absence of added ATP. Since the red blood cells are known to be impermeable to exogenous ATP (69,82), the lack of an effect of added ATP on the phosphorylation of glucose was not unexpected. However, the effect of ATP in diminishing the inhibition by fluoride is more difficult to account for. Perhaps it may be explained by the solubilizing action of ATP on magnesium fluorophosphate (cf.83).

A closer examination of Fig I B reveals that at the higher concentrations of ATP, the rate of glucose phosphorylation decreases, progressively, with increasing concentration of substrate. Evidently, the curve is not of the usual Michaelis-Menten type (vid. 19), and it is possible that hexokinase was inhibited by an excess of substrate as suggested by Hofstee (84). However, it is more likely that the enzyme was inhibited by the ADP and AMP present as contaminants in the stock solutions of ATP (p. 34).

The optimum concentration of ATP was found to be of the order of 6×10^{-3}M. Weil-Malherbe and Bone (37) reported an optimum of $1.5\text{-}2.0 \times 10^{-3}$M ATP for the hexokinase of brain tissue, and Long (31) reported an optimum of 5×10^{-3}M ATP for the crude homogenates of various rat tissues. The apparent K_{ATP} for the enzyme of the erythrocyte was calculated using the coordinates only of the initial, rising portion of the curve in Fig. I B. The values were plotted as in Fig. I C and the apparent K_{ATP} was found to be of the order of 6×10^{-3}M. Slein et al. (18) reported a $K_{ATP} = 9.5 \times 10^{-5}$M for yeast hexokinase, and Saltman (33), a value of 8.7×10^{-4}M for the hexokinase of wheat germ. It is evident that the apparent K_{ATP} for the enzyme of the red blood cell, as found by the writer, is relatively high. ADP and AMP, whether added from the ATP stock solution, or released in the course of the

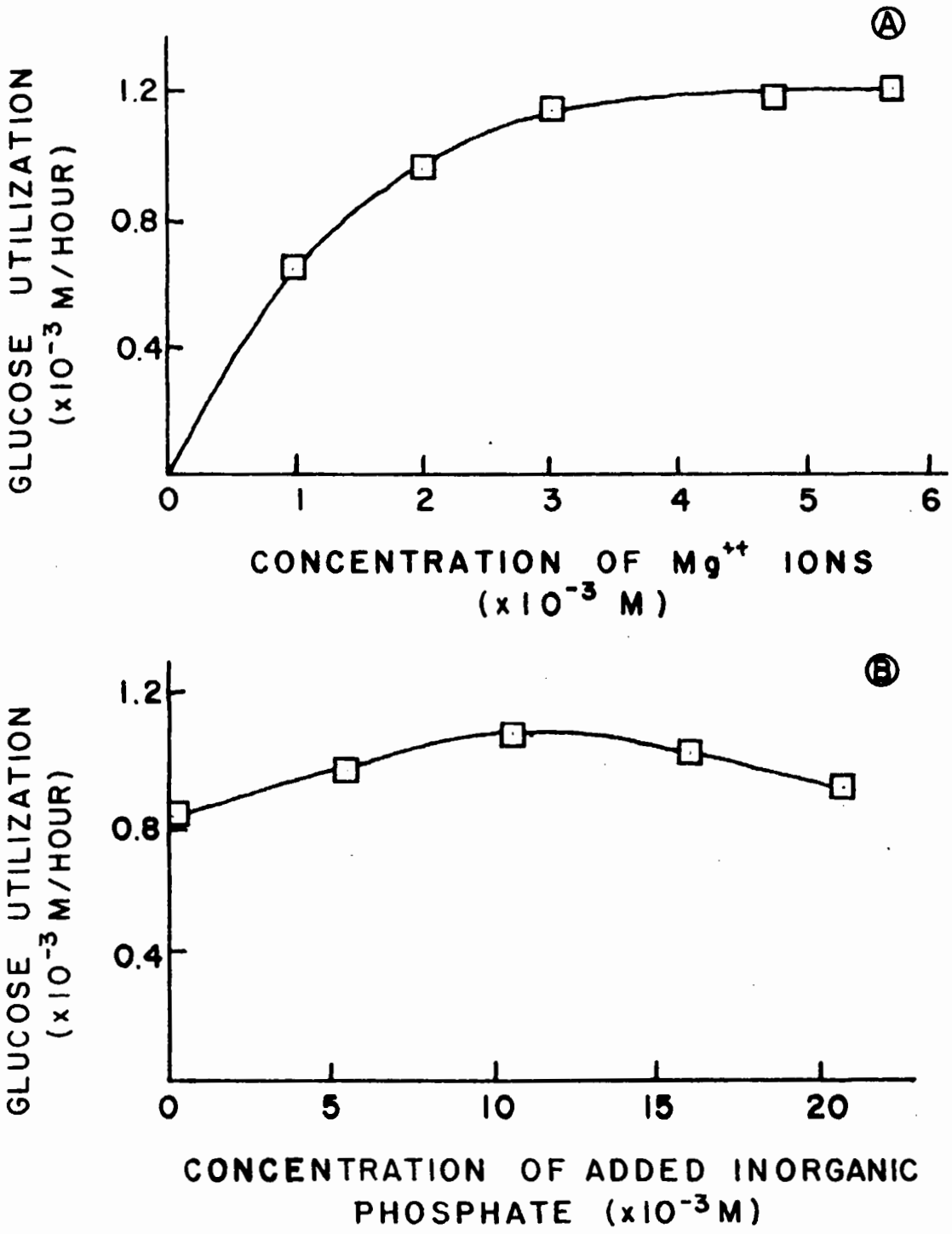
FIGURE 2

The effect of inorganic ions on the activity of hexokinase of the rabbit erythrocyte.

Conditions:-

0.5 ml. hemolyzate incubated with 1.33mM phosphate buffer, or as indicated in the Figure, and 8mM TRIS buffer, pH 8; 5mM $MgCl_2$, or as indicated in the Figure; 2.2mM glucose; 5mM ATP; 0.154 M KCl to make the final volume up to 1.5 ml. Incubation temperature, 37°C. Glucose was measured by the method of Nelson (71).

FIGURE 2


Ⓐ
GLUCOSE UTILIZATION (x10⁻³ M/HOUR)
1.2
0.8
0.4
0
1
2
3
4
5
6
CONCENTRATION OF Mg⁺⁺ IONS (x10⁻³ M)
Ⓑ
GLUCOSE UTILIZATION (x10⁻³ M/HOUR)
1.2
0.8
0.4
0
5
10
15
20
CONCENTRATION OF ADDED INORGANIC PHOSPHATE (x10⁻³M)

phosphorylation of glucose, would tend to inhibit the enzyme and could thereby lead to an increase in the numerical value of the K_{ATP}.

(iii) The effect of inorganic ions.

(a) Magnesium ions. It is evident from Fig.2A that the activity of hexokinase is dependent on the concentration of Mg^{++} ions. Actually, the concentration of Mg^{++} ions in the enzyme preparations was not determined, but it is evident from the shape of the curve that Mg^{++} ions are essential for the activity of hexokinase. Indeed it has been found that phosphorylation reactions involving ATP generally require Mg^{++} ions. In this respect, it is interesting that fluoride ions did not inhibit hexokinase (see p.48). Evidently, fluoride cannot be regarded as an inhibitor of all magnesium-requiring enzymes (cf. 14).

Maximal activity of hexokinase was obtained when the concentration of Mg^{++} ions was about 4×10^{-3} M ATP. The former value compares well with the values reported by Sols and Crane (39) for brain hexokinase, and by Long (31) for the hexokinase of rat intestinal mucosa, heart and kidney. It may be seen, also, that the hexokinase activity of the red blood cell is optimal when the concentrations of Mg^{++} ions and ATP are about equal. Liebecq (85) found the hexokinase activity of rat muscle to be optimal when the ratio of Mg^{++} to ATP was unity. It would appear then,

as suggested by Hers (28), that a magnesium-ATP complex, rather than Mg^{++} ions and ATP separately, is the active substrate of hexokinase. By applying the dissociation constant for the Mg ATP complex [= 1.9×10^{-4} (83)], it can be calculated that, at the optimal concentration of Mg^{++} ions (viz. Fig 2A), most (ca.88%) of these ions exist in the form of a complex with ATP. The Michaelis constant for Mg^{++} ions is of the order of 1.5×10^{-3}M; the K_{MgATP} calculated from this is almost the same.

With respect to the inhibition of hexokinase by high concentrations of ATP, as indicated in Fig. IB, it may be seen that, at the optimum concentration of ATP, the ratio of ATP to Mg^{++} ions might be the limiting factor at the higher concentrations of ATP. However, when the concentration of Mg^{++} ions was increased to three times that employed in the earlier experiment, the inhibition by ATP was still observed and the optimum concentration for ATP remained essentially the same as that indicated in Fig IB. This would be expected only if the enzyme was already saturated with respect to MgATP at the lower level of Mg^{++} ions. It is apparent, therefore, that the diminution in the activity of the enzyme noted in Fig. IB was not attributable to a lack of Mg^{++} ions, but must have been associated, rather, with the increase in the concentration of ATP.

The reader will have observed that the value of 6 x 10^{-3}M reported above for the K_{ATP} is considerably higher than that reported for the K_{MgATP}. Assuming that the MgATP complex is the actual substrate for hexokinase, then, since the Mg^{++} concentration was maximal in the earlier experiments, one would expect the K_{ATP} and K_{MgATP} to be the same. They were not. The K_{MgATP} found, is in closer agreement with the values reported for the hexokinase from other sources (see above), and is probably more reliable since the determination of the constant was not complicated by increasing concentrations of ATP (cf. Fig. 2B).

(b) Phosphate ions. Colowick (14) reported that inorganic phosphate ions can prevent the inactivation of muscle hexokinase in slightly acid media and also during dialysis. In the present experiments, therefore, inorganic phosphate was added to the incubation media as a protective measure. It is evident from Fig. 2B that the hexokinase activity of the rabbit red blood cell was considerably influenced by the concentration of inorganic phosphate. Since the enzyme preparations had not been dialyzed, a considerable amount of endogenous inorganic phosphate was present in the media. However, analyses for the endogenous phosphate was not carried out and, as a result, the data as presented in Fig. 2B are plotted with the concentration of <u>added</u> inorganic phosphate as the abscissa. The

high level of activity at "zero" concentration of phosphate, therefore, was to be expected.

The activity of hexokinase was found to be optimal in the range of 9 - 13 x 10^{-3}M added inorganic phosphate. [At the pH level employed in these experiments (pH 7.8) the phosphate ions exist almot entirely in the dibasic form. Because of the poor buffering capacity of this phosphate solution, glycylglycine buffer was used in the incubated media to maintain the proper pH]. The diminution of the activity of hexokinase at the higher levels of inorganic phosphate is of especial interest. Since the concentration of Mg^{++} ions used in this experiment was 5 x 10^{-3}M, the ratio of inorganic phosphate to Mg^{++} ions, at the optimal concentration of inorganic phosphate (ca. 10 x 10^{-3}M), was 2:1. However, since the concentration of added inorganic phosphate, only, was considered, this ratio represents a minimum value. It is conceivable, therefore, that at the higher concentration of inorganic phosphate, the phosphate ions will tend to bind the Mg^{++} ions in an inactive complex, thereby bringing about a reduction in the activity of the enzyme. Indeed the solubility product ($K_{sp}=[Mg^{++}][PO_4^{=}]$, calculated from the above concentrations of magnesium and phosphate, exceeds 3.2 x 10^{-5}, the solubility product of solid $MgHPO_4$ (83), and it is to be expected, therefore that, at the supra-optimal concentrations of phosphate, many of the

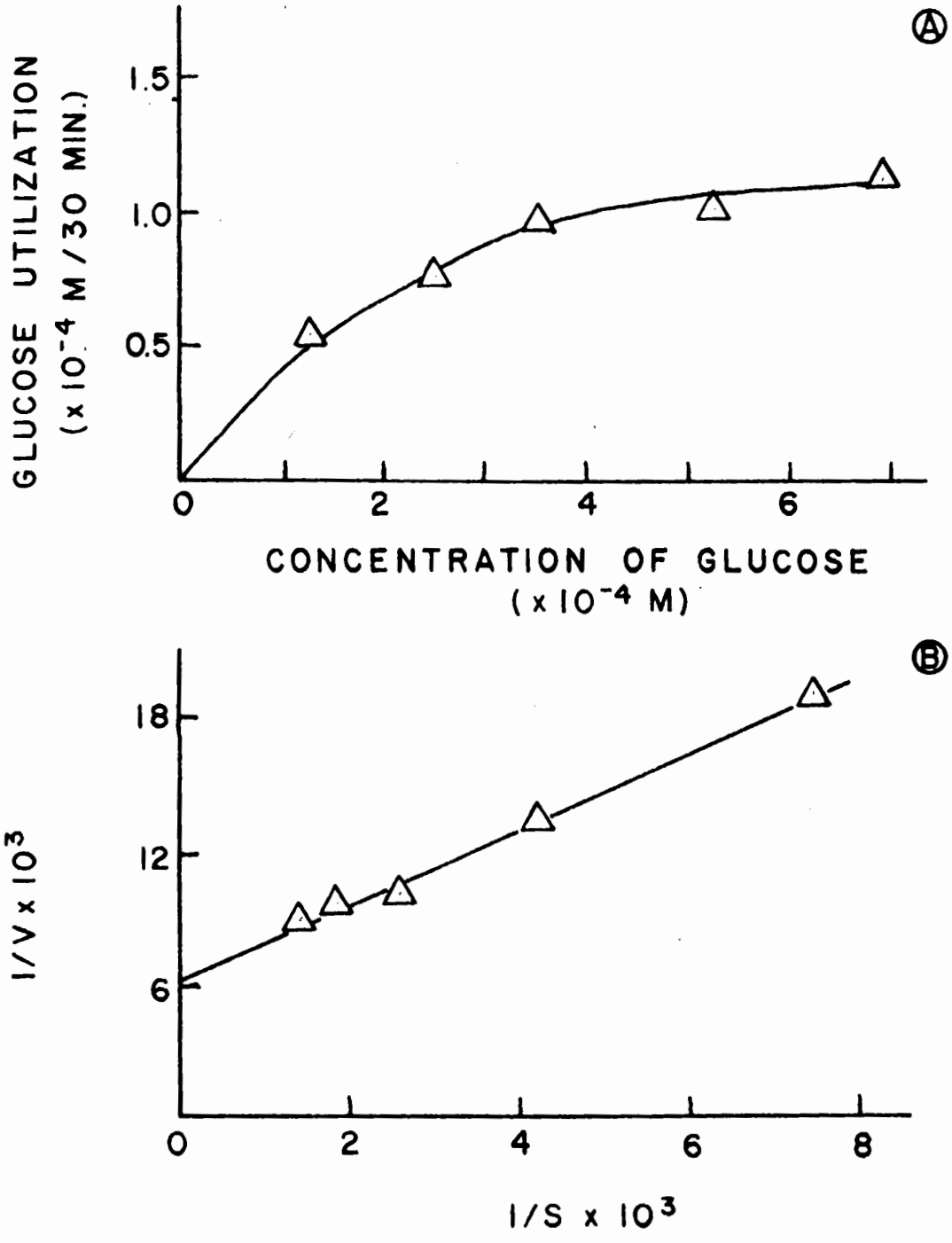
FIGURE 3

Glucose concentration.

Conditions:-

0.2 ml. SFH incubated with 1.33mM phosphate buffer and 2.67mM glycylglycine buffer, pH 8; 5mM $MgCl_2$; glucose as indicated in the Figure; 5mM ATP; 0.154 M KCl to make the final volume up to 1.5 ml. Incubation temperature, 37°C. Glucose was measured by the method of Nelson (71).

FIGURE 3


Ⓐ
GLUCOSE UTILIZATION
(x 10⁻⁴ M / 30 MIN.)
1.5
1.0
0.5
0
2
4
6
CONCENTRATION OF GLUCOSE
(x 10⁻⁴ M)
Ⓑ
1/V x 10³
18
12
6
0
2
4
6
8
1/S x 10³

Mg^{++} ions will no longer exist in the free state.

(iv) Glucose concentration.

A change in the concentration of glucose was found to produce a corresponding change in the activity of hexokinase. This effect is depicted in Fig. 3A. The curve is of the usual Michaelis-Menten type and the $K_{glucose}$ calculated from the data, when represented as in Fig. 3B, is 2.8×10^{-4}M. This value is about one-fifth of that reported for the K_{MgATP} (1.5×10^{-3}M). Because of the low value of the $K_{glucose}$ it was necessary to use relatively low concentrations of glucose in the determination of the constant. The micro-procedure of Park and Johnson (72) was used, therefore, to determine glucose, instead of the Nelson method (71) . However, when these experiments had been completed, it was found that the rate of glucose utilization by hexokinase remained constant from about 5×10^{-4}M to at least 2.6×10^{-3}M glucose. The routine use of 2.2×10^{-3}M glucose was adopted, therefore, and at this concentration the method of Nelson was found to be applicable.

The value given above for the $K_{glucose}$ is somewhat higher than that reported for the hexokinase from other sources. Thus, Sols and Crane (20) found the $K_{glucose} = 1 \times 10^{-5}$M with brain hexokinase. Weil-Malherbe (37) reported the $K_{glucose} = 5 \times 10^{-5}$M for the hexokinase of rat

brain and Saltman reported a value of 4.4×10^{-4}M for wheat hexokinase.

With reference to the estimation of the $K_{glucose}$, there existed the possibility that a hexose phosphatase might be present in the erythrocyte preparations and that this might interfere with the estimation of hexokinase activity. However, when glucose-6-phosphate ($1.3 - 4.0 \times 10^{-3}$M) was incubated with SFH, there was no measurable release of free glucose. This is in agreement with the observations of Roche (86) who found that the optimum pH for the hydrolysis of hexose phosphates occurs in the range of pH 6.0 - 6.7; there is little phosphatase activity in the vicinity of pH 8. The present experiments were carried out at pH 7.8.

(v) The effect of pH.

The effect of pH on the activity of hexokinase is indicated in Fig. 4. It is interesting that the enzyme exhibits two optima of activity. A lesser optimum occurs at pH 6 and a higher one at pH 7.8 - 7.9; a minimum occurs at about pH 6.6. In view of what was said above about the pH optima of the hexose phosphatases in blood (86), it was thought possible that the minimum of hexokinase activity at pH 6.6 might be due to an increased activity of the phosphatases in this pH region. However, the writer's experiments were carried out in the presence of 1×10^{-2}M

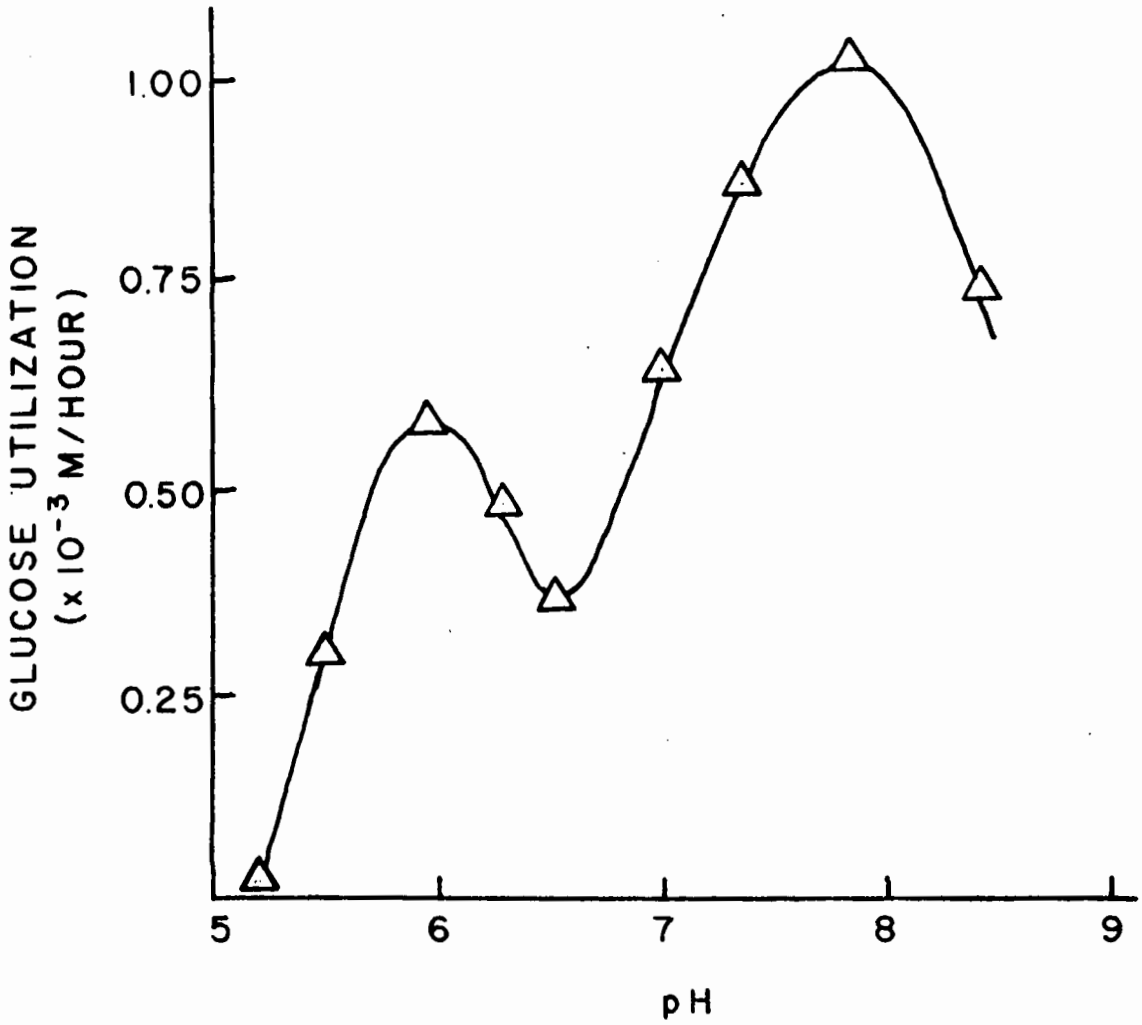
FIGURE 4

The effect of pH on the hexokinase of the rabbit erythrocyte.

Conditions:-

0.5 ml. SFH incubated with 1.33mM phosphate and acetate buffers, and 2.67mM glycylglycine, buffer, pH as indicated in the Figure; 5mM $MgCl_2$; 2.2mM glucose; 5mM ATP; 0.154 M KCl to make the final volume up to 1.5 ml. Incubation temperature, 37°C. Glucose was measured by the method of Nelson (71).

FIGURE 4



fluoride and, indeed, Long (31) observed that 5 x 10^{-2}M KF inhibits the hexose monophosphatase activity of many rat tissues. When 2.2 x 10^{-3}M G-6-P was incubated with SFH in the presence of fluoride, the phosphatase activity was found to be negligible throughout the entire pH range from 4.7 to 8.5. It would appear that the biphasic response of hexokinase is characteristic of the enzyme itself and is not due to the pH-dependence of the associated hexose phosphatases. In this respect, it must be understood that, associated with the change in pH, there may also occur a change in the dissociation, and hence the availability of the substrates (i.e., MgATP). For the moment, however, the latter possibility must be included in the overall effect of pH on the enzyme.

Long (31) and Liebecq (85) reported that the hexokinase activity of rat muscle, kidney and intestinal mucosa is optimal between pH 7.5 and 8.0. These authors, however, did not measure the activity of the enzyme below pH 6.2. Sols and Crane (39) found that the hexokinase activity of their purified brain preparation was optimal within the range pH 6 - 8, although the activity was slightly greater at pH 6 than at pH 8. The present finding that the hexokinase of the erythrocyte exhibits two distinct optima of activity appears, therefore, to be unprecedented.

The effect of pH on the relative rates of utilization of glucose, fructose, mannose and galactose by hexo-

kinase, was studied. Equimolar amounts of these sugars were incubated, separately, with the enzyme at pH 7.8 and 6.6, and the amount of hexose utilized was estimated in each case. The results are listed in Table III. It is evident that the rate of utilization of the four hexoses varies with the pH. At pH 7.8 the rates may be represented in descending order of magnitude as follows:

glucose > mannose > fructose > galactose.

At pH 6.6., on the other hand, the relative position of glucose in the series is shifted:

mannose > fructose > glucose > galactose.

