CHEMICAL CHANGES IN STORED BLOOD

by

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Thesis

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TABLE OF CONTENTS

		Page
INTRODUCTION		1
General		1
Historical		2
A.	Studies on cell preservation	3
B.	Studies on chemical changes in stored blood	10
	(a) Glycolysis in blood under physiological conditions	10
	(b) Factors influencing glycolysis in blood	12
	(c) Glycolysis and associated phenomena in stored blood	14
	i. Glucose	14
	ii. Acid soluble phosphates	14
	iii. Potassium	16
	iv. Sodium	18
	v. pH	18
METHODS		20
A.	Collection of the blood	20
B.	Preservative solutions	20
C.	Resuspension of the red cells	21
D.	Solution used for the resuspension of the red cells	21
E.	Dispensing of the blood	22
F.	Storage of the blood samples	23
G.	Washing of the cells	23
H.	Chemical methods	26
	(a) Total acid-soluble phosphate	26
	(b) Partition of organic acid-soluble phosphates	26
	(c) Glucose	29
	(d) Non-fermentable reducing substances	29
	(e) Glycogen	30
	(f) Lactic acid	30
	(g) Pyruvic acid, total keto acids and total hydrazones	30
	(h) Potassium	31
	(i) Survival of erythrocytes after transfusion	31
I.	Calculation of analytical results	31
RESULTS		33
A.	Inorganic phosphate	33
B.	Total acid-soluble phosphate	37
C.	Labile phosphate	41

TABLE OF CONTENTS (Cont'd.)

		Page
D.	Hexosediphosphate	44
E.	Stable phosphate	47
F.	Potassium	52
G.	Glucose	53
H.	Non-fermentable reducing substances	59
I.	Glycogen	59
J.	Lactic acid	60
K.	Pyruvic acid, total keto acids and total hydrazones	61
L.	Survival of erythrocytes after transfusion	64
DISCUSSION		69
	Chemical changes in red cells during storage	69
	Chemical changes in red cells in relation to cell survival	82
SUMMARY		91
BIBLIOGRAPHY		94

INTRODUCTION

General

The investigation to be described in this thesis forms part of a larger research on blood preservation and plasma substitutes that has been carried on in the Department of Biochemistry since the first year of the war with the financial support of the National Research Council. The present study was begun during the fourth year of the war and has been supported largely by a grant from the John and Mary Markle Foundation in New York. The chemical work has been closely integrated with the other aspects of the research and particularly with the studies carried on by Dr. Dorothy Osborne on the survival of preserved blood cells after transfusion, which were partly conducted in collaboration with Harvard University and the Massachusetts Institute of Technology.

The present study was conceived at a time in the evolution of blood preservation when there was little agreement amongst workers on this Continent and in Great Britain on the matter of so-called preservative solutions for the preservation of erythrocytes. Special committees had been formed in the early years of the war under the Research Councils in Canada and the United States to deal with matters pertaining to blood preservation, plasma substitutes and the treatment of shock. This move in enlisting all the research personnel in a collaborative and systematic study in which each research

group was assigned a particular phase of the problem marked a beginning of a real progress in the improvement of blood preservation and the treatment of shock.

Historical

Blood preservation had, as far as one can tell, its inception in the experiments of Weil and of Rous and Turner in the early years of World War I. Weil in 1915 successfully infused citrated blood that had been stored in the cold for several days. Rous and Turner (1916), who were interested in preserving rabbit erythrocytes for serological purposes, made the important observation that the addition of dextrose to citrated rabbit blood greatly improved the preservation of the cells during storage in the cold. Robertson, (1918) toward the end of the war, used the mixture of Rous and Turner for preserving human blood at one of the British Casualty Clearing Stations in France. He stored blood for as long as 22 days in this solution and transfused it into battle casualties with good results. Unfortunately, no attention appears to have been given to Robertson's important demonstration and it remained practically forgotten in the years between the two World Wars. Renewal of interest in blood preservation must be credited to the Russian workers who rediscovered the successful use of preserved blood for transfusion purposes. Interest in the use of preserved blood gradually spread to other countries from Russia, and during the Spanish War preserved blood was used extensively in the treatment of

wounded. The outbreak of World War II then provided a mighty impetus to research on blood preservation.

A. Studies on cell preservation

Within the past few years numerous reports have appeared concerning the influence of various conditions of storage on the preservation of erythrocytes. The influence of the conditions has been judged either from the chemical or physical changes in the stored blood, or from the survival of the stored red cells after transfusion. The results of these studies lead to the belief, which was quite prevalent until a little over a year ago, that cells could be stored for a certain time without suffering any loss of viability, while longer storage would result in a progressive deterioration of the cells. The length of time during which the stored cells would remain as viable as fresh cells was found by many workers to depend greatly upon the nature of the preservative used. Thus it has been reported that cells preserved in citrate solution will survive as well as fresh cells when stored for less than one week (Wiener and Schaefer, 1939, 1940; Mollison and Young, 1941, 1942), while further storage will result in a rapid loss of cell viability. Cells transfused after about three weeks of storage in simple citrate solution were stated to be promptly eliminated from the recipient's circulation within a few hours (Wiener and Schaefer, 1940; Mollison and Young, 1941, 1942).

However, Mollison (1940), Mollison et al. (1940, 1941), Denstedt et al. (1943, 1944) and others have claimed that if

glucose is present in the preservative solution, the cells remain as viable as fresh cells for two to three weeks of storage.