From the ratios of the reaction velocities at pH 7.8 and 6.6, it is apparent that the differential in the utilization of the hexoses at the two pH levels is greatest with glucose. Thus, the utilization of glucose at the higher pH is about three times as great as that at the lower pH, while with fructose, mannose and galactose, the ratios are of a smaller magnitude, and are similar for all three sugars. It is significant that neither fructose nor mannose give rise to G-6-P (14) and that galactose may be phosphorylated to glucose -1,6-diphosphate without passing through the G-6-P stage (91). With glucose, however, the accumulation of G-6-P in the medium, at pH 6.6, could bring about an inhibition of hexokinase (vid. p. 65). Such an inhibition would

TABLE III

Utilization of hexoses in the presence of rabbit hexokinase at pH 7.8 and 6.6

Hexose	Hexose utilization			Relative velocity*	
	pH 7.8	pH 6.6	Ratio	pH 7.8	pH 6.6
	(µg. per hour)				
Glucose	318	115	2.8	100	100
Fructose	195	126	1.5	61	110
Mannose	287	172	1.7	90	150
Galactose	160	104	1.5	50	90

* The rate of glucose utilization was taken as 100.

Conditions:-

0.3 ml. SFH or packed erythrocytes incubated with 2.2mM hexose; 1.33mM potassium phosphate and acetate, and 2.67mM glycylglycine buffers, pH 6 and 8; 5mM $MgCl_2$; 5mM ATP; 3.33mM KF; 0.154 M KCl to give a final volume of 1.5 ml. Incubation temperature 37°C. Reducing sugars were measured by the method of Nelson (71).

result in a relatively greater diminution in the rate of glucose phosphorylation than in the rate of phosphorylation of the other sugars, at the lower pH, and the above findings would be explained.

From the above results (Table III) it is difficult to decide whether a separate galactokinase is present in the rabbit erythrocyte. It is significant, though, that the hexokinases of yeast and brain have been shown not to phosphorylate galactose (20,34). Irving (87), and Spicer and Clark (88) also found that the rabbit erythrocyte utilizes galactose.

(vi) Adenylate kinase.

The presence of adenylate kinase in the rabbit erythrocyte was demonstrated as follows: A preparation of SFH containing added glucose was incubated, in one experiment with 7.5 μmoles ATP, and in another, with 6.6 μmoles ADP, and the rate of glucose utilization was determined in both cases. In the former sample the amount of glucose phosphorylated in the course of one hour was equal to 2.2 μmoles, while with the ADP-containing sample the amount of glucose phosphorylated was only 0.7 μmoles. Thus, about 30% of the ATP but only 10% of the ADP was utilized in the phosphorylation of the sugar. However, the fact that ADP was utilized by hexokinase was taken as proof of the existence of adenylate kinase in the rabbit erythrocyte. A further discussion concerning this

enzyme will be found in the section describing adenylate kinase in the human erythrocyte.

(vii) Inhibitors of hexokinase.

(a) ADP and AMP. A small degree of inhibition of hexokinase was obtained with 1 to 8 x 10^{-3}M ADP, in the presence of an optimal concentration of ATP. However, the results were not consistent and it is possible that the inhibition was caused by the AMP present as a contaminant in some of the preparations of ADP. Indeed, other experiments showed that AMP produces a considerable inhibition of the enzyme. The results are presented in Table IV. It is evident that the percentage inhibition of hexokinase is dependent on the concentration of both AMP and ATP. Thus at any given level of ATP, an increase in the concentration of AMP led to an increase in the degree of inhibition of the enzyme and, conversely, an increase in the concentration of ATP brought about a diminution of the inhibition produced by AMP. Such behavior is consistent with a competitive type of inhibition (cf. 89). It is possible, however, that AMP was converted to ADP, in the presence of adenylate kinase and ATP. Such a conversion would result in the depletion of ATP, with the consequence that the activity of hexokinase would be influenced, then, by the concentration of either AMP or ATP. In view of these considerations, the difficulty in making a kinetic analysis of the results will be appreciated.

TABLE IV

Inhibition of rabbit hexokinase by AMP

	AMP ($\times 10^{-3}$M)	ATP ($\times 10^{-3}$M)	Glucose utilization (μg./hour)	% inhibition
1a.	0	1.3	115	-
	4	1.3	38	67
	8	1.3	13	89
1b.	0	2.7	138	-
	4	2.7	85	38
	8	2.7	30	78
2.	0	5.0	334	-
	4	5.0	306	8
	5	5.0	300	10
	8	5.0	286	14

Conditions:-

0.3 ml. SFH incubated with 1.33mM phosphate buffer and 2.67mM glycylglycine buffer, pH 8; 5mM $MgCl_2$; 2.2mM glucose; ATP and AMP as above; 0.154 M KCl to make the final volume up to 1.5 ml. Incubation temperature, 37°C. Glucose was measured by the method of Nelson (71).

(b) Glucose-6-phosphate. Hexokinase is inhibited by G-6-P, as can be seen in Table V. The inhibition produced by 2 to 5.3 x 10^{-3}M G-6-P was relatively small but it was obtained consistently. It is significant that Crane and Sols (21) showed that the inhibition of hexokinase by hexose monophosphate is due solely to the presence of G-6-P and does not involve F-6-P. Furthermore, Francoeur (90) demonstrated the presence of an active phosphohexose isomerase in the erythrocyte. (The writer confirmed this finding). As a result, the inhibition of hexokinase by added G-6-P may have been limited by the conversion of G-6-P to F-6-P through the action of the isomerase.

(viii) Distribution of hexokinase in the rabbit erythrocyte.

The present studies indicate that the hexokinase is found only in the soluble or cytoplasmic fraction of the red blood cell. The experimental results are listed in Table VI. From the data in the Table it may be seen that the specific activities of the whole hemolyzate, SFH and the unwashed stroma are all of the same order. This indicates that the hexokinase activity is proportional to the amount of hemoglobin or cytoplasmic material in the enzyme preparation. Indeed, when the stroma was washed, both the hemoglobin and the enzyme activity dropped to a very low value.

Not withstanding the results of the above experiments the possibility still existed that the apparent confinement of hexokinase to the SFH might be an artefact resulting from

TABLE V

Inhibition of rabbit hexokinase by G-6-P

	G-6-P ($\times 10^{-3}$ M)	Glucose utilization (μg./hour)	% inhibition
1.	0	152	-
	2	129	15
	4	107	30
2.	0	92	-
	2.7	77	16
	4.0	67	27
	5.3	56	39

Conditions:-

0.3 ml. SFH incubated with 1.33mM phosphate buffer and 2.67mM glycylglycine buffer, pH 8; 5mM $MgCl_2$; 2.2mM glucose; 6mM ATP; G-6-P as above; 0.154 M KCl to make the final volume up to 1.5 ml. Incubation temperature, 37°C. Glucose was measured by the method of Nelson (71).

TABLE VI

Distribution of hexokinase in the rabbit erythrocyte

Enzyme preparation	Glucose utilization (µg./ml.enzyme per hour)	Hemoglobin (mg./ml. enzyme)	Specific activity (µg./mg.Hb per hour)
1. Hemolyzate	560	88.2	6.4
SFH	504	70.8	7.1
Once-washed stroma	negligible	3.0	negligible
Erythrocytes	36	95.4	0.4
2. Hemolyzate	542	82.6	6.6
SFH	404	65.7	6.4
Unwashed stroma	127	16.7	7.6
Packed erythrocytes	133	232.4	0.6
3. Hemolyzate	424	-	-
SFH	336	-	-
Unwashed stroma	108	-	-

Conditions:-

0.3-0.9 ml. enzyme incubated with 1.33mM phosphate buffer and 2.67mM glycylglycine buffer, pH 8; 5mM $MgCl_2$; 2.2mM glucose; 5mM ATP; 10mM KF (but no fluoride in No.1, "erythrocytes"); 0.154 M KCl to make the final volume up to 1.5 ml. Incubation temperature, 37°C. Glucose was measured by the method of Nelson (71) and hemoglobin, by the method of Evelyn and Malloy (73).

the treatment given the erythrocytes in the process of separating the stroma from the SFH. It is reasonable to suppose that if the hexokinase actually is contained in, or on the membrane of the red cell, then the enzyme should be inhibited by AMP added to the medium surrounding the cell. Such an inhibition, however, could not be obtained. Thus, when 5mM AMP was added to a suspension of red cells, the rate of uptake of glucose remained unaffected. Furthermore, the addition of ATP appeared to have no effect on the rate of utilization of glucose. It was concluded, therefore, that hexokinase in the red blood cell is confined to the cytoplasm.

A comparison was made of the relative rates of utilization of glucose, fuctose, mannose and galactose by intact erythrocytes and by cell-free preparations of the erythrocytes. The results of the experiments are presented in Table VII, which is in effect an extension of Table III. It may be observed that the relative rates of utilization of the hexoses by either the intact cells or the SFH could be represented in the following descending order of magnitude:

glucose > mannose > fructose > galactose.

The extent of hexose utilization by the intact cells was much smaller than that of the SFH. However, the ratio of hexose utilization in the SFH to that in the erythrocyte was essentially constant. It is possible, therefore, that the hexokinase system constitutes the rate-limiting process in the

TABLE VII

A comparison of the rates of utilization of various hexoses by intact rabbit erythrocytes and SFH

Hexose	Hexose utilization		Ratio of Activity of SFH to Activity of Cells
	SFH	Erythrocytes	
	(μg./100mg. Hb/hour)		
Glucose	664	88	7.5
Fructose	402	60	6.7
Mannose	600	81	7.4
Galactose	332	42	7.9

Conditions:-

0.5 ml. enzyme incubated at pH 7.8 as in Table III.

metabolism of hexoses by the erythrocyte. The fact that the pH optimum of glycolysis, in the red cell, and that of hexokinase are identical (pH 7.8) is also noteworthy, in this respect (cf. 92).

SUMMARY

Evidence is presented for the presence of hexokinase in the rabbit erythrocyte. The enzyme is restricted to the soluble, cytoplasmic fraction of the cell.

The rate of glucose phosphorylation by the enzyme was shown to be directly proportional to the concentration of the enzyme. Under the conditions of the experiments, the reaction catalyzed by hexokinase was of a zero order.

The activity of hexokinase is dependent on the concentration of ATP, the optimum concentration of the nucleotide being of the order of 6×10^{-3}M.

Mg^{++} ions were found to be essential for hexokinase activity. The maximal concentration of Mg^{++} ions is of the order of 4×10^{-3}M. Assuming the substrate for hexokinase to be a MgATP complex, it was found that the K_{MgATP} is of the order of 1.5×10^{-3}M.

Hexokinase is not imhibited by fluoride ions.

The addition of inorganic phosphate ions tends to increase the activity of hexokinase, the optimum concentration of phosphate being of the order of 9 to 13 $\times 10^{-3}$M.

The Michaelis constant for glucose was found to be 2.8×10^{-4}M, a value considerably lower than the K_{MgATP}.

Hexokinase activity is optimal at pH 6.0 and 7.8 -7.9; a minimum occurs at pH 6.6. This response is not due to the excessive hydrolysis of ATP or G-6-P at the lower pH levels.

Hexokinase catalyzes the phosphorylation of hexoses, at the pH optimum, 7.8, in the following descending order:

glucose > mannose > fructose > galactose.

At pH 6.6 the order is represented as follows:

mannose > fructose > glucose > galactose.

The phosphorylation of glucose is more sensitive to change in pH than that of the other sugars. The writer's findings suggest that the accumulation of G-6-P at the lower pH, could account for this effect.

AMP and G-6-P were found to inhibit hexokinase, but the modes of inhibition of the enzyme are probably complex.

Evidence is presented for the existence of adenylate kinase in the rabbit erythrocyte. Indirect evidence was obtained, also confirming the presence of an ATPase in the stroma of the erythrocyte. The ATPase is inhibited by fluoride.

B. Hexokinase of the human erythrocyte.

(i) The effect of added ATP.

Hexokinase was readily demonstrated in the human erythrocyte. Under the same conditions as previously outlined for the experiments with rabbit erythrocytes (vd. Table III), the human red cells were found to take up a considerable portion of the glucose added to the medium. As in the case of the rabbit erythrocytes, the hemolyzates of the human red blood cells also proved to be more active in the phosphorylation of glucose than the intact cells (Fig. 6A).

Phosphorylation of glucose in the hemolyzate preparations was found to be possible only in the presence of ATP. With increasing concentrations of the nucleotide, hexokinase activity was found to increase, until an optimal level of ATP was reached, and any further increase in the concentration of ATP was accompanied by a diminution in the activity of the enzyme (vid. Fig. 5). The optimal concentration of ATP was found to be about 5×10^{-3}M. These results are similar to those obtained with the hexokinase of the rabbit erythrocyte and the same comments, with regard to the inhibition by ATP, probably apply.

(ii) The influence of time and enzyme concentration on the rate of glucose phosphorylation.

In order to determine the kinetics of the phosphorylative reaction catalyzed by hexokinase, intact red blood cells and hemolyzates were incubated, separately, with glucose

FIGURE 5

The dependence of hexokinase of the human erythrocyte on the concentration of ATP.

Conditions:-

0.2 ml. hemolyzate (prepared by alternately freezing and thawing washed erythrocytes) incubated as in Figure I.

FIGURE 5

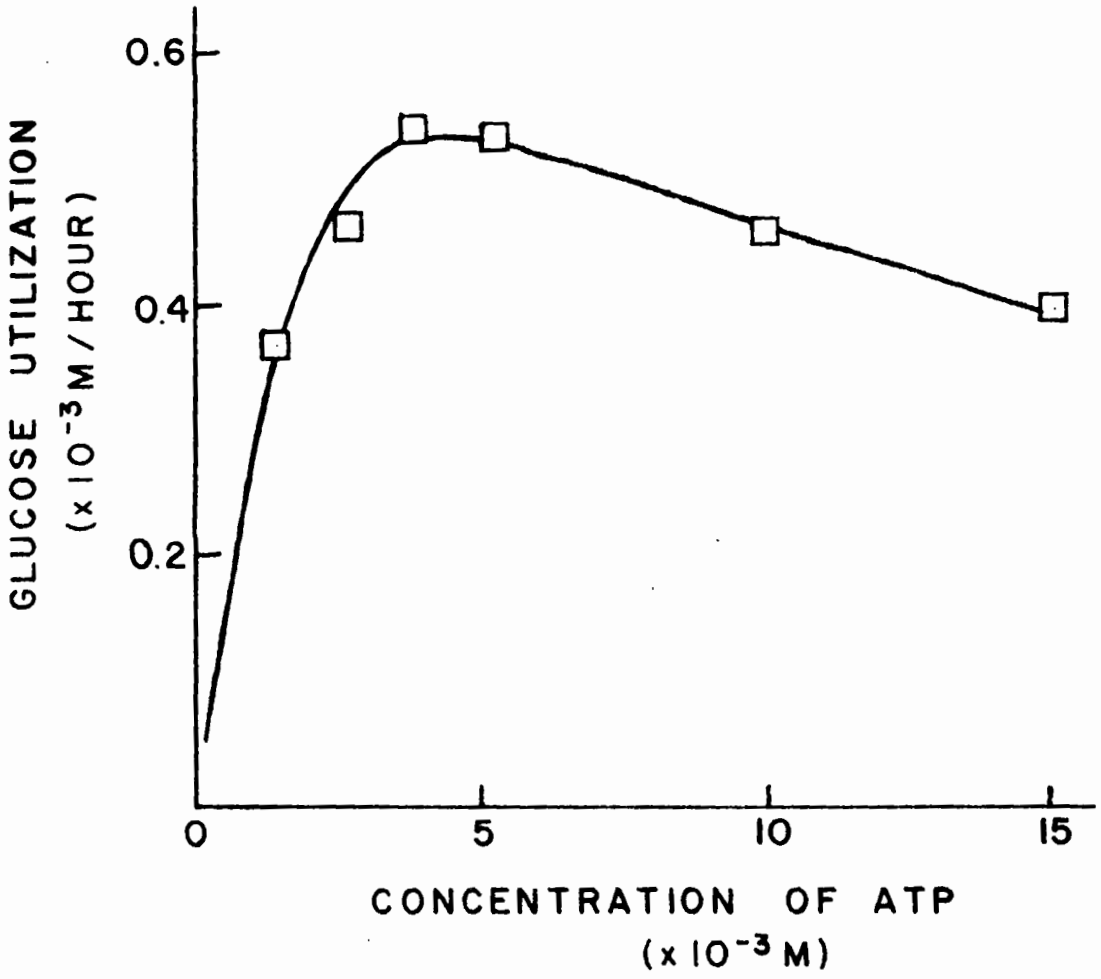

GLUCOSE UTILIZATION
(x 10⁻³ M / HOUR)
0.6
0.4
0.2
0
5
10
15
CONCENTRATION OF ATP
(x 10⁻³ M)

FIGURE 6

Influence of time and enzyme concentration on the rate of glucose phosphorylation.

(○) - Intact erythrocytes.

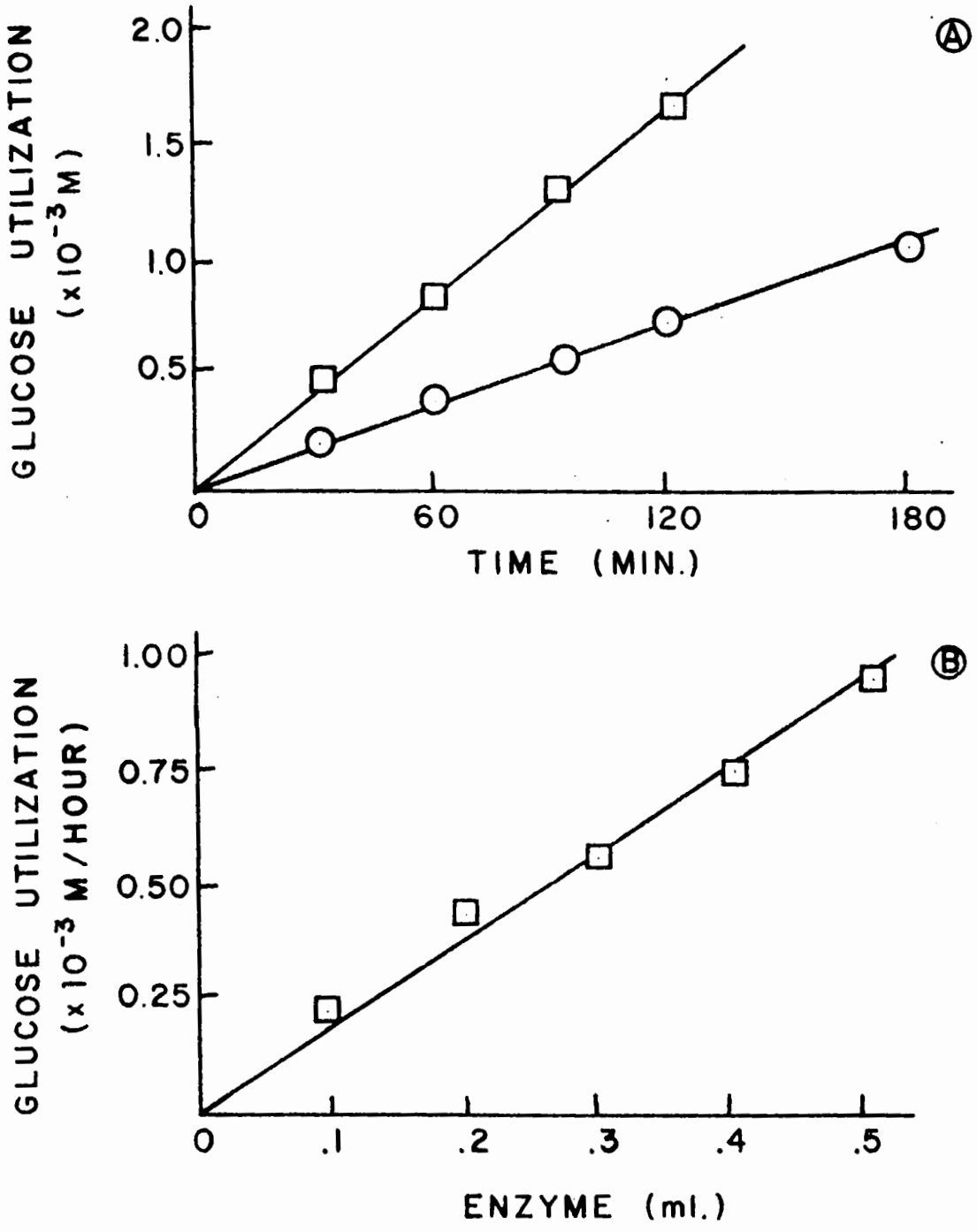
(□) - Hemolyzate.

Conditions:-

A. 0.5 ml. washed erythrocytes or 0.3 ml. hemolyzate (prepared by alternately freezing and thawing washed erythrocytes) incubated with 1.33mM phosphate buffer and 2.67mM glycylglycine buffer, pH 8; 5mM $MgCl_2$; 2.2mM glucose; 5mM ATP; 0.154 M KCl to make the final volume up to 1.5 ml. Incubation temperature, 37°C. Glucose was measured by the method of Nelson (71).

B. 0.1-0.5 ml. hemolyzate (prepared by alternately freezing and thawing washed erythrocytes) incubated as in A.

FIGURE 6


GLUCOSE UTILIZATION
(x10⁻³M)
2.0
1.5
1.0
0.5
0
60
120
180
TIME (MIN.)
Ⓐ
GLUCOSE UTILIZATION
(x10⁻³M/HOUR)
1.00
0.75
0.50
0.25
0
.1
.2
.3
.4
.5
ENZYME (ml.)
Ⓑ

and ATP, for various periods of time, and the rates of glucose utilization were determined. The results are presented in Fig. 6A. It is evident that the rates of glucose utilization, with both enzyme preparations, were linear throughout the experimental period. Thus the reaction is of a zero order, at least until 67% or 40% of the glucose is utilized by the hemolyzate or the intact cells, respectively.

Experiments were carried out, also, employing various amounts of the erythrocyte preparations. The purpose here was to determine whether the rate of glucose phosphorylation is directly proportional to the quantity of enzyme added. The results are presented in Fig. 6B. From 0.1 to 0.5 ml. of a hemolyzate preparation were employed in this study. It was found that the rate of glucose phosphorylation varies directly with the amount of enzyme added, until at least 35% of the glucose present is utilized. In subsequent experiments the amount of enzyme added usually did not exceed the maximum amount used above. Hence, on the basis of the linear relationship observed between the concentration and activity of the enzyme, it is permissible to compare directly the results from different experiments, provided only the concentration of the enzyme is altered in the experiments.

(iii) Distribution of hexokinase in the human erythrocyte.

Washed erythrocytes were hemolyzed with cold water and the stroma was separated from the SFH by centrifugation (vid. P.37). Since the stroma was still red in color after

this initial separation, the material was washed until fairly free of hemoglobin. A hemolyzate also was prepared by alternately freezing and thawing a sample of washed erythrocytes, and a small fraction of the stroma was obtained by centrifugation (vid. p. 37). The stroma was washed to remove hemoglobin. The various erythrocyte preparations were assayed for hexokinase activity, and the results obtained were listed as in Table VIII.

The specific activities of the whole hemolyzate and of the SFH were the same. Evidently, since removal of the stroma brought about no change in the specific activity of the enzyme preparation, and since the activity of the enzyme was proportional to the hemoglobin content of the sample, it must be concluded that hexokinase is associated with the soluble or cytoplasmic elements of the erythrocyte. This conclusion was supported by the fact that the specific activity of the stroma preparations did not increase upon removal of hemoglobin, as might have been expected if hexokinase were bound to the stroma. Indeed, the enzyme activity was found to decrease to zero with purification of the stroma. The results of Crane and Sols (32) are suggestive of the present findings. These authors found that almost all of the hexokinase activity of the hemolyzates of rat erythrocytes could be recovered in the SFH.

Despite the above findings that hexokinase is apparently restricted to the cytoplasm of the red blood cell,

TABLE VIII

Distribution of hexokinase in the human erythrocyte.

Enzyme preparation	Glucose utilization (μg./ml. enzyme per hour)	Hemoglobin (mg./ml. enzyme)	Specific activity (μg./mg. Hb per hour)
1. Hemolyzate	70	-	-
SFH (turbid)	66	-	-
Stroma	0	-	-
2. Hemolyzate	201	44.7	4.5
SFH	150	35.2	4.3
Stroma	54	14.6	3.7
3. Hemolyzate	140	42.6	3.3
SFH	95	28.2	3.4
Washed stroma	0	3.8	-

Conditions:-

0.2-0.7 ml. enzyme incubated with 1.33mM phosphate buffer and 2.67mM glycylglycine buffer, pH 8; 5mM $MgCl_2$; 2.2mM glucose; 5mM potassium ATP; 0.154 M KCl to make the final volume up to 1.5 ml. Incubation temperature, 37°C. Glucose was measured by the method of Nelson (71), and hemoglobin, by the method of Evelyn and Malloy (73).

Experiment No. 1, hemolyzate prepared by alternately freezing and thawing washed, intact erythrocytes.

Experiments No. 2 and 3, hemolyzates prepared by lysing washed intact erythrocytes with cold water.

there remained the possibility that hexokinase, though actually present in the membrane, is released into the SFH as a result of the treatment given the red cell in the preparation of the hemolyzate fractions. The following experiments were undertaken to investigate this possibility. The characteristics of glucose utilization by the red cell, and of glucose phosphorylation by hexokinase were compared in an attempt to define the location of hexokinase in the intact erythrocyte, and to determine its possible role in the transport of glucose across the red cell membrane.

(iv) Thermal activiation of glucose phosphorylation.

Washed erythrocytes and SFH were incubated, separately, at temperatures ranging from 20 to 40°C. and the rates of glucose utilization were determined at each temperature. The results are indicated in Fig. 7. The curve in Fig. 7A illustrates the activating effect of temperature on hexokinase and on the glucose utilization by the intact red cells. With the SFH and with the intact cells the Q_{10}(20-40°C.) was found to be about 1.9. The Q_{10} actually is slightly higher in the range 20-30°C., and slightly lower from 30-40°C. However, this method of plotting the results is not very informative. The $\log_{10}$ of the rate of glucose utilization at the different temperatures, therefore, was plotted against the reciprocal of the absolute temperature, T (cf. 93). This curve is presented in Fig 7B. According to the Arrhenius equation, on which the above method of expressing the results is based, the $\log_{10}$ of the reaction

FIGURE 7

Thermal activation of glucose phosphorylation.

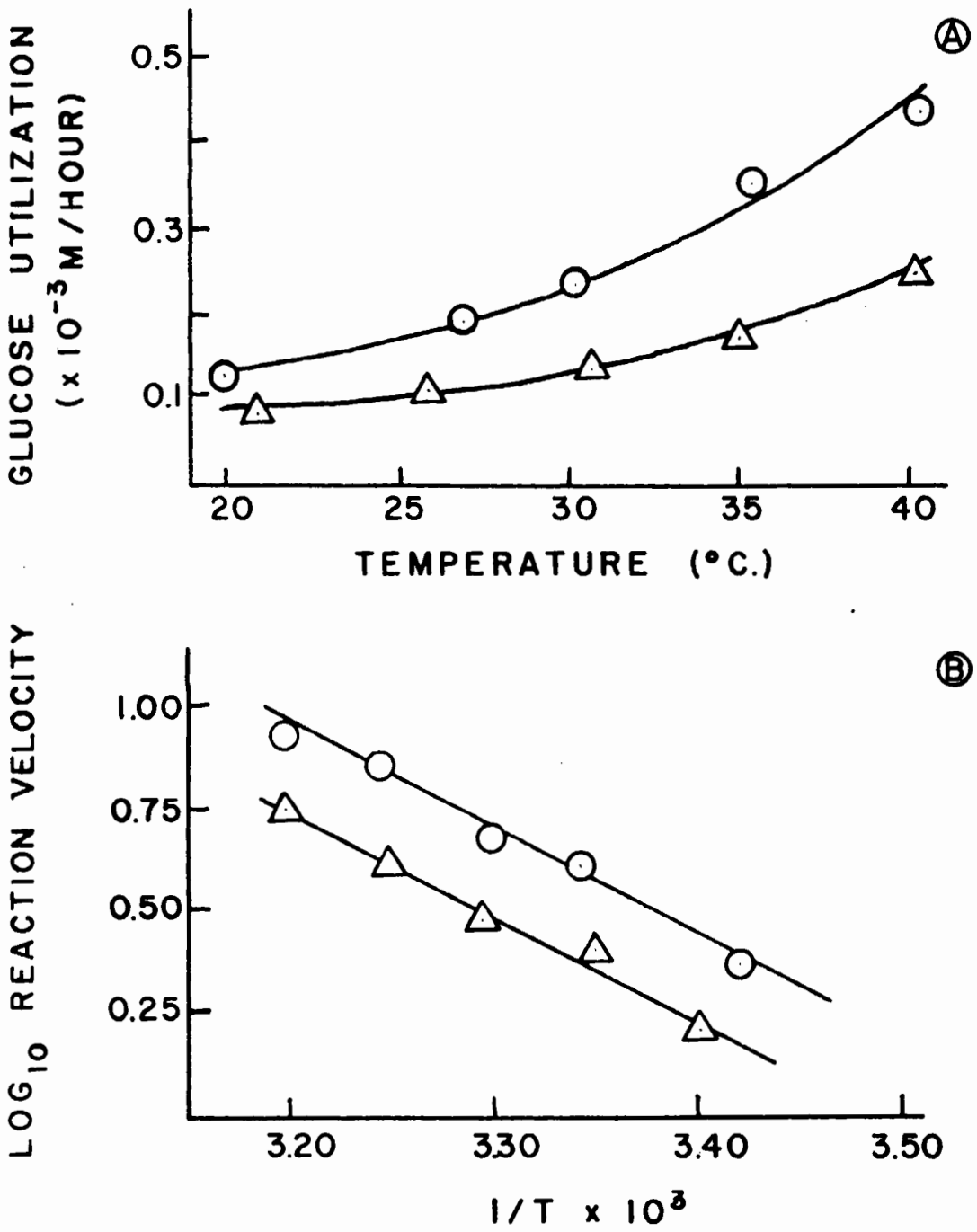
(○) - Intact erythrocytes.

(△) - Stroma-free hemolyzate.

Conditions:-

0.5 ml. washed erythrocytes or SFH (prepared after hemolyzing the erythrocytes with cold water) incubated as in Figure 6, but erythrocytes incubated without ATP. Incubation temperature, 20-40°C.

FIGURE 7


GLUCOSE UTILIZATION
(x 10⁻³ M/HOUR)
0.5
0.3
0.1
20
25
30
35
40
TEMPERATURE (°C.)
A
LOG10 REACTION VELOCITY
1.00
0.75
0.50
0.25
3.20
3.30
3.40
3.50
1/T x 10³
B

velocity constant, k, should be used rather than the $\log_{10}$ of the reaction velocity. However, since the phosphorylation of glucose by the SFH and the intact red blood cells was found to follow zero order kinetics (p.75), then the velocity constant of the reaction is proportional to the reaction velocity:

$$-\frac{dC}{dt} = kEC \qquad \text{(IV)}$$

where C = concentration of substrate,
t = time,
E = enzyme concentration, and
k = velocity constant.

It was permissible, therefore, to use the $\log_{10}$ reaction velocity in the Arrhenius plot and to calculate the activation energy, μ, of the reaction directly from the graph. Furthermore, since the value of μ is calculated from the slope of the line,

$$\mu = 4.6 \frac{(\log_{10}k_2 - \log_{10}k_1)}{(1/T_1 - 1/T_2)} \qquad \text{(V)}$$

where k_1 and k_2 are the reaction rates at the absolute temperatures T_1 and T_2 respectively,

the absolute value of $\log_{10}$ reaction velocity does not influence the final answer. By calculation, the activation energy for the phosphorylation of glucose by the hexokinase in the SFH, and for the phosphorylation of glucose by the intact red blood cells was found to be of the order of 11,500 cal./mol.

The activation energy, μ, may be defined, briefly, as the minimum energy which is required for the formation of

an active enzyme-substrate complex. In an integrated biochemical system the μ value of the system is that of the slowest reaction in the sequence (cf. 19,93). In the present instance, then, the fact that the activation energies of the intact cell and of hexokinase were found to be of the same order, may be interpreted as indicating either that hexokinase is the only factor involved in the transfer of glucose across the erythrocyte membrane, or if another mechanism is involved in this transfer, that the transfer occurs at least as rapidly as the reaction catalyzed by hexokinase.

(v) Inactivation of hexokinase by lipase, pepsin and taurocholate.

The effect of lipase, pepsin and taurocholate on the rate of glucose phosphorylation was determined as follows: 3 ml. aliquots of washed erythrocytes were incubated, separately, with 1 ml. of a lipase or pepsin solution (50 mg.%), or 1 ml. of a 2×10^{-3}M taurocholate solution. After 15 minutes, the cells were washed and made to 3 ml. with isotonic KCl. No hemolysis occurred in any of the samples. The rate of glucose phosphorylation of the treated red cells was then compared with that of a sample of untreated cells. The results are presented in Table IX. 0.5 ml. aliquots of an SFH preparation also were incubated with the above solutions, but the subsequent assays of hexokinase activity had to be carried out in the presence of the inactivators (vid. Table IX).

It is apparent from the data in Table IX that the

TABLE IX

Inactivation of hexokinase of the human erythrocyte by lipase, pepsin and taurocholate.

Inactivating agent	Erythrocytes		SFH	
	Glucose utilization (µg./hour)	% inactivation	Glucose utilization (µg./hour)	% inactivation
None	77	-	108	-
Lipase	51	34	39	64
Pepsin	65	15	85	21
Taurocholate	47	39	86	20

Conditions:-

3 ml. of washed erythrocytes incubated with 1 ml. of lipase or pepsin (50 mg.% solutions) or 2 x 10^{-3}M taurocholate, at 37°C. for 15 minutes. The cells were washed then and made to 3 ml. with isotonic KCl. 0.8 ml. treated and untreated erythrocytes incubated as in Table VIII, but with 5mM ADP instead of ATP.

0.5 ml. SFH, prepared by lysis of erythrocytes with cold water, incubated with phosphate and glycylglycine buffers, $MgCl_2$ and KCl as in Table VIII, but with 0.4 ml. of the inactivating agents. After 30 minutes incubation, glucose and ATP were added and the samples incubated for 60 minutes. Incubation temperature, 37°C.
Glucose was measured by the method of Nelson (71).

hexokinase in the SFH and in the intact erythrocyte was inactivated by lipase, pepsin and taurocholate. These substances were used because of their well-known effect on lipids, proteins and lipoproteins. The results suggest, therefore, that lipid and protein are involved in the activity of hexokinase and that the enzyme may be a lipoprotein in nature. Crane and Sols (32) concluded, similarly, that brain hexokinase is a lipoprotein. Finally, it must be noted that the enzymes, lipase and pepsin, and taurocholate, probably enter into the erythrocyte (cf.94). This observation may explain the effect of these substances on hexokinase in the intact cell.

(vi) Phosphorylation and transfer of glucose.

A study was undertaken to determine the relative rates of the phosphorylation and transfer of glucose by the erythrocyte. The term "transfer" is used here to designate the movement of glucose from the surrounding medium into the intact red blood cell, regardless of the method by which this is accomplished and, also, regardless of the fate of glucose within the cell. If it could be established that glucose is transferred into the red cell faster than it can be phosphorylated, one could infer the involvement of a transport mechanism, other than hexokinase, in the transfer of glucose into the erythrocyte.

Washed red blood cells were incubated with glucose, and, after incubation, aliquots of the incubated samples were centrifuged and the glucose content of the supernatant fluid determined. The glucose content of the whole, incubated

samples also was determined. The former value was considered as a measure of the rate at which glucose was transferred into the erythrocytes, while the latter value afforded a measure of the rate of phosphorylation of glucose by the cells. The results of these experiments are presented in Table X.

It is evident from the data in Table X that the rate of glucose phosphorylation was greater than the rate of transfer of glucose into the red blood cells. This was made possible by the presence of endogenous glucose in the cells and, indeed, the amount of glucose phosphorylated never exceeded the total number of μg. of glucose present in the cells, i.e., endogenous glucose plus transferred glucose. It is noteworthy that the "transferred glucose" does not represent a static quantity, but is, rather, a rate value. It was only for the purpose of calculation that the value for the "transferred glucose" was considered to be a fixed amount of glucose present in the cells. Since almost all of the glucose transferred into and present in the red blood cells was phosphorylated in the course of the experiment, it must be concluded that the sugar molecules were phosphorylated immediately upon entry into the cells. At pH 7.3 the phosphorylation rate was reduced (cf. p. 86) and the rate of phosphorylation just kept pace with the rate of transfer.

From the results presented in Table X it may be seen, furthermore, that the rate at which glucose was transferred into the cells and the rate of glucose phosphorylation were

TABLE X

Phosphorylation and transfer of glucose by human erythrocytes.

	pH	Changes per ml. of incubated sample				
		µg.glucose added	µg.glucose in cells*	µg.glucose transferred into cells	Total µg. glucose in cells	µg.glucose phosphorylated
1.	7.8	155	70	107	177	166
		310	70	67	137	119
		620	70	58	128	75
2.	7.8	160	67	154	221	210
		320	67	191	258	240
		640	67	163	230	204
3.	7.3	142	50	107	157	94
		284	50	83	133	88
		568	50	67	117	73

*µg. equivalents of reducing material found in the erythrocytes. For the purpose of calculation this material was assumed to consist entirely of glucose. Indeed, it was found that, in the absence of added glucose, the reducing substance could be phosphorylated by the cells.

Conditions:-

1.5 ml. washed erythrocytes incubated as in Table VIII in a total volume of 4.5 ml., but with 5mM ADP instead of ATP. After incubation, 1 ml. of the incubated sample was removed for glucose analysis. The remainder was centrifuged at room temperature, and 1 ml. of the supernatant was removed for glucose analysis.

TABLE X (Cont'd.)

The volume of the erythrocytes in the incubated samples was determined in hematocrit tubes, and the values were corrected for the volume of supernatant fluid trapped between the packed cells (vid.94a). The amount of glucose in the supernatant of 1 ml. of whole, incubated sample was obtained by multiplying the number of μg. of glucose per ml. of supernatant fluid by the factor, [1 - (total volume of cells)].

progressively diminished with increasing concentrations of the sugar. SFH was incubated with various concentrations of glucose and it was found that at the higher concentrations of glucose (above 1.7mM), the activity of hexokinase also was diminished. It is significant that the process of glucose transfer in the intact red blood cell and the activity of hexokinase were similarly affected with higher levels of added glucose.

(vii) The effect of pH.

The activity of hexokinase in the SFH and in the intact erythrocyte at various pH levels is indicated in Fig. 8. It is evident that the response was the same for both enzyme preparations. Actually, it was the stability of the enzyme itself, and not the effect of pH on the enzyme-catalyzed reaction, that was determined in these experiments. Washed erythrocytes were incubated in phosphate-glycylglycine buffer at various pH's, in the presence of glucose. After thirty minutes, the erythrocytes were washed with isotonic KCl to remove the buffer and the glucose. The erythrocytes were then incubated at pH 7.8 in the presence of added glucose, and the rate of utilization of the sugar was determined. The SFH preparations were incubated at the various pH levels for 60 minutes without added glucose or ATP. At the end of this period, the substrates were added, without attempting to alter the pH, and the activity of hexokinase was determined.

FIGURE 8

The effect of pH on the hexokinase of the human erythrocyte.

(○) - Intact erythrocytes.

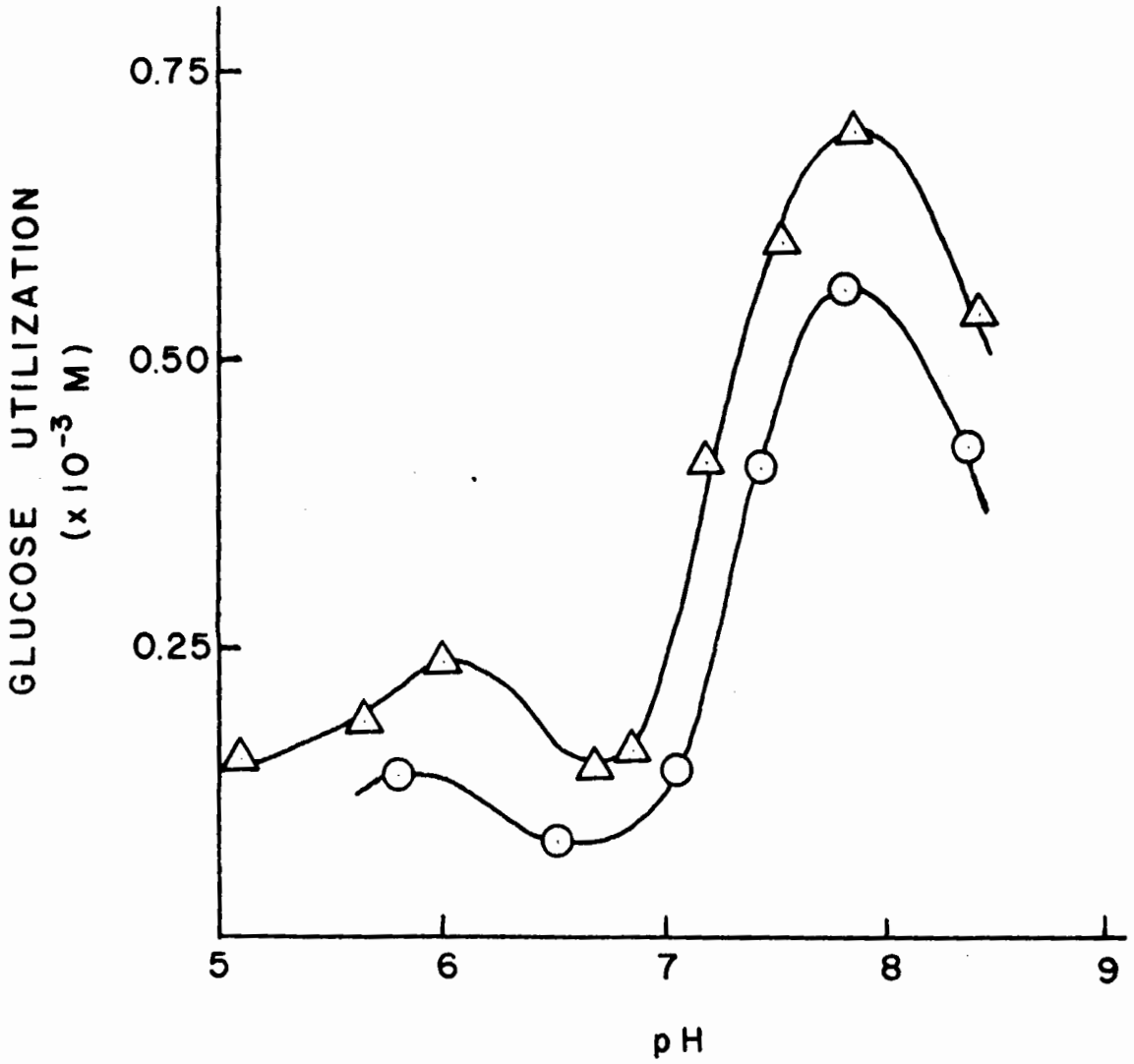
(△) - Stroma-free hemolyzate.

Conditions:-

1.0 ml. washed erythrocytes incubated with 3.3mM phosphate, acetate and glycylglycine buffers, pH as indicated in the Figure; 2.5mM $MgCl_2$; 9.9mM glucose and 0.154 M KCl to make the final volume up to 3.0 ml. Incubated at 37°C. for 30 minutes. The erythrocytes were then washed and made up to 1.0 ml. with 0.154 M KCl. 0.3 ml. of the treated erythrocytes were incubated as in Figure 6.

0.5 ml. SFH (prepared after hemolyzing washed erythrocytes with cold water) incubated with 1.33mM phosphate buffer and 2.67mM glycylglycine buffer, pH as indicated in the Figure; 5mM $MgCl_2$; 0.154 M KCl to make the final volume up to 1.5 ml. Incubated at 37°C. for 60 minutes, then added 2.2mM glucose and 5mM ATP, and incubated further at 37°C. for 60 minutes. Glucose was measured by the method of Nelson (71).

FIGURE 8



The results indicate that hexokinase is maximally stable at pH 7.8-7.9. A second, though smaller, peak of stability occurs at about pH 6. The observations thus are comparable with those obtained with the hexokinase of the rabbit erythrocyte (Fig. 4).

(viii) The effect of adenine nucleotides; adenylate kinase.

SFH was incubated with adenosine mono-, di- and triphosphate, and with various combinations of these nucleotides, and the rate of glucose utilization was determined in each case. The results are presented in Table XI. It may be seen from these results that ADP can be utilized by hexokinase, in place of ATP. This finding suggested that the SFH contains an adenylate kinase. Indeed, when ADP was incubated with SFH and yeast hexokinase, the utilization of glucose was greater than with SFH alone (Table XII). The hexokinase activity of the SFH evidently was low and thus constituted the rate limiting factor in the system. However, when yeast hexokinase was added, hexokinase no longer was the limiting factor and the ATP, formed from ADP by adenylate kinase, could readily be used for the phosphorylation of glucose. Furthermore, it is apparent from the data in Table XII that, whereas hexokinase of the SFH was readily destroyed at 55°C., adenylate kinase was much more stable, and was still active after heating for 30 minutes at 60°C.

From the results shown in Table XI, it is evident,

TABLE XI

Effect of adenine nucleotides on the hexokinase of human erythrocytes.

	Nucleotides	Glucose utilization (µg./hour)	% inhibition
1.	ATP	73	-
	ADP	52	-
	ATP + ADP	48	34
2.	ATP	80	-
	ADP	62	-
	AMP	0	-
	ATP + ADP	43	46
	ATP + AMP	67	16
	ADP + AMP	35	43

Conditions:-

0.5 ml. SFH incubated as in Table VIII. The concentration of all the adenine nucleotides was 5mM.

The percentage inhibition was calculated on the basis of the activity obtained with added ATP. For the experiment with ADP + AMP, the inhibition was calculated on the basis of the activity with added ADP alone.

also, that ADP inhibits hexokinase. AMP also inhibited the enzyme and, indeed, it caused a diminution in the rate of glucose phosphorylation in the presence of ADP. This latter inhibition could possibly have been due to the inhibition either of adenylate kinase or of hexokinase, or both (cf. p.63). It is interesting, therefore, that Colowick and Kalckar (12) found that AMP inhibits adenylate kinase but not yeast hexokinase. As might be expected, the writer found that the hexokinase from the SFH does not utilize AMP for the phosphorylation of glucose.

(ix) Adenylate kinase in the intact erythrocyte.

From the above results it seemed reasonable to suppose that, if hexokinase is present in the membrane of the erythrocyte, then AMP and ADP should inhibit the uptake of glucose by the intact cell. Accordingly, washed erythrocytes were incubated with ATP, ADP and AMP, and also with various combinations of the nucleotides, and the rate of glucose phosphorylation was determined in every case. The results obtained were entirely unexpected (Table XIII).

It may be seen from Table XIII that the addition of ADP to intact red blood cells increased the glucose utilization of the cells to about 1½ times that obtained in the absence of the nucleotide. ATP and AMP, on the other hand, caused a diminution in the rate of glucose phosphorylation. The two latter nucleotides also reduced the increase in glucose phosphorylation brought about by ADP. Evidently

TABLE XII

Adenylate kinase

Enzyme	Nucleotide	Glucose utilization (µg./30 min.)
SFH	ADP	31
Heated SFH'	"	0
Heated SFH'	ATP	0
YH	ADP	23
SFH + YH	"	675
Heated SFH' + YH	"	493
Heated SFH'' + YH	"	25

Conditions:-

0.5 ml. SFH or heated SFH and 0.06 ml. yeast hexokinase (YH) were incubated as in Table VIII. 5mM ATP or ADP was added to the medium.

(') : SFH was heated at 55°C. for 15 minutes.

('') : SFH was heated at 60°C. for 30 minutes.

TABLE XIII

Effect of adenine nucleotides on the glucose utilization of intact human erythrocytes.

Experiment	1	2	3
Nucleotide	Glucose utilization (µg./hour)		
-	57	75	142
ATP	21	38	127
ADP	88	124	250
AMP	-	19	78
ATP + ADP	-	-	213
ATP + AMP	-	-	108
ADP + AMP	-	-	213

Conditions:-

In experiments 1 and 2, p.3 ml. washed erythrocytes, and in experiment 3, 0.8 ml. washed erythrocytes were incubated as in Table VIII. 5mM of each nucleotide was added to the medium.

ADP underwent a conversion to ATP and this conversion was inhibited by ATP and AMP. To explain these findings it was necessary to postulate the operation, in the erythrocyte, either of adenylate kinase, or of a mechanism which catalyzes the phosphorylation of ADP with inorganic phosphate.

These possibilities were examined as follows: Washed erythrocytes were incubated with ADP in the presence of yeast hexokinase and it was found that the rate of glucose phosphorylation was greatly increased in the presence of the yeast enzyme (Table XIV). Evidently, the ATP formed from ADP by the erythrocytes can pass into the external medium where it can act as a substrate for the yeast hexokinase. AMP was found to inhibit the formation of ATP also in this system. Washed stroma could be substituted for the intact red cells and the activity of both these preparations was relatively stable to heat (Table XIV). As with the adenylate kinase of the SFH (viz. Table XII), the ATP-forming activity of the erythrocytes and stroma was retained after heating at 60°C. for 30 minutes.

That the formation of ATP from ADP was not brought about by the simple phosphorylation of the latter with inorganic phosphate was indicated by the following observations: (1) no decrease in the concentration of inorganic phosphate could be demonstrated during the experiment, and (2) neither arsenate nor iodoacetate inhibited the formation of ATP. Evidently, adenylate kinase was responsible for the trans-

TABLE XIV

Conversion of ADP to ATP by intact human erythrocytes.

Enzyme	Glucose utilization (µg./30 min.)
YH	67
Erythrocytes	29
YH + erythrocytes	482
YH + heated erythrocytes'	132
YH + heated erythrocytes''	58
Washed stroma	0
YH + washed stroma	207
YH + heated stroma'	49

Conditions:-

0.1 ml. erythrocytes or 0.4 ml. washed stroma, plus 0.06 ml. yeast hexokinase (YH) were incubated with 5mM ADP as in Table VIII.

(') : indicates heating at 58°C. for 20 minutes.
('') : indicates heating at 60°C. for 30 minutes.

formation of ADP to ATP.

The above results indicate that at least some of the adenylate kinase of the erythrocyte is present in the membrane. Preliminary evidence was obtained indicating that ATP cannot pass from the interior of the erythrocyte to the surrounding medium; Szekely et al. (69) found that ATP cannot enter the erythrocyte. It must be assumed therefore, that the adenylate kinase is so situated within the membrane that the ATP which is formed by the enzyme can move freely either into the interior of the cell, or into the fluid which surrounds the erythrocyte.

SUMMARY

The presence of hexokinase in the human erythrocyte was demonstrated. Under the conditions of the experiments, the reaction catalyzed by the enzyme, whether in the intact erythrocyte or in the hemolyzate, could be described by zero order kinetics. The rate of glucose phosphorylation by the enzyme was found to be proportional to the concentration of the enzyme.

Hexokinase is absent from the stroma and is found entirely in the SFH.

The activation energy for the phosphorylation of glucose by hexokinase is of the order of 11,500 cal./mol., both in the case of the SFH and the intact erythrocyte.

Lipase, pepsin and taurocholate inactivate hexokinase.

Glucose phosphorylation in the erythrocyte proceeds at least as rapidly as the transfer of glucose from the external medium into the interior of the cells. At pH 7.8, the phosphorylative process is more rapid than the transfer process.

The rate of glucose transfer into the erythrocyte, and the activity of hexokinase in the SFH, are diminished in the presence of high concentrations of glucose.

The stability of hexokinase in the intact erythrocyte and in the SFH varies with the pH of the medium. The enzyme is most stable at pH 7.8-7.9. Another, smaller, peak of stability occurs at pH 6.

AMP and ADP inhibit hexokinase.

Adenylate kinase is present in the SFH. The enzyme is relatively stable to heating at 60°C., whereas hexokinase is rapidly destroyed at this temperature.

Addition of ADP to intact erythrocytes stimulates the rate of glucose utilization by the cells, while AMP and ATP antagonize the stimulatory action of ADP. Evidently, adenylate kinase is responsible for the observed effect of ADP. At least some of the enzyme is present in the erythrocyte membrane.

C. Preservation of human blood[5]; effect on hexokinase.

(i) Instability of hexokinase.

While studying the hexokinase of the human erythro-

---

5. A major part of this study was carried out in cooperation with Dr. David Rubinstein of this laboratory. The author

cytes, the writer observed that the activity of the enzyme in hemolyzate and SFH preparations, and even in washed, intact erythrocytes, diminished fairly rapidly during storage of the preparations for several days in the refrigerator. The addition of glucose to the enzyme specimens did not prevent the progressive fall in the activity of the enzyme. It was noted, furthermore (vid. Fig. 8), that hexokinase, both in the SFH and in the intact erythrocyte, is most stable at about pH 7.8 and that, as the pH of the medium is lowered to 6.6, the activity of the enzyme is decreased considerably. It is significant that the pH of the blood samples preserved in CD or ACD media is usually within the range pH 6.5 to 7.8 (cf.96). The writer's studies have indicated that the hexokinase of the red blood cell is unstable and tends progessively to diminish in activity during the storage of blood.

Samples of blood were stored in CD and ACD media, and, periodically, aliquots of the blood were removed, the red blood cells were washed with isotonic KCl, and the hexokinase activity of the red cells was determined. Hemolyzates of the washed erythrocytes also were prepared and assayed. The results of these experiments are presented in Table XV and in Fig. 9. The specific activity of the enzyme was calculated on the basis of the hemoglobin content of the samples

---

(5. cont'd.)

gratefully acknowledges the technical assistance of Mrs. Sheila M. Gosselin, who carried out the chemical analyses on the samples of preserved blood.

Part of the work presented here has been published in the Canadian Journal of Biochemistry and Physiology (95).

TABLE XV

Behavior of hexokinase during storage at 4°C.

Enzyme	Duration of storage (Days)	Specific activity (Δ µg. glucose/100 mg. Hb/hour)			
		CD medium	ACD medium	CD+Ad medium	ACD+Ad medium
Intact erythrocytes	1	101	84	79	91
	4	54	79	67	73
	8	17	63	47	53
	13	4	83	14	35
	18	20	79	33	39
	21	21	37	17	51
	27	-	15	-	53
	32	15	-	-	49
Hemolyzate	1	328	251	209	225
	4	232	216	242	236
	8	173	214	185	187
	13	165	211	172	187
	18	165	179	181	177
	21	157	173	170	176
	27	155	168	188	155
	32	144	149	167	141

Conditions:-

Stored erythrocytes were washed with isotonic KCl and about one half of the washed erythrocytes were hemolyzed by alternate freezing and thawing. 0.5 ml. aliquots of the enzyme preparations were incubated as in Table VIII, but with 5mM ADP instead of ATP in the case of the intact erythrocytes.

FIGURE 9

Behavior of hexokinase during storage at 4°C.

a - Hemolyzate.

b - Intact erythrocytes

Curves A (○) - In ACD medium, without adenosine.

Curves B (△) - In ACD medium, with adenosine (400 mg.%).

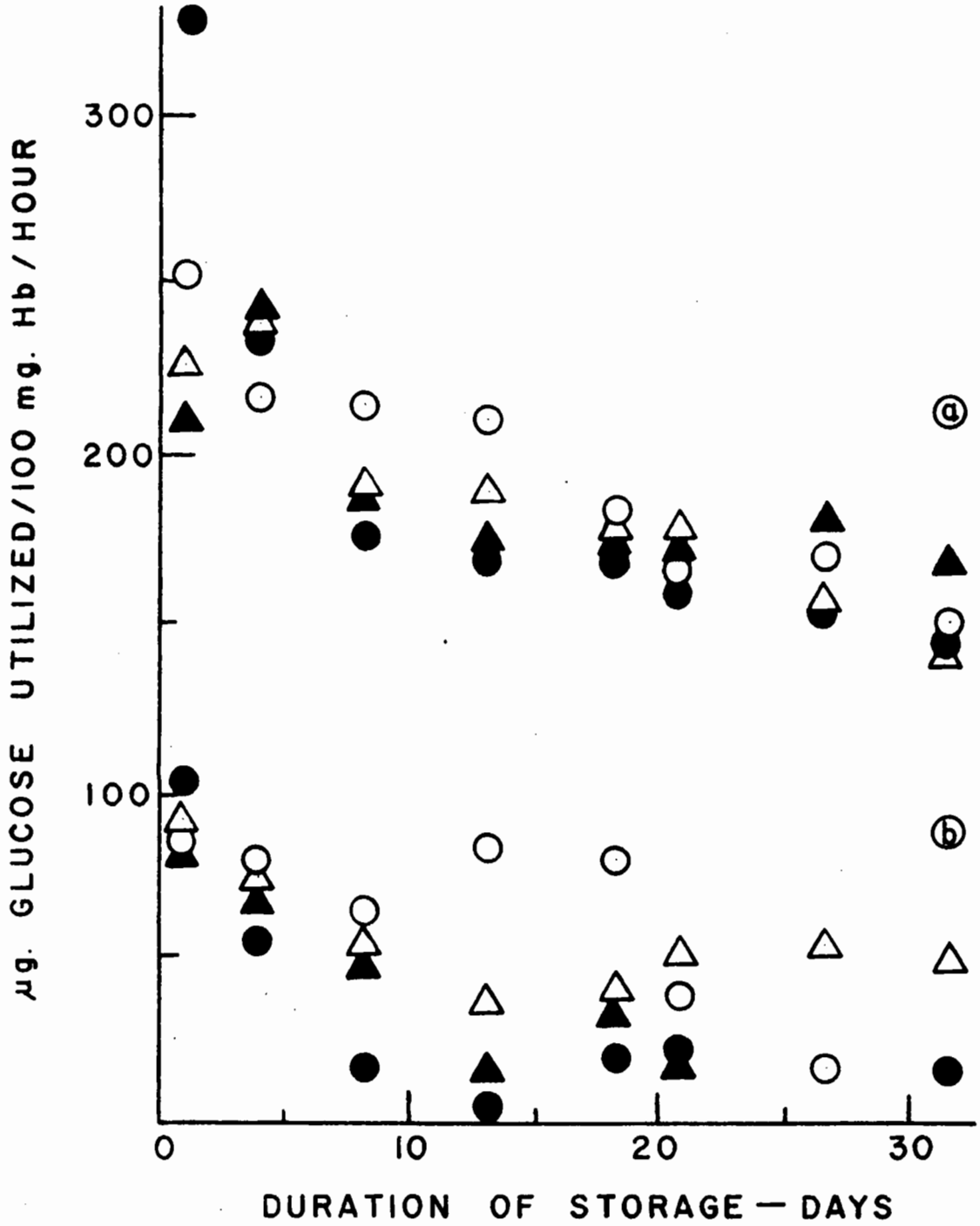
Curves C (●) - In CD medium, without adenosine.

Curves D (▲) - In CD medium, with adenosine (400 mg. %).

Conditions:-

See Table XV.

FIGURE 9


μg. GLUCOSE UTILIZED/100 mg. Hb/HOUR
300
200
100
0
10
20
30
DURATION OF STORAGE — DAYS
@
ⓑ

so that any loss of red blood cells due to hemolysis during storage or resulting from the washing procedure, and any changes in the volume of the erythrocytes during storage (cf.96) did not influence the assay of hexokinase activity. It is evident from Table XV and Fig. 9 that the specific activity of hexokinase fell rather rapidly during the storage period. The fall in enzyme activity was observed both in the CD and ACD specimens. However, the number of samples was too small to permit a comparison of the rates of inactivation of hexokinase in the erythrocytes stored in the two media.

The lability of hexokinase in stored blood was demonstrated in yet another way. SFH was prepared from fresh erythrocytes and from erythrocytes which had been stored in an ACD medium at 3°C. for 22 days. When the SFH preparations were incubated at 37°C. for 60 minutes, in the absence of glucose and ATP (cf. p.87), it was found that, whereas the SFH from the fresh erythrocytes retained enzyme activity through the incubation period, the hexokinase activity of the older sample was entirely abolished. The addition of glucose during the incubation period did not prevent the inactivation of the enzyme in the latter preparation.

(ii) Effect of adenosine.

Finch and coworkers (97,98) found that when adenosine was added to blood, the viability of the erythrocytes during storage was greatly improved. Associated with this

improvement in the condition of the erythrocytes there was an increase in the concentration of the organic phosphate esters, and a corresponding drop in the level of inorganic phosphate in the blood. These findings were confirmed and extended in the present study.

Adenosine (in a final concentration of 400 mg.%) was added to two samples of blood, one of which was stored in CD and the other in an ACD medium. For comparison, control samples of blood were stored in CD and ACD media but without the addition of adenosine. The analytical results are presented in Tables XVI and XVII, and in Figures 10-13. The results obtained with CD and ACD, in the absence of adenosine, confirmed those of Pappius et al. (78) and Rapoport (96). It is apparent that the concentration of inorganic phosphate in these samples increased at the expense of the organic phosphate esters. In the blood sample preserved in the CD medium, the liberation of P_i from the phosphate esters did not occur until about the 12th day of storage, while in the ACD sample, it commenced from the very beginning of storage. The fall in the level of DPG corresponded with the increase in P_i. In both specimens the hydrolysis of DPG accounted for about 75% of the increase in the P_i fraction during the 32-day period of storage. In the samples to which adenosine had been added, on the other hand, the P_i was almost completely esterified and the content of DPG was increased (Table XVI, Fig. 10).

TABLE XVI

Changes in the phosphate fractions of blood stored in CD and ACD, with and without adenosine.

Phosphate fraction	Duration of storage (Days)	µmoles phosphate/ml. whole blood			
		CD medium	ACD medium	CD+Ad medium	ACD+Ad medium
P_i	0	0.79	1.22	0.82	0.76
	4	0.58	1.91	0.68	0.56
	8	0.34	2.73	0.14	0.03
	13	1.22	3.92	0.41	0.30
	18	1.78	4.57	0.26	0.18
	21	2.40	4.82	0.17	0.36
	26	3.55	5.50	0.31	0.30
	32	4.70	5.97	0.15	0.43
P_l	0	1.71	1.81	1.27	1.81
	4	1.47	1.65	1.15	1.28
	8	0.93	1.80	1.10	1.68
	13	0.75	1.75	1.22	1.42
	18	0.81	1.88	1.25	1.71
	21	0.65	1.67	1.16	1.40
	26	0.68	1.21	1.11	0.98
	32	0.87	1.20	1.34	1.18

TABLE XVI (cont'd.)

Phosphate fraction	Duration of storage (Days)	µmoles phosphate/ml. whole blood			
		CD medium	ACD medium	CD+Ad medium	ACD+Ad medium
P_M	0	1.13	1.05	1.53	1.45
	4	1.78	1.19	1.97	2.37
	8	1.88	1.23	2.33	2.68
	13	0.98	0.67	2.22	2.34
	18	1.04	0.67	2.40	2.13
	21	1.13	0.49	2.09	1.65
	26	0.96	0.87	2.24	1.56
	32	0.58	0.49	2.06	1.45
P_s	0	4.65	4.24	4.48	4.90
	4	4.17	3.69	4.07	4.42
	8	3.84	2.46	3.77	3.22
	13	4.93	1.60	3.92	4.97
	18	4.43	1.34	4.04	5.33
	21.	4.14	1.18	4.75	7.05
	26	3.06	1.07	4.55	7.49
	32	1.89	0.89	4.82	7.92

TABLE XVII

Changes in glucose, lactate and pyruvate in blood stored in CD and ACD, with and without adenosine.

Metabolite	Duration of storage (Days)	µmoles/ml. whole blood			
		CD medium	ACD medium	CD+Ad medium	ACD+Ad medium
Glucose	0	33.2	39.9	29.6	36.8
	4	28.2	37.2	28.2	33.2
	8	24.8	34.7	24.3	28.3
	13	24.7	33.9	20.9	24.7
	18	21.2	29.5	18.0	23.1
	21	21.0	29.5	16.7	20.7
	26	19.0	28.4	14.2	22.2
	32	16.9	26.6	11.3	20.2
Lactate	0	2.2	1.4	2.5	1.9
	4	6.9	5.6	8.3	8.9
	8	12.9	10.4	14.7	16.2
	13	17.7	17.0	22.8	24.6
	18	21.6	22.0	30.0	31.0
	21	26.0	27.5	34.5	37.7
	26	29.5	29.2	39.4	40.3
	32	37.1	33.9	52.0	48.0

TABLE XVII (cont'd.)

Metabolite	Duration of storage (Days)	µmoles/ml. whole blood			
		CD medium	ACD medium	CD+Ad medium	ACD+Ad medium
Pyruvate	0	0.1	0.1	0.1	0.1
	4	0.2	0.2	0.3	0.3
	8	0.1	0.3	0.3	0.3
	13	0.1	0.4	0.4	0.1
	18	0.4	0.4	0.4	0.1
	21	0.6	0.4	0.4	0.1
	26	1.0	0.4	0.4	0.2
	32	1.4	0.5	0.4	0.5

FIGURE 10

Changes in the concentration of DPG and inorganic phosphate in stored blood.

Curve A - Fraction P_s (DPG), without adenosine.

Curve B - Fraction P_s (DPG), with adenosine.

Curve C - Fraction P_i, without adenosine.

Curve D - Fraction P_i, with adenosine.

FIGURE 10

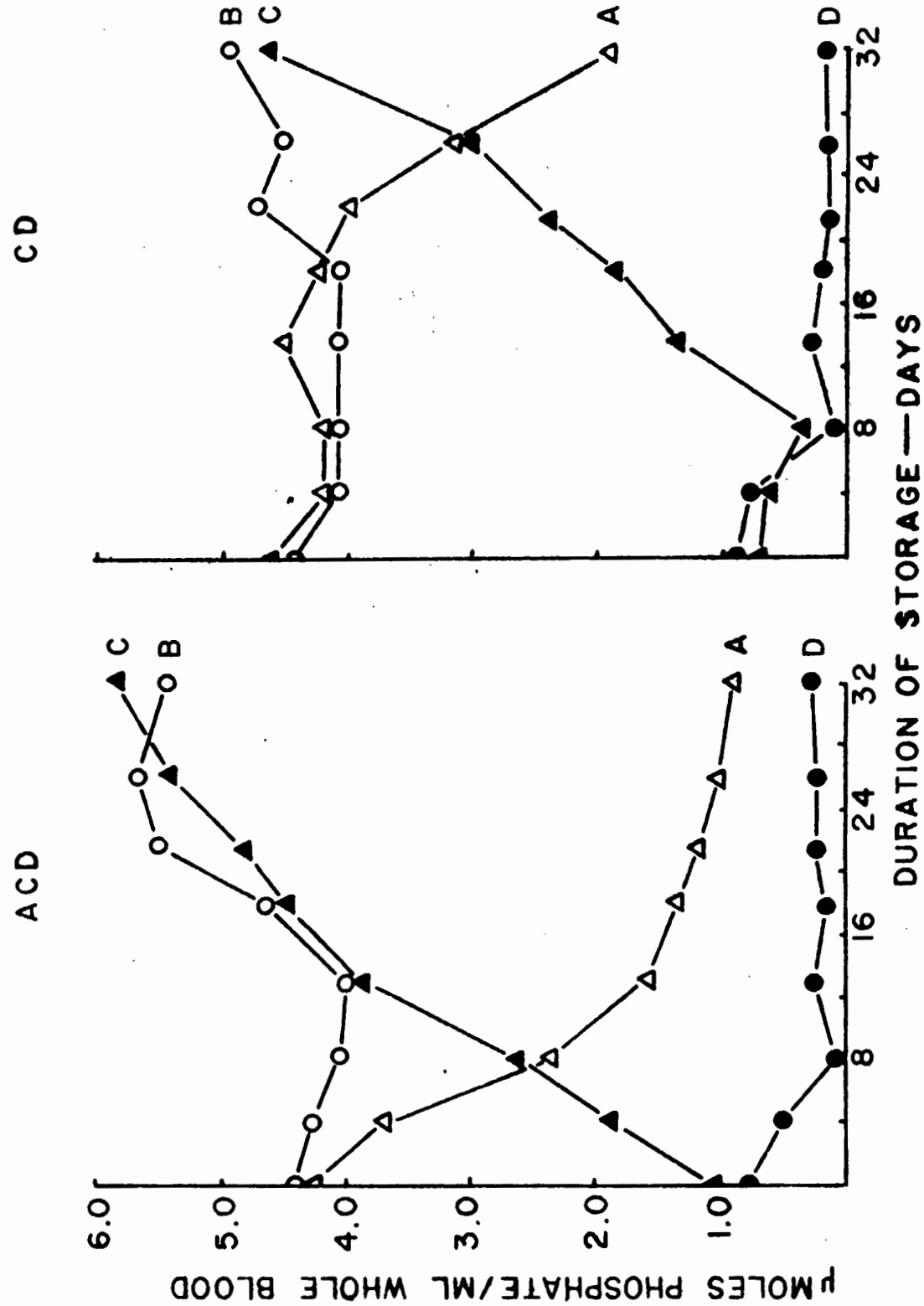

ACD
CD
μMOLES PHOSPHATE/ML WHOLE BLOOD
6.0
5.0
4.0
3.0
2.0
1.0
8
16
24
32
DURATION OF STORAGE—DAYS
A
B
C
D

FIGURE 11

Changes in the concentration of ATP and hexose phosphates in stored blood.

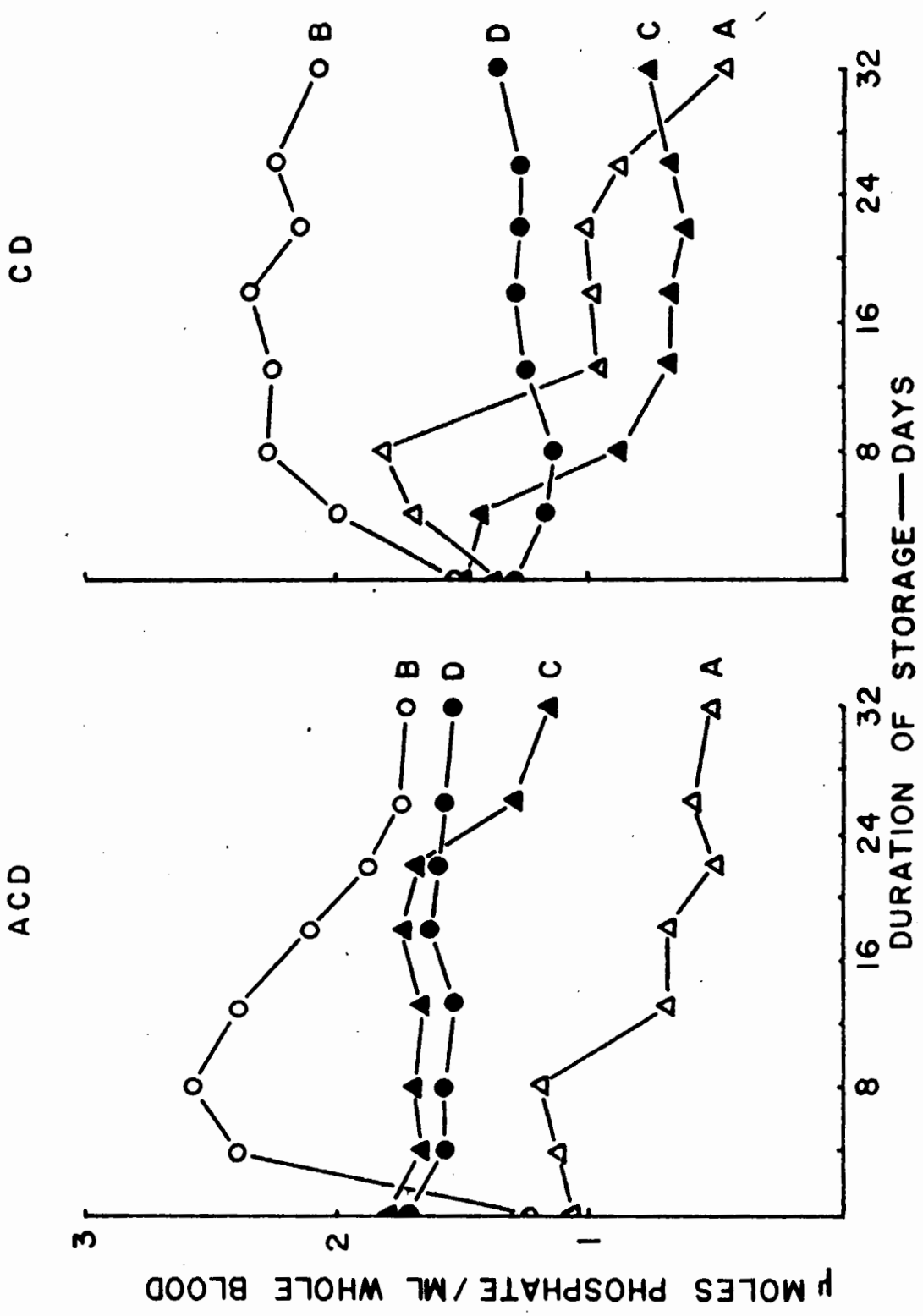
Curve A - Hexose phosphates, without adenosine.

Curve B - Hexose phosphates, with adenosine.

Curve C - ATP, without adenosine.

Curve D - ATP, with adenosine.

FIGURE II


CD
ACD
B
D
C
A
3
2
1
8
16
24
32
DURATION OF STORAGE — DAYS
μ MOLES PHOSPHATE / ML WHOLE BLOOD

The results in Table XVI and Fig. 11 show that the level of ATP fell in the blood samples preserved either in CD or ACD. It is noteworthy, however, that the fall in the concentration of ATP was considerably delayed in the ACD specimen, whereas in the CD specimen the diminution in ATP occurred almost from the beginning of storage. In both media the fall in ATP appeared to be the inverse of that of DPG. This behavior is consistent with the finding that ATP is relatively stable in blood at a pH below 7.3 (96,99,100). Above this pH the hydrolysis of the terminal phosphate of ATP and the synthesis of DPG are favored. Indeed, Rapoport (100, 101) has presented evidence that the levels of ATP and DPG are closely related in the intact erythrocyte. Therefore, in the ACD medium (initial pH 7.1) the level of ATP remained essentially unchanged during the first 21 days of storage, while that of DPG fell. At the relatively higher pH of the CD specimens (initial pH 7.4), ATP was hydrolyzed more rapidly, while DPG was maintained until about the 21st day. In the presence of adenosine both ATP and DPG were maintained during the entire period of storage in the CD and ACD media. In addition, adenosine induced a synthesis of hexose phosphates in the stored blood; in the samples without adenosine, a considerable degree of hydrolysis of these esters was found to occur.

The rate of glucose utilization by the erythrocytes during storage, as indicated in Table XVII and Fig. 12,

FIGURE 12

Glucose utilization in stored blood.

Curve A - In CD medium, without adenosine.

Curve B - In CD medium, with adenosine.

Curve C - In ACD medium, without adenosine.

Curve D - In ACD medium, with adenosine.

FIGURE 12

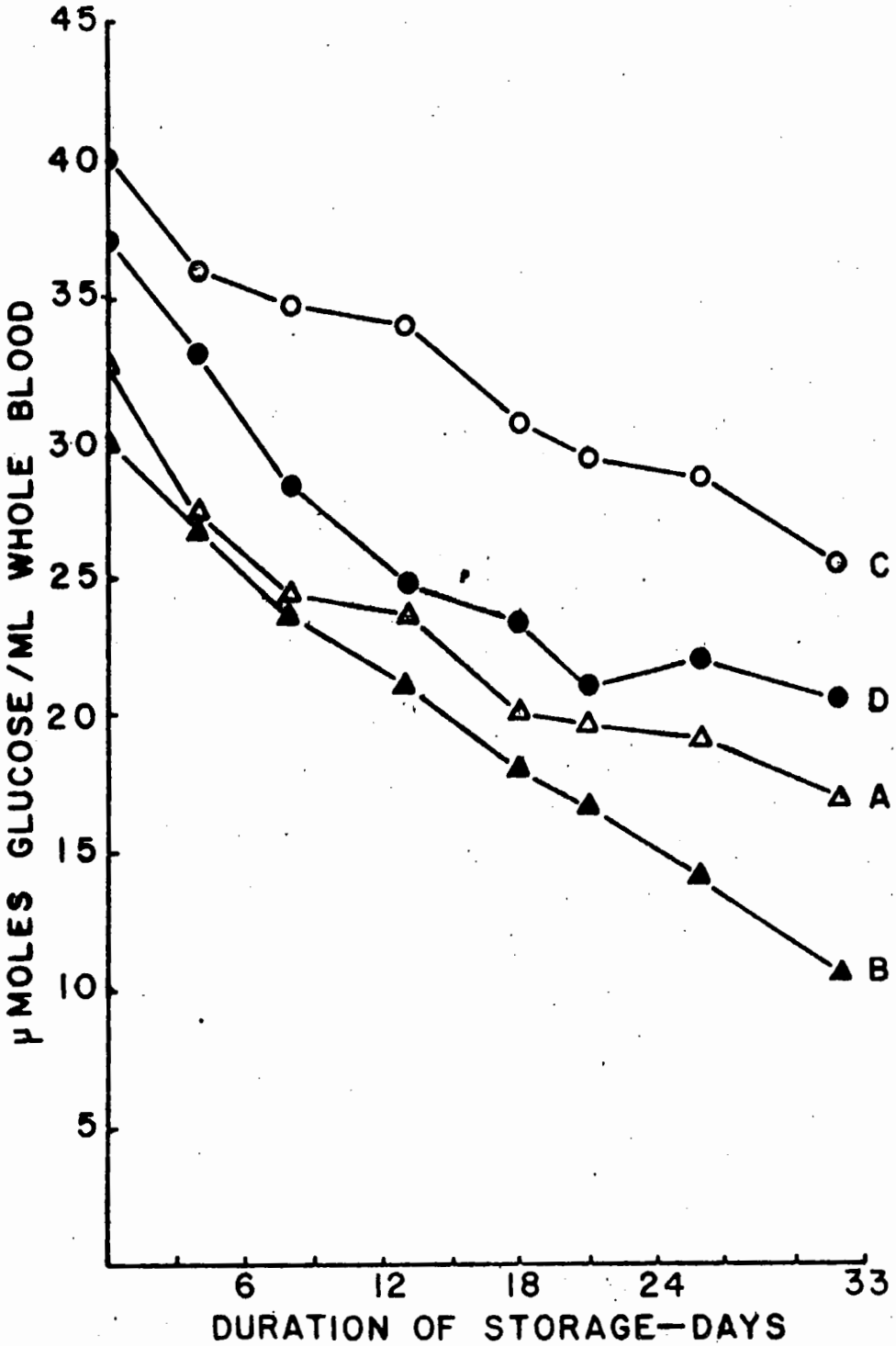

45
40
35
30
25
20
15
10
5
μ MOLES GLUCOSE/ML WHOLE BLOOD
C
D
A
B
6
12
18
24
33
DURATION OF STORAGE—DAYS

FIGURE 13

Changes in the concentration of lactate and pyruvate in stored blood.

Curve A - Lactate, without adenosine.

Curve B - Lactate, with adenosine.

Curve C - Pyruvate, without adenosine.

Curve D - Pyruvate, with adenosine.

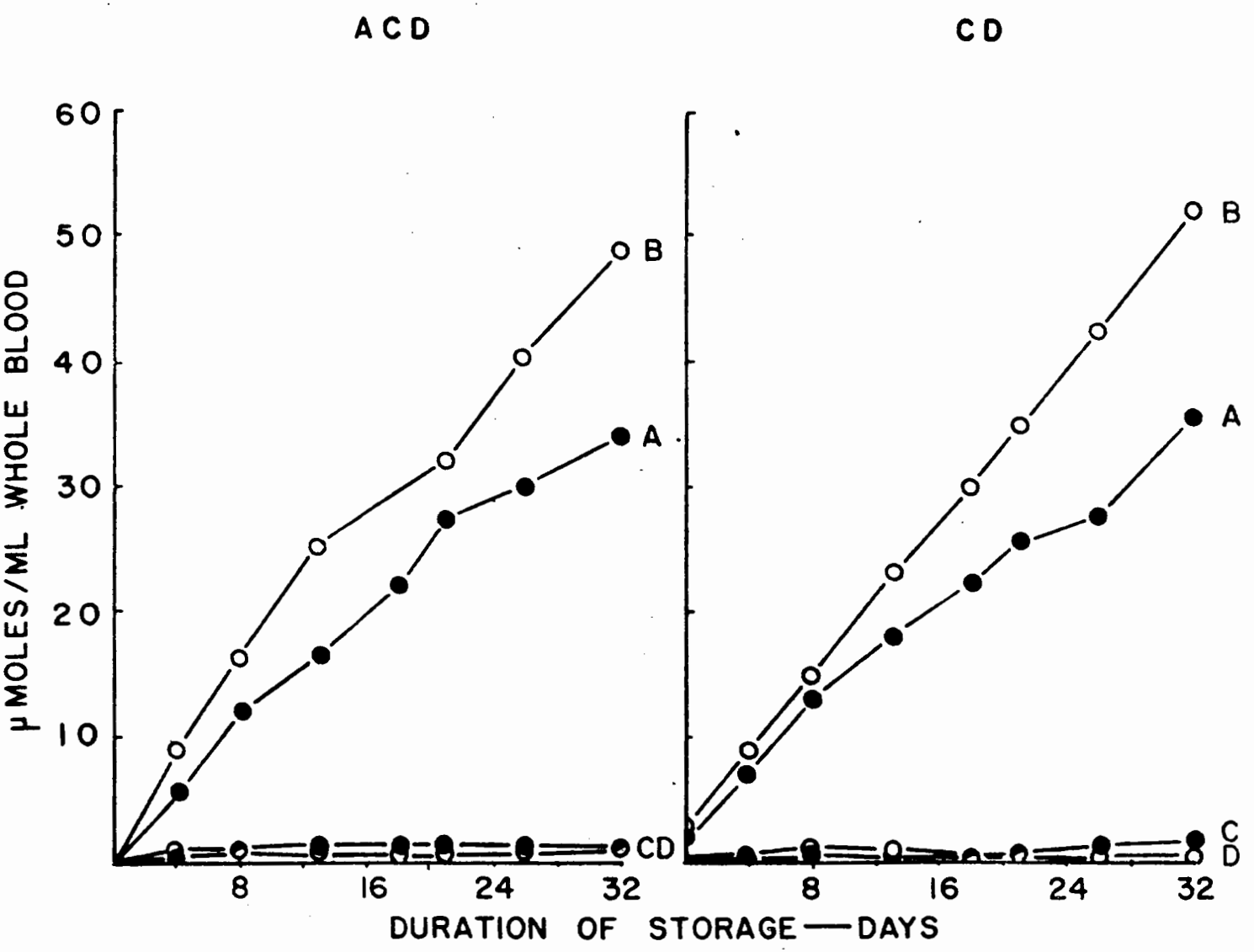

ACD
CD
60
50
40
30
20
10
μ MOLES/ML WHOLE BLOOD
B
A
CD
C
D
8
16
24
32
DURATION OF STORAGE — DAYS

FIGURE 13

appeared to be slightly stimulated by the presence of adenosine in the CD or ACD medium However, the rate of formation of lactate in the stored erythrocytes was increased by about 40% in the presence of adenosine (Table XVII, Fig. 13). Pyruvate was slightly increased in the blood specimens preserved without adenosine, while in the samples containing the nucleoside, the increase in the concentration of pyruvate was almost entirely abolished. In all specimens, the increase in pyruvate was small (usually less than 1 μmole per ml. of whole blood) compared with that of lactate (about 35 μmoles per ml. of whole blood).

A "carbon balance" represented by the amounts of glucose utilized and glycolytic products formed in the blood specimens stored at 4°C., is given in Table XVIII. The values in the Table are derived from the data given in Tables XVI and XVII, and represented in Figures 10-13. The values for the amounts of glucose utilized and hexose phosphates formed were multiplied by 2 in order ro represent them as μmoles of 3-carbon equivalents per ml. of whole blood. In the blood specimens preserved without adenosine, the total of the lactate and pyruvate formed was nearly equal to the combined amounts of glucose and other metabolites utilized. However, in the samples containing adenosine, the total of the lactate and pyruvate formed amounted to almost 150% of the combined amounts of the metabolites utilized. The 50% excess represents the portion of lactate and pyruvate which

TABLE XVIII

Carbon balance of metabolites during blood storage.

Metabolite	Δ μmoles of 3-carbon equivalents per ml. of whole blood/32 days			
	CD medium	ACD medium	CD+Ad medium	ACD+Ad medium
Glucose	-32.6	-26.6	-36.6	-33.2
DPG	- 2.8	-3.4	+0.3	+3.0
Hexose-PO_4	-1.1	-1.1	+1.0	0
Total metabolite utilized	-36.5	-31.1	-35.3	-30.2
Lactate	+34.9	+32.5	+49.5	+46.1
Pyruvate	+1.3	+0.4	+0.3	+0.4
Total metabolite produced	+36.2	+32.9	+49.8	+46.5
Balance of metabolites	-0.3	+1.8	+14.5	+16.3

must have been formed from adenosine. Indeed, later experiments showed that the ribose moiety of adenosine can be used as a substrate in the metabolism of the erythrocytes (vid.p.118).

To ascertain whether the lack of inorganic phosphate may have limited the effectiveness of adenosine in promoting the synthesis of phosphate esters, specimens of blood were stored at 4°C. with added inorganic phosphate and adenosine. As indicated in Fig. 14 a greater uptake of inorganic phosphate occurred in these samples than in the control samples containing adenosine alone. A slight increase in the concentrations of hexose phosphate and lactate were evident in the presence of added inorganic phosphate, while the level of ATP remained virtually unchanged under these conditions. Contrasted with these smaller changes, the concentration of DPG was increased by about 300% in the presence of added phosphate. Evidently, DPG serves as a reservoir of organic phosphate in the erythrocyte.

(iii) Conversion of adenosine to inosine.

As indicated in Table XIX there was an increase in the pH of the blood specimens containing adenosine. This increase did not occur immediately upon addition of adenosine to the blood, but took place during the first few days of storage. Thereafter, the pH fell, as in the specimens containing no nucleoside, owing to the formation of lactic acid (cf.96). The rise in pH was assumed to result from the progressive deamination of adenosine, since Conway and Cooke

FIGURE 14

Influence of added inorganic phosphate on the synthesis of DPG induced by adenosine in stored blood.

Curve A - DPG, without added phosphate.

Curve B - DPG, with added phosphate.

Curve C - P_i, without added phosphate.

Curve D - P_i, with added phosphate.

FIGURE 14

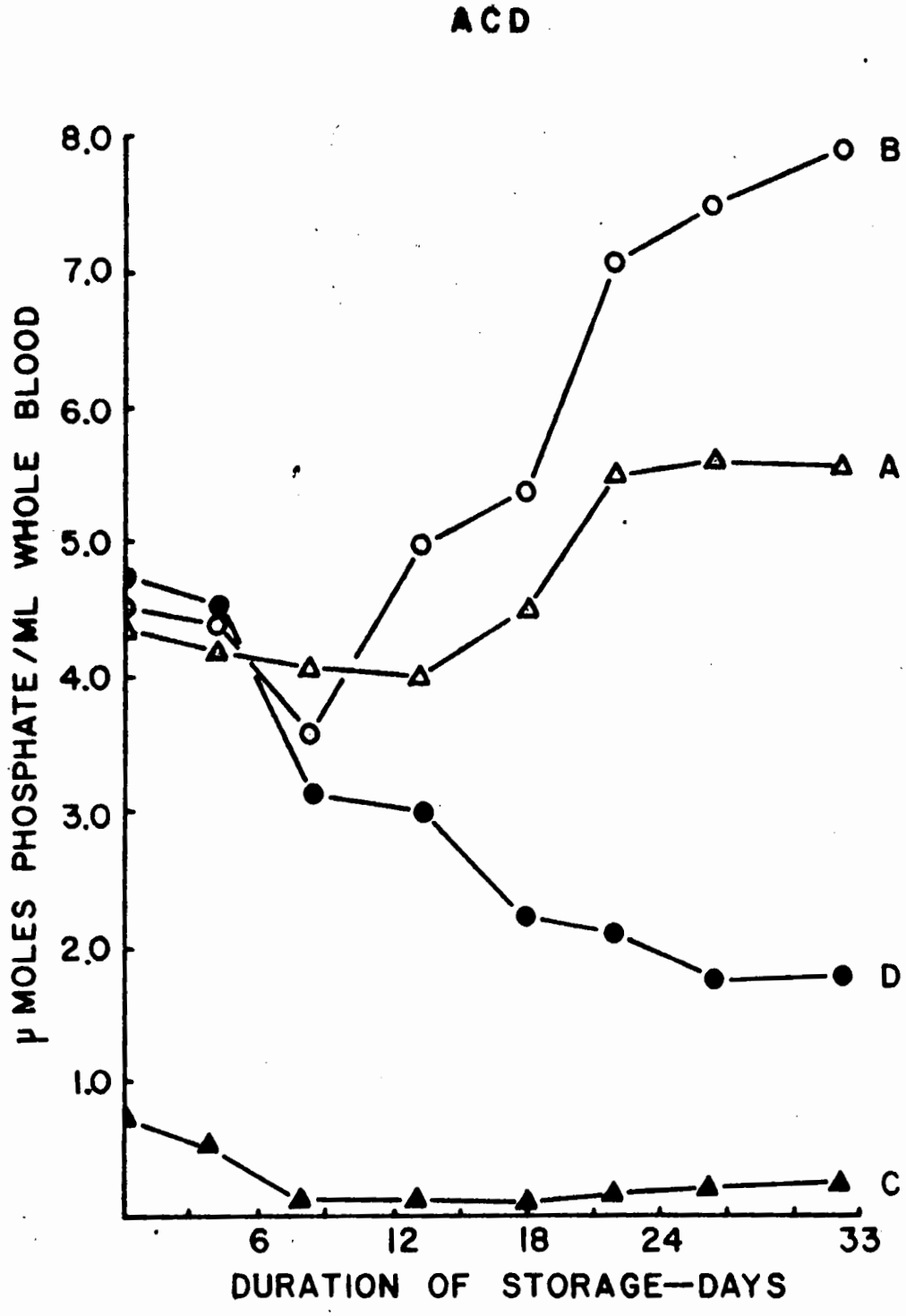

ACD
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
μ MOLES PHOSPHATE/ML WHOLE BLOOD
B
A
D
C
6
12
18
24
33
DURATION OF STORAGE—DAYS

TABLE XIX

The influence of adenosine on the pH of blood stored at 4°C.

Duration of storage (Days)	pH			
	CD medium	ACD medium	CD+Ad medium	ACD+Ad medium
0	7.5	7.0	7.6	7.2
1	7.5	7.1	7.9	7.5
3	7.4	7.0	7.9	7.4
5	7.3	6.9	7.7	7.3
8	7.2	6.9	7.7	7.2
13.	7.1	6.8	7.4	7.0
21	2.0	6.7	7.3	6.9
32	6.8	6.5	7.0	6.6

(102), and Alivisatos (103) in our laboratory, had succeeded in demonstrating the presence of a powerful deaminase in red blood cells.

To test the validity of this explanation, the following experiment was carried out. Adenosine was added to two aliquots of blood and the two specimens were incubated separately, at 37°C. and 4°C. Periodically, samples of the blood were removed and the pH was determined. These samples were then deproteinized and the optical density of the filtrates was determined at 265 mμ in the Beckman DU spectrophotometer. At this wavelength, the optical density diminishes as adenosine undergoes deamination to form inosine (104).

The results are presented in Fig. 15. It is evident that a rapid rise in pH occurred in the specimen at 37°C., reaching a maximum in about two hours. Thereafter, the pH fell gradually as the formation of lactic acid proceeded. At the same time, the absorption at 265 mμ decreased reaching a steady level within two to three hours. In the specimen maintained at 4°C., on the other hand, the pH continued to rise for about 10 hours and then remained constant. Concurrently, the absorption at 265 mμ decreased. [The residual absorption at 265 mμ for either specimen may be attributed to the absorption of inosine (104).] The absorption of the "control" samples (one part of ACD and four parts of isotonic KCl) with added adenosine, remained unchanged. The difference between the initial pH value of the specimen kept at 4°C. and

FIGURE 15

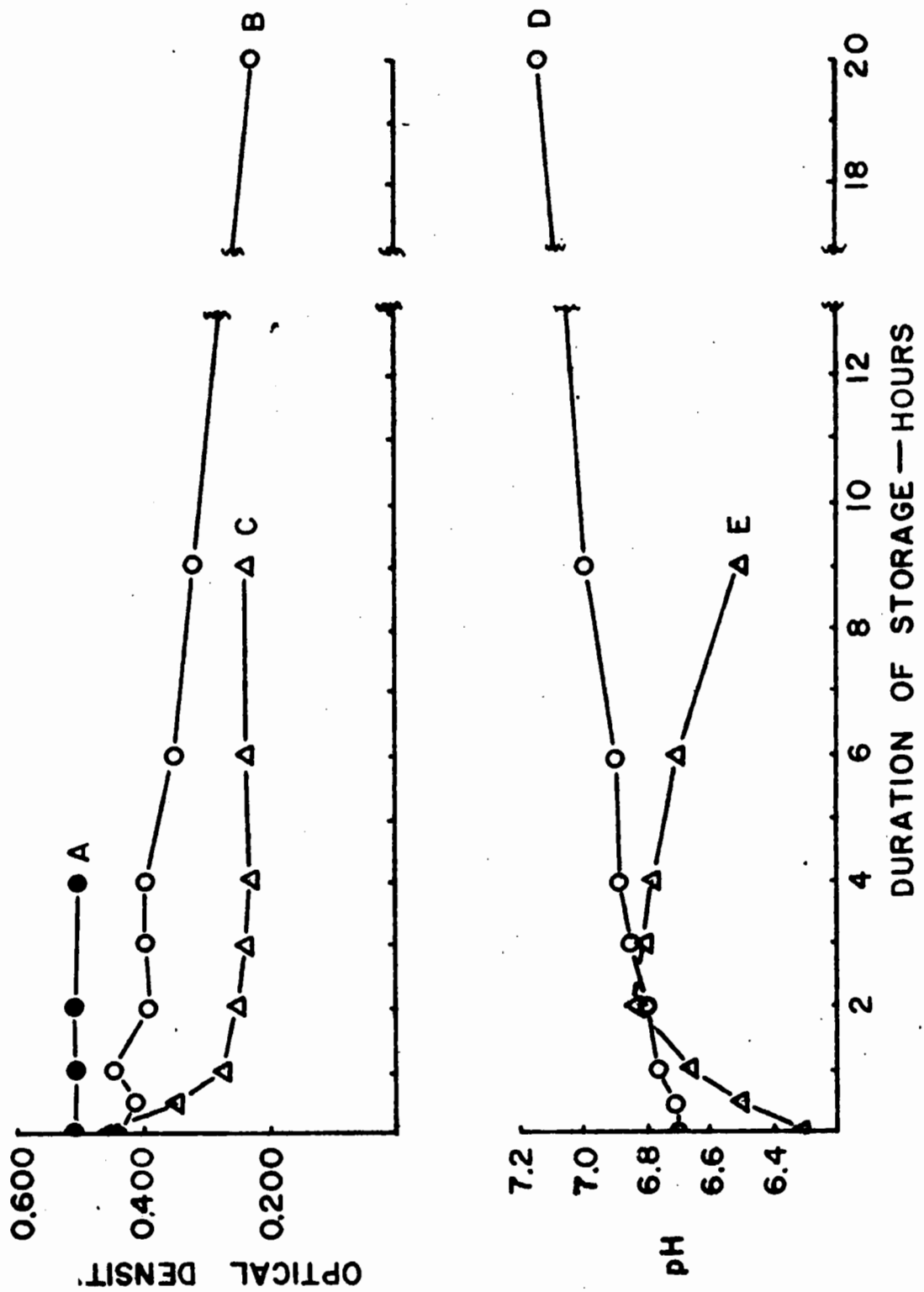
Changes in pH and optical density, at 265 mμ, upon addition of adenosine to blood.

Curve A - "Control", adenosine (400 mg.%) in ACD solution at 37°C.

Curves B and D - Blood specimens preserved in ACD at 4°C.

Curves C and E - Blood specimens preserved in ACD at 37°C.

FIGURE 15


A
B
C
D
E
0.600
0.400
0.200
OPTICAL DENSITY
7.2
7.0
6.8
6.6
6.4
pH
2
4
6
8
10
12
18
20
DURATION OF STORAGE—HOURS

that at 37°C. may be attributed mainly to the influence of temperature on the hydrogen ion concentration (the pH determinations were made on the samples before they attained room temperature), and partly to the effect of the lactic acid formed during the 30 minute preincubation treatment at 37°C. given the erythrocytes (vid. Fig. 15).

A portion (0.05 ml.) of the deproteinized blood filtrate from the incubated specimens was subjected to paper ionophoresis (vid. p.43). The results are reproduced in Fig. 16. It may be seen that after one-half hour at 37°C. (Fig. 16A) the intensity of the adenosine band was greatly diminished, while a new, stronger band had appeared at the position corresponding to that of inosine in the "marker" strip. In addition, a faint band appeared at the position corresponding to hypoxanthine. The apparent conversion of adenosine to inosine and then to hypoxanthine proceeded until, at the end of two hours, the adenosine was depleted. By the ninth hour most of the inosine had been converted to hypoxanthine. In the blood specimen stored at 4°C., the behavior of adenosine followed the same pattern as at 37°C., except that the changes occurred more slowly at the lower temperature; the adenosine was not depleted until about the 45th hour. In neither of the samples, however, was there any evidence of the formation of free adenine. It is apparent, therefore, that adenosine is rapidly deaminated in blood to yield ammonia and inosine. That inosine is split to yield

FIGURE 16

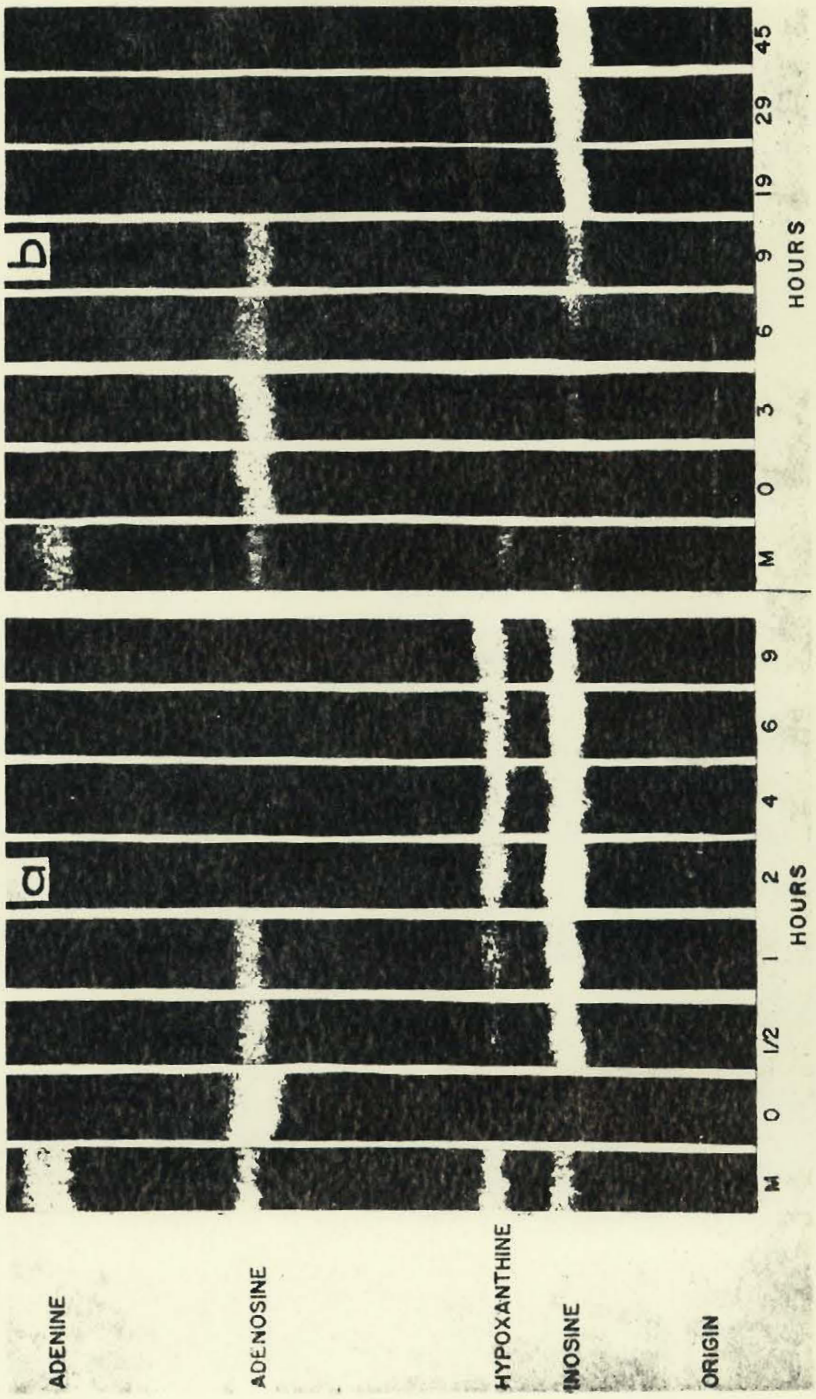
Electrophoresis patterns indicating the conversion of adenosine to inosine and the splitting of the latter to hypoxanthine in blood during preservation in ACD.

a - pattern from sample stored at 37°C.

b - pattern from sample stored at 4°C.

M designates "marker".

FIGURE 16


a
b
M
0
1/2
1
2
4
6
9
3
19
29
45
HOURS
ADENINE
ADENOSINE
HYPOXANTHINE
INOSINE
ORIGIN

hypoxanthine and a ribose moiety was indicated, furthermore, by the fact that ribose disappeared from blood samples stored at 4°C. in the presence of the nucleoside (Table XX). Evidently, the ribose moiety of the nucleoside is metabolized to lactic acid in the red blood cells, and thereby brings about the observed esterification of inorganic phosphate and the accumulation of phosphorylated intermediates (Tables XVI to XVIII and Figs. 10-14). Guanosine and xanthosine were found to be metabolized in a similar manner in the erythrocyte.

(iv) Comparison of the effect of inosine and adenosine.

It was of interest to compare the effect of inosine and adenosine on the metabolism of the erythrocyte during storage at 4°C. Accordingly, two samples of blood were stored in a CD medium for 85 days, one sample containing 800 mg.% (3×10^{-2}M) inosine and the other with the same concentration of adenosine. A third sample of blood was stored in CD alone to serve for purposes of reference. The effect of these nucleosides on the esterification of inorganic phosphate and on the levels of the various phosphate esters in the blood, was essentially the same with inosine as with adenosine; actually the level of organic phosphate esters was found to be slightly higher in the sample containing adenosine. However, the most remarkable difference in the effect of the nucleosides was found to be on the relative rates of utilization of ribose

TABLE XX

Comparison of the effects of inosine and adenosine on blood stored at 4°C.

Metabolite	Duration of storage (Days)	µmoles/ml. blood		
		CD medium	CD+In. medium	CD+Ad. medium
Glucose	0	20.0	20.8	19.7
	8	16.2	19.5	17.2
	21	11.9	17.2	11.9
	35	9.6	16.7	6.5
	50	9.1	17.2	3.1
	63	8.1	16.6	2.3
	85	7.6	16.7	2.0
Difference*		-12.4	- 4.1	-17.7
Ribose	0	2.5	40.7	-
	8	3.0	38.5	48.0
	21	2.0	31.9	40.6
	35	0.9	25.4	36.0
	50	1.3	21.7	37.4
	63	1.2	17.0	34.5
	85	0.9	11.6	29.4
Difference		-1.6	-29.1	-18.6

* Change in µmoles of metabolite/ml. of blood during 85 days of storage.

TABLE XX (cont'd.)

Metabolite	Duration of storage (Days)	µmoles/ml. blood		
		CD medium	CD+In. medium	CD+Ad. medium
Lactate	0	2.2	1.9	2.6
	8	10.9	10.6	11.9
	21	20.0	21.3	27.5
	35	29.5	31.0	37.5
	50	31.2	33.0	43.3
	63	29.4	37.3	53.3
	85	29.7	46.7	60.8
Difference*		+27.5	+44.8	+58.2
P_1	0	1.37	1.11	1.19
	8	0.79	0.87	0.91
	21	0.41	0.70	0.92
	35	0.36	0.57	0.74
	50	0.22	0.50	0.67
	63	0.16	0.49	0.63
	85	0	0.41	0.49
Difference		-1.37	-0.70	-0.70

* Change in µmoles of metabolite/ml. of blood during 85 days of storage.

FIGURE 17

Comparison of the effects of inosine and adenosine on blood stored at 4°C.

a - Glucose utilization

b - Ribose utilization

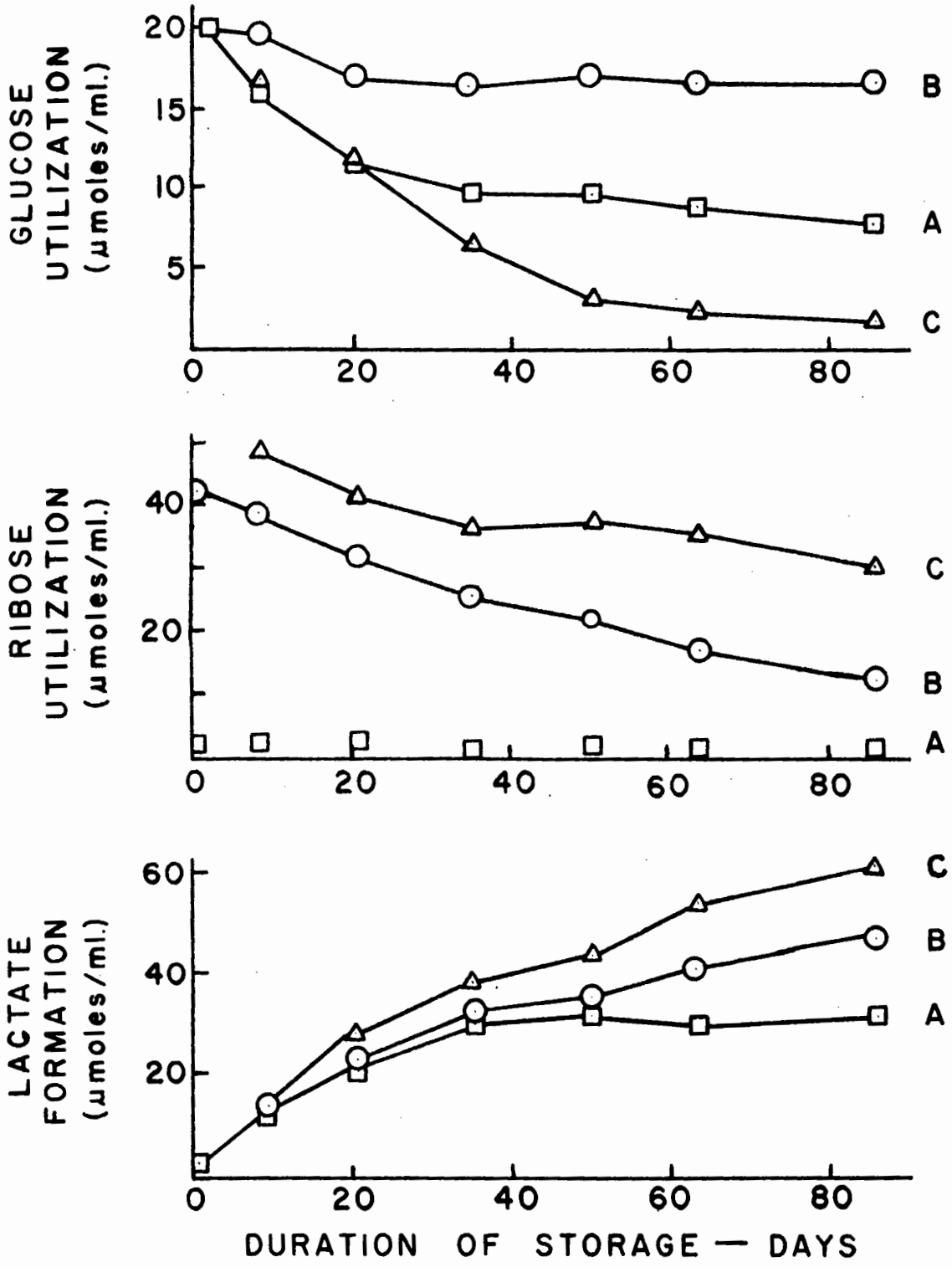
c - Lactate production

Curve A (□) - In CD medium, without nucleoside.

Curve B (○) - In CD medium, with inosine (800 mg.%).

Curve C (△) - In CD medium, with adenosine (800 mg.%).

FIGURE 17


GLUCOSE UTILIZATION (µmoles/ml.)
20
15
10
5
0
20
40
60
80
B
A
C
RIBOSE UTILIZATION (µmoles/ml.)
40
20
0
20
40
60
80
C
B
A
LACTATE FORMATION (µmoles/ml.)
60
40
20
0
20
40
60
80
C
B
A
DURATION OF STORAGE — DAYS

and glucose, and on the formation of lactate, by the erythrocytes. The results are presented in Table XX and Fig. 17.

Glucose utilization in the blood specimen containing adenosine was greater than in any of the other specimens. In the sample containing inosine, on the other hand, the rate of glucose utilization was greatly depressed, while the rate of ribose utilization was greater than in the sample stored in CD alone, or in that containing adenosine. Indeed, in the presence of inosine, glucose utilization became negligible after the first few days of storage. In the sample stored in CD alone, glucose utilization was intermediate between that observed in the presence of inosine and adenosine. Thus, inosine depressed the rate of glucose utilization whereas adenosine stimulated it. It would appear, therefore, that an increase in the rate of utilization of either substrate was compensated for by a decrease in the rate of utilization of the other.

The rate of lactate formation was greatly increased in the presence of either nucleoside, although considerably more lactate was produced with adenosine than with inosine. Lactate formation remained quite active till the 85th day of storage in the samples containing nucleoside, thus indicating the continued utilization of ribose in these samples. In the blood sample stored in CD alone, on the other hand, lactate formation and also glucose utilization came almost completely to a standstill after the 40th day of storage.

These results may be explained partially by the

effect of pH on the relative rates of utilization of glucose and riboside. Aliquots of fresh blood in a CD medium were incubated (at 37°C. and 4°C.) in the pH region 6 to 7.8, with and without inosine, and the rates of glucose and ribose utilization were determined. It was found that, whereas glucose utilization at pH 7.8 was from two to three times higher than at pH 6, the rate of ribose utilization was hardly affected by the change of pH between 6 and 7.8. It was significant, therefore, that the pH of the blood specimens in the CD,CD+inosine and CD+adenosine media was 7.4, 7.4 and 7.9 initially, and 6.9, 6.8 and 7.2, respectively, at the 56th day of storage. Thus, at the relatively higher pH of the CD+adenosine medium glucose utilization predominated over that of ribose. However, at the pH of the CD+inosine medium (pH 7.4), glucose utilization was considerably depressed and soon came to a stop, while the utilization of ribose was increased to compensate for the lack of substrate.

A study was made[3] of the possible toxic effects of the various mucleosides on the dog and rat. Inosine, guanosine, xanthosine and hypoxanthine, in 50 mg. quantities, were injected intravenously into a dog, and it was found that none of these compounds produced a change in the blood pressure of the animal. However, when 50 mg. of adenosine were injected into the animal, a drop in the blood pressure from 150 to 50 mm. Hg was immediately observed. An equivalent amount of ammonia (neutralized solution) produced only a slight drop in the

blood pressure. Furthermore, when a small amount of human blood which had been stored in a CD+adenosine medium at 4°C. for 60 days, was injected into the dog, the blood pressure did not change. However, if adenosine was added to the stored blood immediately before injection, a prompt drop in the blood pressure was produced. In the rat, the intravenous administration of 10 mg. adenosine proved fatal. However, injection of 200 mg. of inosine into the animal produced no observable effect.

(v) The lack of effect of nucleosides on the stability of hexokinase.

When inosine was added to preserved blood specimens at the 20th or 41st day of storage, as when added at the start, the nucleoside brought about a rapid esterification of inorganic phosphate and a corresponding rise in the levels of ATP and DPG in the erythrocytes. However, in all samples, the rate of glucose utilization was found to be diminished almost immediately upon the addition of the nucleoside. These results confirm those obtained earlier (vid. Tables XVI - XVII, XX).

A study of the hexokinase activity of the stored erythrocytes to which nucleoside had been added at various times during storage, was undertaken. Two aliquots of blood were stored at 4°C. in ACD media. Adenosine (400 mg.%) was added to one sample at the 24th day of storage, while the other was stored without the addition of nucleoside. The ability of the erythrocytes to utilize glucose was determined at intervals during the storage period. The results are presented in Fig. 18. It is apparent that the ability of the

FIGURE 18

Utilization of glucose in blood stored with and without adenosine.

Curve A (●) - Erythrocytes preserved in an ACD medium with adenosine (400 mg.%) added at the 24th day of storage.

Curve B (○) - Erythrocytes preserved in an ACD medium, without adenosine.

Conditions:-

Preserved erythrocytes were washed with isotonic KCl and 0.5 ml. aliquots of the washed erythrocytes were incubated as in Table VIII, but with 5mM ADP instead of ATP.

FIGURE 18

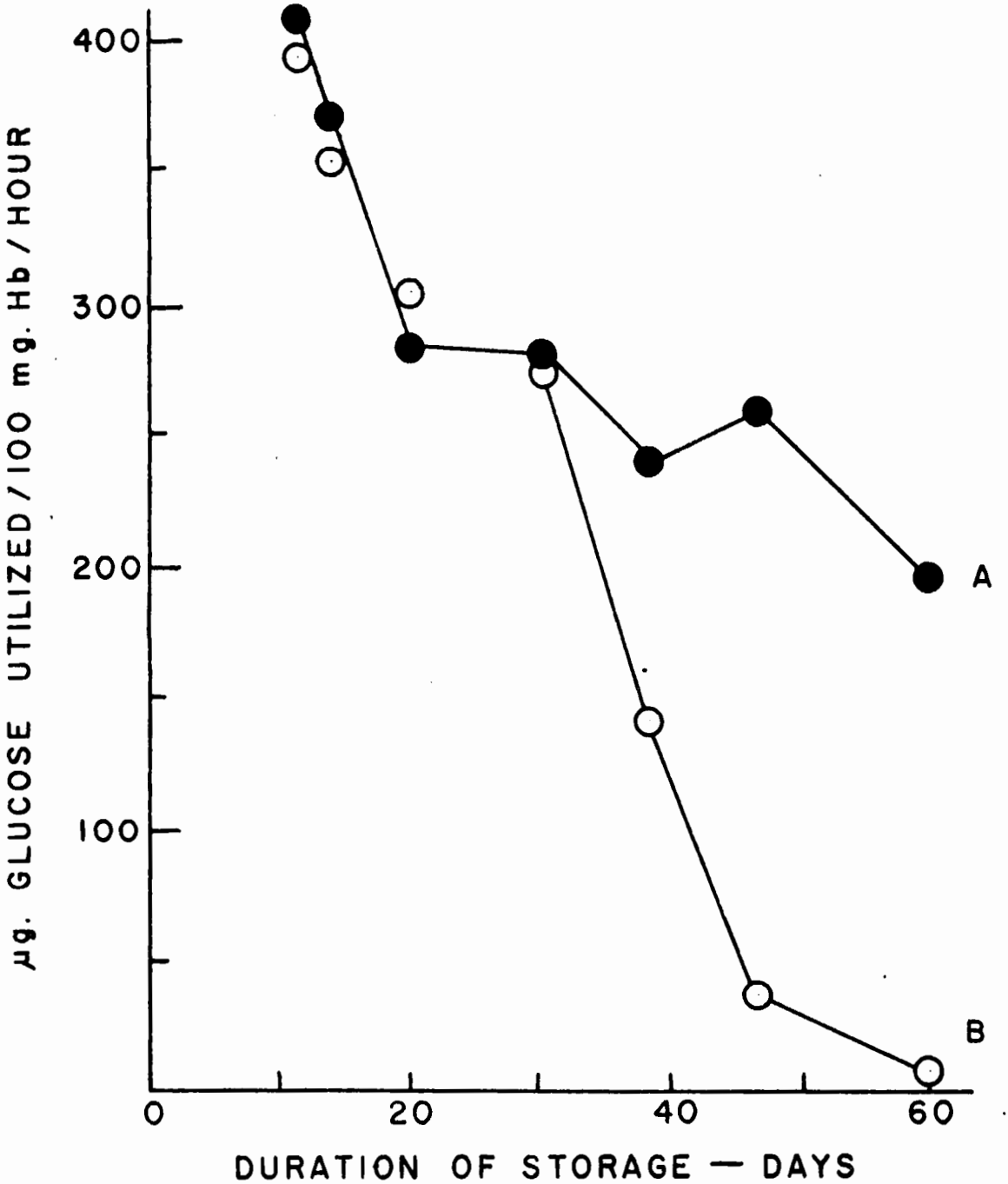

400
300
200
100
μg. GLUCOSE UTILIZED / 100 mg. Hb / HOUR
A
B
0
20
40
60
DURATION OF STORAGE — DAYS

FIGURE 19

Behavior of hexokinase stored with and without adenosine.

Curve A (○) - Erythrocytes preserved in an ACD medium, without inosine.

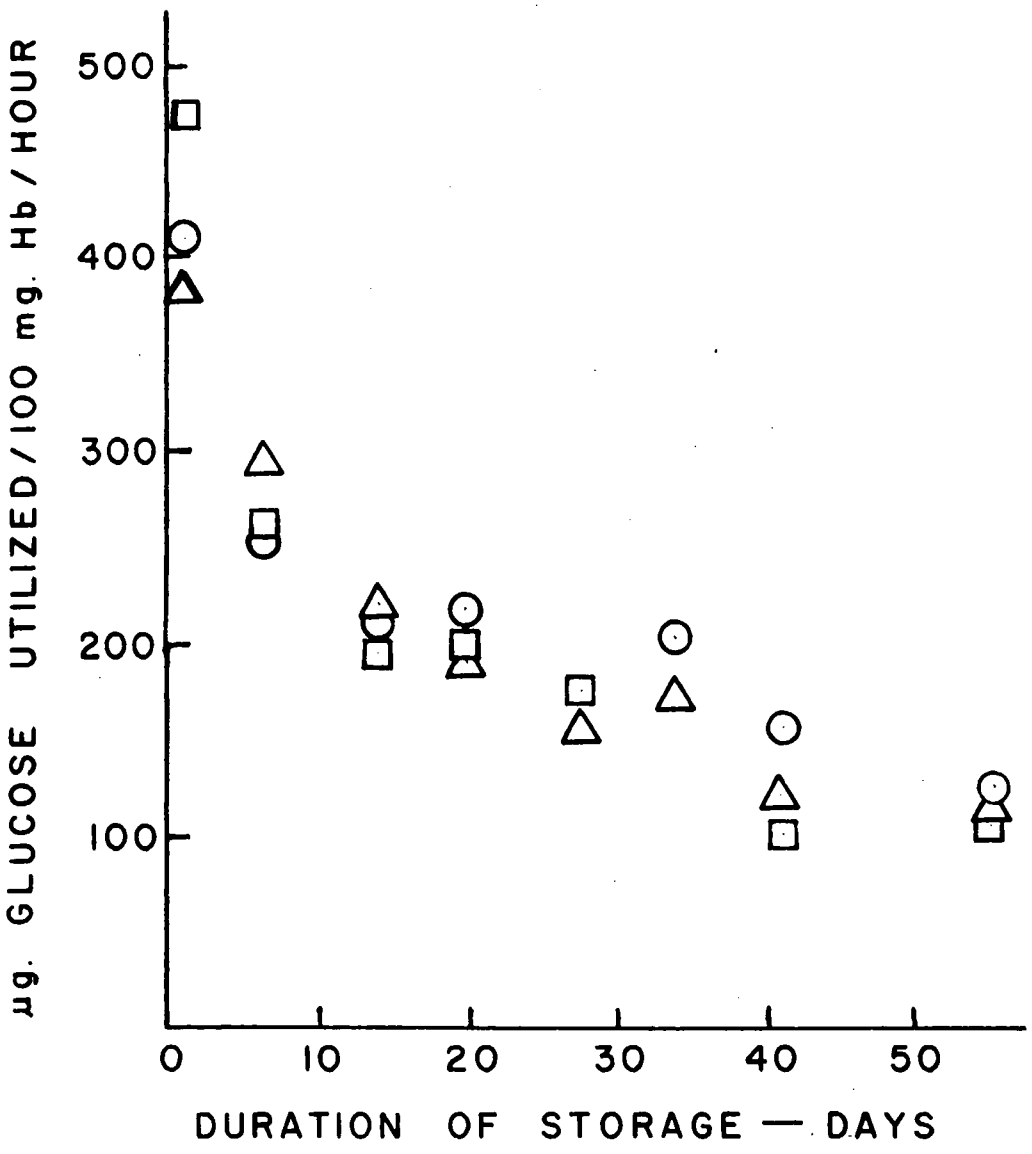
Curve B (△) - In ACD medium, with inosine (800 mg.%) added at the beginning of storage.

Curve C (□) - In ACD medium, with inosine (800 mg.%) added at the 20th day of storage.

Conditions:-

Preserved erythrocytes were washed with isotonic KCl and the washed erythrocytes were hemolyzed by alternate freezing and thawing. 0.5 ml. hemolyzate incubated as in Table VIII.

FIGURE 19


µg. GLUCOSE UTILIZED / 100 mg. Hb / HOUR
500
400
300
200
100
0
10
20
30
40
50
DURATION OF STORAGE — DAYS

erythrocytes to utilize glucose decreased progressively during storage (cf. Fig. 9). However, after the 30th day, the rate of decrease was much more rapid in the sample stored in ACD alone than in the sample to which adenosine had been added. In fact, at the 60th day of storage the glucose-utilizing activity of the erythrocytes stored in ACD alone was almost entirely abolished. In another experiment, the glucose-utilizing activity of erythrocytes which had been stored in a CD medium for 70 days, was determined. Only those erythrocytes which had been stored in the presence of adenosine or inosine were capable of utilizing glucose; the ability of the cells stored in CD alone to utilize glucose was negligible.

The hexokinase activity of the hemolyzates prepared from stored erythrocytes, on the other hand, diminished, during storage, at about the same rate in the samples stored with inosine as in those stored without added nucleoside (vid. Fig.19). It is evident, therefore, that the nucleosides do not prevent the deterioration of the hexokinase of the erythrocyte during storage (vid. Table XV, Fig. 9). However, it is significant that there still was a considerable degree of hexokinase activity in the hemolysates of the erythrocytes even at the 55th day of storage.

SUMMARY

The hexokinase of the human erythrocyte is unstable and undergoes a progressive diminution in activity during

the storage of blood at 4°C. in the CD or ACD medium. The inactivation of the enzyme during storage is not prevented by the addition of adenosine or inosine to the blood. However, the nucleosides prevent the complete loss of the ability of the intact erythrocytes to use glucose, such as ordinarily occurs by the 60th day with erythrocytes stored in the ACD medium alone. The hexokinase activity in the preserved erythrocytes persists, to a considerable degree, at least until the 55th day of storage.

The addition of adenosine or inosine to preserved blood induces a synthesis of organic phosphate esters in the erythrocyte. In blood stored in the CD or ACD medium, the concentrations of ATP, DPG and hexose phosphate tend to fall during storage. In the presence of the nucleosides there is an initial increase in the level of DPG and hexose phosphate, and the fall in ATP is retarded. If inorganic phosphate is added to the blood specimen, the increase in the concentration of DPG, brought about by adenosine, is augmented. Thus, DPG appears to serve as a reservoir of esterified phosphate in the erythrocyte.

Adenosine added to blood rapidly undergoes deamination, in the erythrocyte, to yield inosine. The latter is split, in turn, to hypoxanthine and a ribose moiety which is metabolized to lactic acid. Guanosine and xanthosine similarly contribute to lactic acid formation and phosphate esterification in the erythrocyte.

Because of the liberation of ammonia by the deamination of adenosine, the pH of the blood samples containing adenosine tends to increase during the initial few days of storage.

Inosine depresses the rate of glucose utilization by the erythrocytes during storage in the cold, while adenosine increases it. Ribose utilization, however, is greater in the blood samples containing added inosine than in the samples containing adenosine. The differences may be partially attributable to the pH of the stored blood samples. Thus glucose utilization by the erythrocytes is increased at the higher pH levels, while the utilization of ribose remains unaffected by the change in pH.

Adenosine, when injected into a dog, produces a severe vasodepression. Inosine and hypoxanthine, on the other hand, are harmless in this respect.

DISCUSSION

Studies on the metabolic behavior of the erythrocyte have revealed that there is a rapid depletion of the organic phosphate esters and a progressive failure of glycolysis in the red cells during storage in the cold (78,96, 105-107). Rapoport (96) attributed the progressive decrease in the rate of lactate production by the erythrocytes to the rapid fall in the ATP content of the cells. He found the glycolytic activity was best maintained in erythrocytes stored in the ACD medium. In these specimens, the ATP concentration still was fairly high at the 40th day of storage, whereas in samples preserved in the CD medium, the ATP content and also the glycolytic activity of the cells had reached a very low level by the 30th day. Pappius et al. (78), on the other hand, found that the DPN content of preserved erythrocytes gradually diminishes during storage, and suggested that the glycolytic failure and the inability of the cells to maintain a high level of phosphate esters, might be the result of the progressive depletion of DPN rather than ATP. However, these authors could not decide which of the two coenzymes is more closely involved in the initiation of the failure of the stored red cell. Fishman (105), in our laboratory, shared the view of Rapoport (96) that the decrease in the concentration of ATP probably is the most important cause of the gradual deterioration of the erythrocyte during storage.

While it has been realized for a long time that the storage of blood is attended by the progressive depletion of coenzymes such as ATP and DPN, in the erythrocyte, little consideration has been given to the possible alteration of the apoenzymes as being contributory to the metabolic failure of the cell. Fishman (108) found that the activity of carbonic anhydrase and cholinesterase in blood is not diminished even on storage, in the cold, for many months. The cholinesterase, however, tends to become detached from the surface of the erythrocyte, and escapes into the plasma. Allison and Burn (109) found that the cholinesterase, catalase and glyoxalase activity of red blood cells decreases as the cells grow older, even in the circulation. Blanchaer et al.(110) found that 3-phosphoglyceraldehyde dehydrogenase, 3-phosphoglycerate kinase and triose phosphate isomerase could still be demonstrated in hemolyzates prepared from red blood cells which had been stored in the ACD medium for 22 days. However, Blanchaer and Weiss (111) showed that the ability of erythrocytes to reduce added pyruvate was progressively diminished when the erythrocytes were stored in the cold. The authors concluded that the defect in the reduction mechanism of the preserved red cells could not be attributed to the fall in the concentration of either DPN or ATP, or to the failure of aldolase, 3-phosphoglyceraldehyde or lactic dehydrogenase. They suggested, rather, that the defect probably was attributable to a progressive failure in the enzymatic steps by which glucose is converted to fructose diphosphate.

The present writer found that the hexokinase of the erythrocyte undergoes a progressive inactivation during storage. It appears that the decrease in the activity of the enzyme is initiated by the fall in the pH of the preserved blood specimens. Thus it was found (Fig. 8) that the stability of hexokinase is influenced by the pH of the medium. The enzyme is most stable at pH 7.8 and, as the pH is lowered to pH 6.5, the rate of inactivation of the enzyme is rapidly increased. Furthermore, the inactivation appears to be irreversible (vid. p. 86).

During preservation of blood at 3°C., the pH of the samples was observed to fall from the initial pH 7.0-7.5 to about pH 6.5-6.8 within a 32-day period (Table XIX). It is reasonable to conclude, therefore, that the falling off of the activity of hexokinase was, in large measure, the result of the change in the pH of the blood samples. Furthermore, the rate of inactivation of the enzyme was found not to be influenced by the level of endogenous ATP. In the CD medium, the concentration of ATP began to fall from the outset of storage, while in ACD, it remained constant during the first three weeks (Fig. 11, Table XVI), nevertheless, the rate of inactivation of hexokinase appeared to be the same in the two media. Indeed, when adenosine was added to the blood, the concentration of ATP remained constant throughout the storage period (Fig.11, Table XVI), but the fall in hexokinase still occurred (Fig.9). Inosine, added at various times during storage, brought about an immediate increase in the level of ATP in the preserved

erythrocytes. However, the rate of inactivation of the enzyme was not affected by the addition of the nucleoside (Fig. 19).

The rate of glucose utilization by the erythrocyte, during storage, apparently is influenced by several factors. During the first three to four weeks of storage, whether the blood samples were preserved in CD or in ACD, the rate of glucose uptake from the preservative medium, remained essentially constant (Fig. 12, Table XVII) (78). However, during this entire period, the hexokinase activity of hemolyzates prepared from the preserved cells, and also the rate of glucose utilization, at 37°C., by the intact cells were found to decrease (Figs. 9, 18, 19). This apparently anomalous behavior can be explained on the assumption that a considerable portion of the total hexokinase present in the erythrocytes is not functional at 4°C. Consequently, as some of the hexokinase of the red cells becomes inactivated during storage, the amount of active enzyme left still is sufficient to permit the utilization of glucose, during storage, to proceed at the normal rate. When the stored cells are incubated at 37°C., however, the rate of glucose utilization is increased, because of the higher temperature (vid. Fig. 7), and reaches the optimal capacity of the enzyme. Under these conditions, the loss of hexokinase through inactivation during storage at 4°C. is clearly evident. This occurs despite the fact that the ATP level in the erythrocytes may have been very well maintained

by the nucleosides added to the medium.

After the first four weeks of storage, the capacity of the erythrocytes to utilize glucose from the preservative medium, at 4°C., progressively became impaired (Table XX). By this time the level of ATP in the erythrocytes had fallen to about one-fifth of its initial concentration. Also, when the preserved erythrocytes were incubated at 37°C., the rate of glucose utilization by the cells progressively diminished, with storage, and became negligible by the 60th day (Fig.18). However, there still persisted a considerable degree of hexokinase activity in the red cells (Fig. 19). Evidently, the failure of the erythrocytes to utilize glucose after the fourth week of storage was due primarily to the depletion of endogenous ATP and not of hexokinase. Thus, in erythrocytes stored in the presence of adenosine, the ATP content of the cells was well maintained and indeed the level of hexokinase activity was high enough to permit the utilization of glucose, at 4°C. and at 37°C., to proceed even at the 60th day of storage (Figs.17 and 18; Table XX). Nevertheless, it still is possible that the depletion of ATP in the erythrocytes, after three weeks of storage (without added nucleoside), was the result of the gradual diminution in the activity of hexokinase; the reverse apparently is not true (cf. p. 131). Thus, it would appear that, while the fall in hexokinase activity during the first 3-4 weeks of storage was not sufficient to affect the metab-

olism of the red cell in the cold (cf. p.132), the reduction in the activity of the enzyme from the fourth week on, was great enough to bring about the observed depletion of ATP in the cells.

It is significant that, although ADP was added to the erythrocytes (preserved without nucleoside) during incubation at 37°C. (cf. pp. 90-95), the utilization of glucose nevertheless fell almost to zero by the 60th day. This would suggest that the adenylate kinase of the erythrocyte, or at least that portion of it contained in the cell membrane, becomes inactivated during storage.

After the transfusion of the preserved erythrocytes the hexokinase capacity of the cells may play a very significant role in the reconstitution and revitalization of the erythrocytes. As far as the red blood cells which remain viable during storage are concerned, the capacity for reconstitution in the circulation after transfusion probably is determined by the concentration of ATP in the preserved cells at the time of transfusion (cf. 96,97,112). Thus if there is sufficient ATP in the preserved erythrocytes, the cells will readily survive in the circulation. However, if the level of ATP is low, it is conceivable that the degree of hexokinase activity remaining in the cells will determine whether the ATP can be replenished or not. In other words, ATP will be replenished only if the hexokinase activity of the cell is high enough. When the erythrocytes have been

preserved with added nucleosides, the level of ATP in the cells is better maintained and, as a result, it probably is easier for the remaining hexokinase to maintain the ATP content of the surviving red cells. By analogy, a gradual depletion of hexokinase in the circulating erythrocyte, with age, may be the cause of the eventual loss of viability and removal of the cell from the circulation (cf. 109).

It has been seen (p. 118) that the nucleosides exert their beneficial effect on the organic phosphate esters by serving as a substrate in the metabolism of the erythrocyte. Their behavior is characterized by the disappearance of ribose, the appearance of hypoxanthine and the accumulation of lactic acid. These findings are consistent with the idea that inosine undergoes phosphorolysis yielding free hypoxanthine and ribose-1-phosphate. If adenosine is added to the blood specimens, it undergoes deamination to inosine before phosphorolysis can proceed. Kalckar (113) found that a nucleoside phosphorylase present in rat liver, can split inosine or guanosine to ribose-1-phosphate plus hypoxanthine or guanine, respectively, in the presence of inorganic phosphate. Prankerd and Altman (114) obtained evidence that human erythrocytes can also phosphorylze adenosine. Recently, Gabrio and Huennekens (115) demonstrated the presence of a nucleoside phosphorylase in human red blood cells. Finally, Dische (116)

and Dische and Pollaczek (117) showed that erythrocyte hemolyzates are capable of splitting adenosine, and that the subsequent disappearance of the ribose moiety of the nucleoside is accompanied by the formation of triose phosphate, hexose mono- and diphosphates and heptulose phosphate. These esters could be obtained also by adding ribose-5-phosphate to the hemolyzates. Evidently, ribose-1-phosphate, obtained from nucleosides, can be metabolized in the erythrocyte by way of the pentose phosphate pathway (vid. 19,118). The results presented in this thesis are consistent with the above findings. Thus, in the metabolism of inosine in preserved erythrocytes, inorganic phosphate is esterified, with the formation of various phosphate esters, and lactic acid is produced. Apparently, the rate of utilization of ribose phosphate by the erythrocytes is so great that the ATP content of the cells is well maintained throughout the storage period, and the levels of hexose phosphate and DPG actually are increased (Figs. 10 and 11, Table XVI). Indeed, with inosine or adenosine present, the concentrations of ATP and hexose phosphate are so well maintained in the preserved erythrocytes, that when extra inorganic phosphate is added, the concentrations of these esters can no longer be increased and all the extra phosphate is esterified in the form of DPG (Fig. 14). It is evident, therefore, that nucleosides can be used as an artificial means of maintaining ATP and the other phosphate esters of the erythrocyte when glucose metabolism is no longer capable of maintaining the

energetics of the cell.

It was found that the addition of inosine tends to decrease, and adenosine to increase the rate of glucose utilization by erythrocytes during storage (Fig.17, Table XX). The former effect may be explained, partially, in terms of the inhibition of hexokinase which results from the accumulation of hexose monophosphate in the red cell, in the presence of added nucleoside (Fig. 11, Table XVI; see also ref. 116). This probably occurs with both inosine and adenosine, but with the latter, the utilization of glucose is stimulated, apparently as a result of the increase in the pH effected by the liberation of ammonia on deamination of the nucleoside. Rubinstein[6], in our laboratory, has obtained evidence which indicates that ammonium ions, per se, increase the rate of glucose utilization by the erythrocyte. The mechanism of this effect is unknown. It is significant, however, that the utilization of glucose and ribose by the erythrocyte bear an inverse relationship to one another. Thus a decrease in the rate of utilization of either of these substrates was compensated for by an increase in the rate of utilization of the other.

The addition of nucleosides to blood specimens to be preserved for clinical use, appears to offer considerable advantage as far as the prolongation of the storage period is concerned. Gabrio et al. (97) have studied the survival, after transfusion, of erythrocytes which had been stored with added

---

6. Private communication.

adenosine, and found that the percentage survival of the cells was greatly improved by the presence of the nucleoside. However, adenosine is known to be toxic when injected into the animal body. The writer confirmed this in the dog and rat (vid. p. 122), but found, on the other hand, that inosine and hypoxanthine produced no detectable adverse effects in these animals, even when injected in relatively large quantities. Inosine, therefore, by virtue of its equally beneficial influence, on red cell viability during storage, and because of its apparent lack of toxicity, definitely is to be preferred for use with blood. Clinical tests are now in progress to ascertain the human tolerance to inosine in the quantities required in the preservation of human blood for clinical use.

There is considerable evidence to indicate that the transfer of glucose into the red blood cell does not occur by a process of simple diffusion. Thus, it has been established that the rate at which glucose enters into the red cell is not proportional to the concentration of the sugar and that, when more than one hexose is present, there is a mutual inhibition of the entry of any one of these into the erythrocyte (119,120). Enzyme inhibitors have been shown to prevent the transfer of sugars (120) and, also, the effect of temperature on the transfer process is different from its effect on the passive diffusion of sugars in water (94). These findings suggest that

specific mechanisms within the erythrocyte are responsible for the transfer of sugars across the red cell membrane. However, the red blood cell will permit only enough sugar to enter it to equalize the concentration of sugar, per unit volume of water, inside and outside the cell. In this respect, the process differs from glucose absorption in the intestine and the renal tubule in that these organs can transfer glucose against a concentration gradient, i.e., from a region of lower concentration to one of higher concentration (cf. 119). As a result, many authors have preferred to use the term "facilitated transfer", to describe the movement of glucose across the red cell membrane, instead of "active transfer", which seems to imply an ill-defined secretory-like activity on the part of the cell.

LeFevre (121,122) and Wilbrant (120) and their coworkers attempted to explain the facilitated transfer of sugars into the red blood cell by postulating that the sugar molecules are transported across the cell membrane in the form of complexes with specific carriers which are present in the membrane. Under these conditions, the sugar-carrier complex is formed at one surface of the membrane, the complex then traverses the membrane and is finally split, at the other membrane surface, with the release of free sugar into the medium. These authors found that their experimental results fitted the equations which were developed on the basis of the above hypothesis. They then suggested that the initial step in the transfer process is the hexokinase-catalyzed formation of G-6-P, and the final step,

that is the release of free sugar into the medium, is catalyzed by a hexose phosphatase (120). The same workers found, however, that G-6-P, G-1-P and fructose diphosphate do not enter into the erythrocyte (cf. 82).

The writer's results indicate that hexokinase is indeed responsible for the transfer of glucose into the red blood cell. It was found that the activation energy for the phosphorylation of glucose by the hexokinase in the SFH, and that for the phosphorylation of glucose by the intact erythrocyte, were of the same order (p.80). This indicates either that the transfer of glucose into the red blood cell is identical with the phosphorylation of glucose by hexokinase or, if some other mechanism is involved, that the velocity of the transfer process is faster than the rate of glucose phosphorylation. However, other experiments (Table X) showed that the rate at which glucose is transferred into the red cell is equal to or slower than the rate at which it is phosphorylated by hexokinase. Thus it would appear that the two types of activity occur by the same mechanism. Furthermore, the effect of pH and glucose concentration on the activity of hexokinase is closely similar to that on the uptake of glucose by the intact cells. These results are consistent with the hypothesis that hexokinase is responsible for the transfer of glucose into the red blood cell. Indeed, the finding by Le Fevre (121, 122) that glucose, mannose, fructose, sorbose and

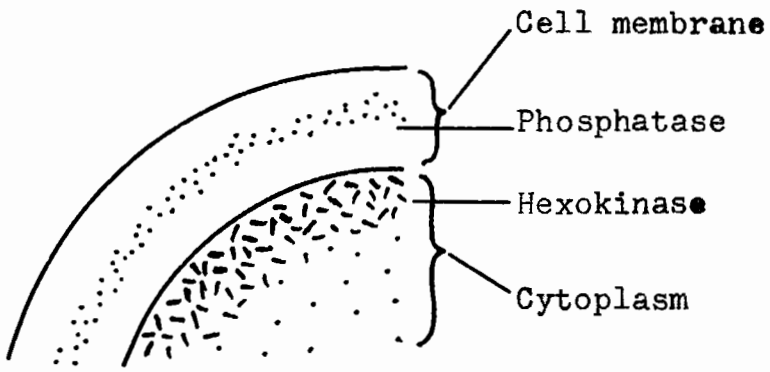
other sugars compete with one another for entry into the erythrocyte (cf. 20), can readily be explained in terms of the involvement of hexokinase in the uptake of the sugars.

Bartlett and Marlow (123), using C^{14}-labeled glucose, found that, under the conditions of their experiments, glucose outside the red blood cell does not equilibrate with the free glucose inside the cell. The observation, made by LeFevre (121) and others, that the extracellular glucose (unlabeled) tends to reach an equilibrium with the glucose in the erythrocyte must be explained, therefore, by assuming that the intracellular glucose is obtained through the hydrolysis of G-6-P in erythrocyte. This is in agreement with the scheme proposed by Wilbrandt (120) who suggested that glucose must be phosphorylated before being transferred into the red blood cell, and that the ester subsequently undergoes hydrolysis to yield free glucose. It is interesting in this respect, that van Crevold and Brinkman (124) found that the red cells of blood which had been collected with extreme precaution so as to prevent even the initiation of clotting, contained no free glucose.

It was generally assumed, in relation to glucose transport, that hexokinase is present in the membrane of the erythrocyte. However, the writer's experiments have shown, on the contrary, that the enzyme probably is confined to the cytoplasm of the cell (Table VIII). On the basis of this finding, it is difficult to imagine that glucose cannot enter freely into the red cell, and that the transfer of glucose

into the cell is dependent on the phosphorylation of the sugar by hexokinase. The difficulty is overcome, however, if one assumes that hexokinase is arranged in the cytoplasm at the inner boundary of the red cell membrane, as indicated in Fig. 20. Such a distribution of the enzyme would be possible if the arrangement of the molecules within the cytoplasm were not wholly random, as in a solution, but were arranged with some degree of regularity, perhaps as in the "short ordered" or semi-fluid state described by Ponder (125).

FIGURE 20



In the transfer of glucose into the erythrocyte, therefore, the sugar molecules apparently traverse the lipid and protein layers of the membrane proper (cf. 125) with little difficulty, and are then phosphorylated by the hexokinase situated at the inner surface of the cell membrane. As a result, glucose enters the main body of the cytoplasm in the form of G-6-P. The glucose ester can then be metabolized, or hydrolyzed to

free glucose by hexose phosphatase. The phosphatase probably is located in the cell membrane since Wilbrandt (120) found that, while phloretin inhibits the phosphatase, P^{32}-labeled phloretin will not enter the red cell interior.

The above-mentioned scheme for the transfer of glucose into the red blood cell simplifies the theoretical basis of the transfer mechanism since it does not involve the formation of a glucose-carrier complex which must travel through a maze of membrane structures before emerging into the interior of the cell (cf. 70,94). Furthermore, it is evident, from this scheme, that G-6-P can undergo immediate metabolism upon entry into the cytoplasm of the erythrocyte. In the previously suggested mechanisms, the phosphorylation of glucose was considered merely a means of facilitating the passage of the sugar molecule through the cell membrane. Thus, the subsequent metabolism of the sugar was supposed to require a separate phosphorylative step.

Concerning the entry of sugars other than glucose into the red blood cell, it is possible that the passage of the sugar molecule through the cell membrane will not be by free diffusion but will depend, rather, on the properties of the sugar molecule and the cell membrane. In such an instance, the translocation process may become the rate-limiting factor in the final entry of the sugar into the erythrocyte. Consequently, the kinetics of the overall transfer process will depend on the relative ease with which the sugar molecule can

penetrate the membrane, and not on the rate of phosphorylation of the sugar by hexokinase. Glucose, evidently, can pass through the red cell membrane with great ease so that the phosphorylation of the molecule by hexokinase is the rate-limiting step in the overall passage of the sugar into the cell. The kinetics of the transfer process, therefore, are the same as those which describe the phosphorylation of glucose by hexokinase.

Finally, it is interesting to note that Best (126) postulated that the hexokinase of yeast is located just within the membrane of the intact cell. Also, Derrick et al. (127) found, with the aid of immunological methods, that phosphatase is located at the outer surface of the yeast cell. The apparent arrangement of the two enzymes in yeast is similar, therefore, to that proposed by the writer for the same enzymes in the intact red blood cell.

CONCLUSIONS

The progressive decrease in the activity of the hexokinase of the red blood cell during storage in the cold, probably is caused by the fall in the pH of the preserved blood specimens. The inactivation of the enzyme apparently is not dependent upon the level of ATP in the erythrocytes, since the rate of decrease in the activity of the enzyme is practically the same in the ACD as in the CD medium, even though ATP is better maintained in the blood specimens preserved in the former medium. Furthermore, the addition of nucleosides such as adenosine and inosine does not retard the rate of enzyme inactivation, despite the beneficial effect of these substances in the maintenance of the concentration of ATP in the preserved erythrocytes.

In the intact erythrocyte, the diminution in hexokinase activity does not appear to affect the rate of glucose utilization by the cells, during storage in the cold. Apparently, the amount of hexokinase left in the red blood cells after some of the enzyme has been inactivated during storage, still is sufficient to permit the glucose utilization by the cells to proceed unaffected. After the fourth week of storage, however, the ability of the erythrocytes to utilize glucose diminishes rapidly as a result of the depletion of the ATP content of the cells. This occurs despite the fact that a considerable degree of hexokinase activity still persists

in the red cells even at the 55th day of storage.

When the stored erythrocytes are incubated at 37°C., the progressive decrease in the activity of hexokinase soon becomes apparent. Thus, when red blood cells which had been preserved in the absence of added nucleoside, are incubated at 37°C., the ability of the cells to utilize glucose is rapidly reduced, following increasing periods of storage, to a negligible value; with added nucleoside, however, both the level of ATP and the glucose-utilizing capacity of the stored cells are better maintained.

An adequate level of ATP appears to be of primary importance for the survival of the preserved erythrocyte. At the same time, the hexokinase activity remaining in the preserved erythrocyte may determine the capacity of the cells to reconstitute and maintain the level of ATP after transfusion.

Inosine is rapidly metabolized in the erythrocyte. The nucleoside undergoes phosphorolysis to yield hypoxanthine and ribose-1-phosphate and the pentose phosphate is metabolized, presumably by way of both the pentose phosphate and glycolytic pathways, to yield lactic acid as an end product. Adenosine is first deaminated to yield inosine before undergoing phosphorolysis.

The nucleosides thereby provide an alternative substrate for the erythrocytes when the capacity of the cells to utilize the normal substrate, glucose, is diminished. The addition of adenosine or inosine to the preserved blood spec-

imens ensures the maintenance of a high level of ATP and other phosphate esters in the erythrocytes during storage.

The presence of inosine diminishes the rate of glucose utilization by the erythrocytes during storage in the cold. This retardation presumably occurs as the result of the inhibition of hexokinase by the accumulation of relatively large amounts of G-6-P from the metabolism of the nucleoside. Adenosine, on the contrary, tends to increase the rate of glucose utilization. It would appear that the release of ammonium ions in the deamination of the nucleoside is in some way responsible for this effect. Apparently a reduction in the rate of utilization of either the glucose or the ribose phosphate results in an increased utilization of the alternate substrate. The utilization of ribose is greatest in the blood samples containing inosine.

Inosine, unlike adenosine, is nontoxic in the animal body. Inosine, therefore, has promise as an adjuvant in the long-term preservation of blood for clinical use.

The transfer of glucose into the red blood cell appears to be mediated by hexokinase. The experimental evidence indicates that the enzyme is located in the cytoplasm of the cell rather than within the membrane. It is suggested, therefore, that the hexokinase is arranged in the cytoplasm at the inner surface of the cell membrane. Evidently glucose can diffuse through the red cell membrane with little difficulty. Having passed through the membrane, the sugar molecule is phos-

phorylated immediately, on contact with hexokinase, and thus enters the cytoplasm as glucose-6-phosphate.

GENERAL SUMMARY

Evidence is presented that hexokinase is present in the rabbit and the human erythrocyte. The enzyme appears to be restricted to the cytoplasmic portion of the red cell.

It is suggested that the hexokinase in the human erythrocyte is arranged in the cytoplasm at the inner surface of the cell membrane. Apparently the transfer of glucose into the red blood cells is mediated by hexokinase.

The activity of hexokinase is dependent upon the concentration of glucose, ATP, Mg^{++} ions and inorganic phosphate ions. The K_{MgATP} is of the order of 1.5×10^{-3}M, and the $K_{glucose} = 2.8 \times 10^{-4}$M. The enzyme is not inhibited by fluoride ions.

Hexokinase is optimally active at pH 7.8-7.9. A second, and smaller optimum of activity occurs at pH 6.0.

The ability of the hexokinase of the rabbit erythrocyte to catalyze the phosphorylation of hexoses, at pH 7.8, may be represented in the following descending order:

glucose > mannose > fructose > galactose.

At pH 6.6 the order is as follows:

mannose > fructose > glucose > galactose.

AMP, ADP and G-6-P inhibit hexokinase.

Lipase, pepsin and taurocholate inactivate the enzyme. It is inferred that hexokinase may be of the nature of a lipoprotein.

The activation energy for the phosphorylation of glucose by hexokinase is of the order of 11,500 cal./mol., both in the case of the SFH and in the intact erythrocyte.

Glucose phosphorylation in the intact erythrocyte occurs at least as rapidly as the transfer of glucose from the external medium into the interior of the cell. The rate of glucose transfer into the red cell, and the activity of hexokinase in the SFH, are diminished in the presence of high concentrations of glucose.

Hexokinase, in the human erythrocyte, is unstable and undergoes a progressive diminution in activity during storage of blood at 4°C., in the CD or ACD medium. The inactivation of the enzyme probably is caused by the fall in the pH of the preserved blood specimens. The addition of nucleosides such as adenosine or inosine to the blood, does not retard the rate of enzyme inactivation, although the nucleosides prolong the ability of the preserved cells to utilize glucose.

After the third or fourth week of storage, the erythrocytes rapidly lose the ability to utilize glucose, in the cold. Apparently, this failure is due to the depletion of the ATP of the cells. The concentration of ATP appears to be of primary importance for the survival of the preserved erythrocyte. However, the hexokinase activity remaining in the pre-

served erythrocyte may determine the capacity of the cells to reconstitute or maintain the endogenous ATP.

The addition of adenosine or inosine to preserved blood samples induces a synthesis of organic phosphate esters, including ATP, in the erythrocyte.

Adenosine is rapidly deaminated in the red blood cells to yield inosine. The latter, in turn, undergoes phosphorolysis to yield hypoxanthine and ribose-1-phosphate. The pentose phosphate then is metabolized to lactic acid, presumably by way of both the pentose phosphate and glycolytic pathways. Guanosine and xanthosine similarly contribute to lactic acid formation and the esterification of phosphate, in the erythrocyte.

The nucleosides thus serve as alternative substrates for the erythrocytes when the ability of the cells to utilize glucose fails.

The pH of the blood samples containing added adenosine tends to increase during the initial few days of storage in the cold, as a result of the liberation of ammonia in the deamination of the nucleoside.

Inosine diminishes, and adenosine increases, the rate of glucose utilization by the erythrocytes during storage in the cold. The effect of inosine may be attributable to the formation of hexose monophosphate in the erythrocytes, in the presence of the nucleoside, and the subsequent inhibition of hexokinase by the phosphate ester. The effect of adenosine

probably is related to the liberation of ammonia resulting from the deamination of the nucleoside. Apparently, a reduction in the rate of utilization of either glucose or ribose phosphate results in an increased utilization of the alternate substrate. The utilization of ribose is greatest in the blood samples containing inosine.

Inosine is free from the harmful effects exhibited by adenosine, when injected into the animal body. Inosine, therefore, appears to be of promise as an adjuvant in the long-term preservation of blood for clinical use.

Adenylate kinase is present in the SFH of the human and the rabbit erythrocyte. The enzyme is relatively stable to heat. At least some of the adenylate kinase of the human erythrocyte is contained in the cell membrane.

Indirect evidence is presented confirming the presence of an ATPase in the stroma of the erythrocyte. The ATPase is inhibited by fluoride.

CLAIMS TO ORIGINALITY

The author considers the following to represent original contributions to knowledge:

(1) The finding that, in the erythrocyte of the human and the rabbit, hexokinase is confined in its distribution to the cytoplasm of the cell. It is inferred that the enzyme is arranged, in an organized manner, at the inner surface of the cell membrane.

(2) The suggestion, based on experimental evidence, that the transfer of glucose into the red blood cell is mediated by hexokinase.

(3) The finding that the activity of hexokinase, in the erythrocyte, is dependent upon the concentration of glucose, ATP, Mg^{++} ions and inorganic phosphate ions, and is sensitive to the pH of the medium. Further, that hexokinase is not inhibited by fluoride.

(4) The finding that the rate at which glucose, mannose, fructose and galactose are phosphorylated in the presence of hexokinase, varies with the sugar, and is influenced by the pH of the medium.

(5) The observation that AMP, ADP and G-6-P inhibit the hexokinase of the erythrocyte. Lipase, pepsin and taurocholate inactivate the enzyme.

(6) The measurement of the activation energy for

the phosphorylation of glucose by hexokinase, which is of the order of 11,500 cal./mol.

(7) The demonstration that the phosphorylation of glucose in the erythrocyte proceeds at least as rapidly as the transfer of glucose from the external medium into the interior of the cells.

(8) The observation that hexokinase in the human erythrocyte is unstable and undergoes a progressive inactivation, during storage of blood at 4°C., in the CD or ACD medium, even in the presence of added nucleosides such as adenosine or inosine. Adenosine sustains the ability of the preserved erythrocyte to utilize glucose, presumably by inducing a synthesis of organic phosphate esters, including ATP, in the cell.

(9) The inference, based on experimental evidence, that the degree of hexokinase activity remaining in the preserved erythrocyte may determine the viability of the cell, by affecting the ability of the erythrocyte to reconstitute or maintain the endogenous ATP.

(10) The finding that adenosine is rapidly deaminated, in blood, to yield inosine which, in turn, undergoes phosphorolysis to yield hypoxanthine and ribose phosphate. The latter is metabolized to lactic acid. Guanosine and xanthosine are similarly metabolized.

(11) The demonstration that inosine diminishes, and adenosine increases the rate of glucose utilization by the erythrocyte during storage of blood in the cold. The utiliz-

ation of ribose is greatest in the blood samples containing inosine.

(12) The observation that the pH of the blood specimens containing added adenosine tends, initially, to increase, as a result of the liberation of ammonia in the deamination of the nucleoside.

(13) The demonstration that inosine is free from the toxic effects produced by adenosine, when injected into the animal body. Inosine, therefore, is to be preferred to adenosine as an adjuvant in the preservation of blood.

(14) The finding that adenylate kinase is present in the human and rabbit erythrocyte, and that, in the human red cell, at least some of the enzyme is present in the cell membrane.

BIBLIOGRAPHY

1. Buchner, H., Munchener med. Wehnschr. 48, 1343 (1897).

2. Garrison, F.H., "An Introduction to the History of Medicine", W.B. Saunders Co., Philadelphia, 4th edition (1929), p. 584; J.S. Fruton and S. Simonds, "General Biochemistry", John Wiley and Sons, Inc., New York (1953), pp. 199-205.

3. Harden, A. and Young, W.J., Proc. Roy. Soc. (London) 32, (Proc. of Nov. 12, 1904) (1905).

4. Kashket, S., M.Sc. Thesis , McGill University (1953).

5. Meyerhof, O., Biochem. Z. 178, 395 (1926).

6. Meyerhof, O., ibid. 183, 176 (1927).

7. Embden, G. and Zimmerman, M., Z. physiol. chem. 167, 114 (1927).

8. Robison, R., Biochem. J. 16, 809 (1922).

9. Meyerhof, O., Biochem. Z. 246, 249 (1932).

10. von Euler, H. and Adler, E., Z. physiol. Chem. 235, 122 (1935).

11. Warburg, O., and Christian, W., Biochem. Z. 254, 438 (1932).

12. Colowick, S.P. and Kalckar, H.M., J. Biol. Chem. 148, 117 (1943).

13. Kalckar, H.M., ibid. 148, 127 (1943).

14. Colowick, S.P., in "The Enzymes", edited by J.B. Sumner and K. Myrback, Academic Press Inc., New York (1951), pp. 114-150.

15. Price, W.H., Cori, C.F. and Colowick, S.P., J. Biol. Chem. 160, 633 (1945).

16. Colowick, S.P., Cori, G.T. and Slein, M.W., ibid. 168, 583 (1947).

17. Gottschalk, A., Adv. Carbohydrate Chem. 5, 49 (1950).

18. Slein, M.W., Cori, G.T. and Cori, C.F., J. Biol. Chem. 186, 763 (1950).

19. Fruton, J.S. and Simmonds, S., "General Biochemistry", John Wiley and Sons, Inc., New York (1953).

20. Sols, A. and Crane, R.K., J. Biol. Chem. 210, 581 (1954).

21. Crane, R.K. and Sols, A., ibid. 210, 597 (1954).

22. Gamble, J.L., Jr. and Najjar, V.A., Science 120, 1023 (1954); J. Biol. Chem. 217, 595 (1955).

23. Meyerhof, O. and Wilson, J.R., Arch. Biochem. 19, 502 (1948).

24. Berger, L., Slein, M.W., Colowick, S.P. and Cori, C.F., J. Gen. Physiol. 29, 379 (1945-46).

25. Eeg-Larsen, N. and Laland, S.G., 8th Scand. Chem. Meeting, Oslo, Norway (1953), cited in Kalckar, H.M. and Klenow, H., Ann. Rev. Biochem. 23, 527 (1954).

26. Vestling, C.S., Mylroie, A.K., Irish,V. and Grant, N.H., J. Biol. Chem. 185, 789 (1950).

27. Mackler, B. and Guest, G.M., Am. J. Physiol. 174, 487 (1953).

28. Hers, H.G., Biochem. Biophys. Acta 8, 416 (1952).

29. Cardini, C.E. and Leloir, L.F., Arch. Biochem. Biophys. 45, 55 (1953).

30. Caputto, R., Leloir, L.F., Cardini, C.E. and Paladini, A.C., J. Biol. Chem. 184, 333 (1950).

31. Long, C., Biochem. J. 50, 407 (1951).

32. Crane, R.K. and Sols, A., J. Biol. Chem. 203, 273 (1953).

33. Saltman, P., ibid. 200, 145 (1953).

34. Kunitz, M. and McDonald, M.R., J. Gen. Physiol. 29,

35. Harpur, R.P. and Quastel, J.H., Nature, London, 164, 693 (1949).

36. Wajzer, J., Compt. rend. Acad. 236, 2116 (1953).

37. Weil-Malherbe, H. and Bone, A.D., Biochem. J. 49, 339 (1951).

38. Ebersole, E.R., Guttentag, C. and Wilson, P.W., Arch. Biochem. 3, 399 (1943-44).

39. Sols, A. and Crane, R.K., J. Biol. Chem. 206, 925 (1954).

40. Fraenkel-Conrat, H., in "Amino Acids and Proteins", edited by D.M. Greenberg, Charles C. Thomas, publisher, Springfield, Ill. (1951), p. 532.

41. Dixon, M. and Needham, D.M., Nature, London, 158, 432 (1946).

42. Bailey, K., Biochem. J. 42, 60 (1948).

43. Griffiths, M., Arch. Biochem. 20, 451 (1949).

44. Lardy, H.A., Wiebelhaus, V.D. and Mann, K.M., J. Biol. Chem. 187, 325 (1950).

45. Broh-Kahn, R.H. and Mirsky, I.A., Science, 106, 148 (1947).

46. Chaikoff, I.L., in "The Harvey Lectures, 1951-1952", Academic Press Inc., New York (1953), pp. 99-125.

47. Cori, C.F. and Cori, G.T., J. Biol. Chem. 94, 581 (1931-32).

48. Krahl, M.E. and Cori, C.F., ibid. 170, 607 (1947).

49. Park, C.R., Phosphorus Metabolism 2, 634 (1952).

50. Park, C.R. and Krahl, M.E., J. Biol. Chem. 181, 247 (1949).

51. Villee, C.A., White, V.K. and Hastings, A.B., ibid. 195, 287 (1952).

52. Beloff-Chain, A., Chain, E.B., Bovet, D., Pocchiari, F., Catanzaro, R. and Longinotti, L., Biochem. J. 54, 529 (1953).

53. Stadie, W.C., Hangaard, N. and Marsh, J.B., J. Biol. Chem. 188, 167 (1951).

54. Stadie, W.C. Hangaard, N. and Vaughn, M., ibid. 199, 729 (1952).

55. Lee, N.D. and Williams, R.H., Endocrinology, 54, 5 (1954).

56. Goldstein, M.S., Henry, W.L., Huddlestun, B. and Levine, R., Am. J. Physiol. 173, 207 (1953).

57. Wick, A.N. and Drury, D.R., ibid. 173, 229 (1953); 174, 445 (1953).

58. Willee, C.A. and Hastings, A.B., J. Biol. Chem. 179, 673 (1949).

59. Bornstein, J., ibid. 205, 513 (1953).

60. Krahl, M.E. and Park, C.R., ibid. 174, 939 (1948).

61. Murlin, J.R., Clough, H.D., Gibbs, C.B.F. and Stokes, A.M., ibid.56, 253 (1923).

62. Sutherland, E.W., Phosphorus Metabolism 2, 577 (1952).

63. McDermott, W.V., Fry, E.G., Brobeck, J.R. and Long, C.N.H., Proc. Soc. Exp. Biol. and Med. 73, 609 (1950).

64. Grossman, M.S., Ryder, C.S. and Pearson, O.H., Fed. Proc. 12, 57 (1953).

65. Weber, G., Allard, C., deLamirande, G. and Cantero, A., Biochem. Biophys. Acta 16, 618 (1955).

66. Luthy, E. and Verzar, F., Biochem. J. 57, 316 (1954).

67. Kornberg, A., J. Biol. Chem. 182, 805 (1950).

68. Bartlett, G.R. and Marlow, A.A., J. App'd. Physiol. 6, 335 (1953).

69. Szekely, M., Manyai, S. and Straub, F.B., Acta Physiol. Hung. 3, 571 (1952).

70. Ponder, E., "Hemolysis and Related Phenomena", Grune and Stratton, New York (1948).

71. Nelson, N., J. Biol. Chem. 153, 375 (1944).

72. Park, J.T. and Johnson, M.J., ibid. 181, 149 (1949).

73. Evelyn, K.A. and Malloy, H.T., ibid. 126, 655 (1938).

74. Meijbaum, W., Z. physiol. Chem. 258, 117 (1939).

75. Friedman, T.E. and Haugen, G.E., J. Biol. Chem. 147, 415 (1943).

76. Barker, S.B. and Summerson, W.H., ibid. 138, 535 (1941), as modified by Lepage, G.A. in "Manometric Techniques", edited by W.W. Umbreit, R.H. Burris and J.F. Stauffer, Burgess Publishing Co., Minneapolis, Minn. (1949), p. 192.

77. Fiske, C.H. and SubbaRow, Y., J. Biol. Chem. 66, 375 (1925).

78. Pappius, H.M., Andreae, S.R., Woodford, V.R. and Denstedt, O.F., Can. J. Biochem. Physiol. 32, 271 (1954).

79. Bartlett, G.R. and Marlow, A.A., J. Lab. Clin. Med. 42, 178 (1953).

80. Clarkson, E.M. and Maizels, M., J. Physiol. 116, 112 (1952).

81. Szekely, M., Manyai, S. and Straub, F.B., Acta Physiol. Hung. 4, 31 (1953).

82. Hevesy, G. and Aten, A.H.W. Jr., Danske Vidensk. Selsk., Biol. Medd. 14, 5 (1939), cited in Mueller, C.B. and Hastings, A.B., J. Biol. Chem. 189, 881 (1951).

83. Jonas, H. and Gourley, D.R.H., Biochem. Biophys. Acta, 14, 335 (1954).

84. Hofstee, B.H.J., J. Biol. Chem. 216, 235 (1955).

85. Liebecq, C., Biochem. J. 54, xxii (1953).

86. Roche, J., ibid. 25, 1724 (1931).

87. Irving, J.T., ibid. 20, 1320 (1926).

88. Spicer, S.S. and Clark, A.M., J. Biol. Chem. 179, 987 (1949).

89. Ebersole, E.R., Guttentag, C. and Wilson, P.W., Arch. Biochem. 3, 399 (1943-44).

90. Francoeur, M., Ph.D. Thesis, McGill University (1953).

91. Paladini, A.C., Caputto, R., Leloir, L.F., Trucco, R.E. and Cardini, C.E., Arch. Biochem. 23, 55 (1949).

92. Beck, W.S., J. Biol. Chem. 216, 333 (1955).

93. Sizer, I.W., Adv. Enz. 3, 35 (1943); J. Biol. Chem. 154, 461 (1944).

94. Davson, H. and Danielli, J.F., "The Permeability of Natural Membranes", the Macmillan Co., New York (1943).

94a. Armstrong, W.D., Singer, L. and Dunshee, B.R., Proc. Exp. Biol. Med. 80, 639 (1952).

95. Rubinstein, D., Kashket, S. and Denstedt, O.F., Can. J. Biochem. Physiol. 34, 61 (1956).

96. Rapoport, S., J. Clin. Invest. 26, 591 (1947).

97. Gabrio, B.W., Hennessey, M., Thomasson, J. and Finch, C.A., J. Biol. Chem. 215, 357 (1955).

98. Gabrio, B.W., Donohue, D.M. and Finch, C.A., J. Clin. Invest. 34, 1509 (1955).

99. Gourley, D.R.H., Arch. Biochem. Biophys. 40, 1 (1952).

100. Rapoport, S. and Luebering, J.L., J. Biol. Chem. 183, 507, (1950).

101. Rapoport, S. and Luebering, J.L., ibid. 189, 683 (1951).

102. Conway, E.J. and Cooke, R., Biochem. J. 33, 457 (1939).

103. Alivisatos, S.G.A., Ph.D. Thesis, McGill University (1952).

104. Kalckar, H.M., J. Biol. Chem. 167, 445 (1947).

105. Gabrio, B.W. and Finch, C.A., J. Clin. Invest. 33, 242 (1954).

106. Maizels, M. and Paterson, J.H., Lancet 239, 417 (1940).

107. Parpart, A.K., Gregg, J.R., Lorenz, P.B. Parpart, E.R. and Chase, A.M., J. Clin. Invest. 26, 641 (1947).

108. Fishman, S., Ph.D. Thesis, McGill University (1953).

109. Allison, A.C. and Burn, G.P., Brit. J. Hematology 1, 291 (1955).

110. Blanchaer, M.C., Williams, H.R. and Weiss, P., Am. J. Physiol. 181, 602 (1955).

111. Blanchaer, M.C. and Weiss, P., J. App'd. Physiol. 7, 168 (1954).

112. Denstedt, O.F., in "Blood Cells and Plasma Proteins", edited by J.L. Tullis, Academic Press, New York (1953).

113. Kalckar, H.M., J. Biol. Chem. 167, 477 (1947).

114. Prankerd, T.A.J. and Altman, K.I., Biochem. J. 58, 522 (1954).

115. Gabrio, B.W. and Huennekens, F.M., Biochem. Biophys. Acta 18, 585 (1955).

116. Dische, Z., Phosphorus Metabolism 1, 171 (1951).

117. Dische, Z. and Pollaczek, E., Resumes des Communications, 11e Congres International de Biochimie (1952), p. 289.

118. Neilands, J.B. and Stumpf, P.K., "Outlines of Enzyme Chemistry", John Wiley and Sons, Inc., New York (1955).

119. Widdas, W.F., J. Physiol. 125, 163 (1954).

120. Wilbrandt, W., Symp. Soc. Exp. Biol. 8, 136 (1954).

121. LeFevre, P.G., ibid. 8, 118 (1954).

122. LeFevre, P.G. and LeFevre, M.E., J. Gen. Physiol. 35, 891 (1952).

123. Bartlett, G.R. and Marlow, A.A., Bull. Scripps Metabolic Clinic 2, No. 5 (1951).

124. van Crevold, S. and Brinkman, R., Proc. Kon. Acad. 23, 1256 (1920).

125. Ponder, E., Blood 9, 227 (1954).

126. Best, J.B., J. Cell. and Comp. Physiol. 46, 29 (1955).

127. Derrick, M.J., Miller, R.E. and Sevag, M.G., Fed. Proc. 12, 196 (1953).