Many factors were believed to influence the preservation of the erythrocytes. The nature of the preservative, the degree of dilution of blood by the preservative, the pH of the solution and the temperature at which the blood was stored have all been suggested to influence the preservation of the blood.

Many preservatives have been advocated on one basis or another, but as yet the only reliable way of determining the relative efficacy of a preservative to retard the loss of cell viability during storage involves the transfusion of stored blood and a study of the rate of elimination of the cells from the recipient's circulation. Such a method was first used by Ashby in 1921 and a modification was later introduced by Schaefer and Wiener (1939). In the Ashby method, group O blood is transfused into a group A recipient. Blood samples are taken from the recipient at intervals and the recipient's cells are selectively agglutinated with group B serum. By this method the donor's cells, which are left free in the sample, can be counted. Or a group B recipient can be employed and the blood samples treated with group A serum. The method of Schaefer and Wiener is similar in principle but in this case, the sub-groups M and N are made use of.

By these methods it is possible to determine the loss of viability of red cells during storage from the percent of the donor's cells which are eliminated within a few hours of transfusion. The rate of subsequent discard can also be followed until practically all the cells have disappeared from the circulation.

The last method, which is more limited in scope, makes use of cells labelled with radioactive iron instead of agglutinogens. The labelled cells are obtained by administering radioactive iron to the prospective donor who utilizes this iron for the formation of haemoglobin. The relative efficacy of different preservatives can be estimated from the proportion of stored cells which are eliminated from the recipient's circulation shortly after transfusion.

The disadvantage of this method is that the studies cannot be carried out for longer than two days as the radioactive iron liberated from the cells which are broken down shortly after transfusion is rapidly incorporated into new cells (Ross and Chapin, 1943). Most of the studies on blood preservation using radioactive iron are to be found in confidential reports of the National Research Councils of Canada, the United States and Great Britain.

Although the results obtained by the radioactive iron and agglutination methods generally agree, they occasionally may differ by as much as 20 percent, with a tendency for the radioactive iron method to show the more rapid rate of destruction of stored cells.

As a means of studying the relative efficacy of preservatives, these methods have many disadvantages. First, they are laborious and time consuming. It has been shown by Ashby (1919) and by Wiener (1934) that the average life span of red cells is about eighty days, but that some may survive for one hundred and thirty days or longer. Therefore, if cell survival after each transfusion of stored blood is to be followed until the time of complete disappearance of the cells from the circulation, it is obvious that this will require about four months in cases where the transfused cells are as viable as fresh cells. Secondly, individual variation in the rate at which the cells are destroyed in the body necessitates a large number of transfusions of blood stored in each preservative before any conclusions can be drawn as to relative merits of the preservatives. Thirdly, these methods for following the rate of disappearance of transfused cells require at least a 200 cc. sample of blood for each transfusion. Therefore, each donation can only be collected in two or three preservatives.

These disadvantages have been appreciated, and various attempts have been made to find some in vitro method of estimating the viability of stored cells. Most investigators have approached this problem by attempting to relate the loss of viability that occurs during storage to some single physical or chemical change in the blood.

Of all the physical changes, the degree of spontaneous haemolysis and alterations in the osmotic fragility of red

cells during storage have been most frequently followed. (Bushby et al., 1940; Maizels and Whittaker, 1940; Denstedt et al., 1941, 1943, 1944; Mollison et al., 1941, 1942; Wurmser et al., 1942; Maizels, 1943-1944; Loutit et al., 1943-1944 and others.) It has been shown that the extent of these changes in stored blood is greatly dependent upon the chemical composition of the preservative used (DeGowin et al., 1939; Dubash et al., 1940; Thistle et al., 1941, and many others), the dilution of the blood with the preservative (Denstedt et al., 1941, 1944), the pH of the solution (Cotter and MacNeal, 1938; Maizels and Whittaker, 1940; Wurmser et al., 1942; Loutit et al., 1943-1944) and the temperature of storage (Thistle et al., 1941).

Maizels and Whittaker on the basis of their studies concluded, however, that the extent of haemolysis in stored blood cannot serve as a guide to the viability of the red cells. Maizels (1941,1943-1944) also found that there was lack of agreement between the various in vitro tests. Mollison and Young (1941 and 1942) and Denstedt et al.(1943) present data on both in vitro and in vivo studies on stored blood which demonstrate that neither the degree of spontaneous haemolysis nor the changes in the osmotic fragility bears any constant relation to the capacity of the cells to survive in vivo. Loutit et al. (1943-1944) have also found that the osmotic fragility of stored cells provides a very fallacious guide to their state of preservation. Gibson (1946) on the other hand, still maintains that the results of the fragility tests agree with the results of the radioactive iron method of studying the efficacy

of various preservatives with respect to indicating the proportion of non-viable cells in a sample of stored blood.

In point of fact, these two in vitro tests have little experimental basis. Most workers have assumed that cells become progressively less resistant to hypotonic solutions with age. The experimental results of Cruz et al. (1941) are diametrically opposed to such a concept. These workers have found, using radioactive iron to label the cells, that the very new blood cells of dogs are the least resistant to hypotonic salt solutions, that this difference disappears after three to four days, but that the very old cells (130 days) are more resistant to hypotonic saline than the circulating cells of a mean age group.

At present, the in vitro methods described above are in a state of disrepute. The determination of the extent of haemolysis in a preserved sample, however, has a certain negative value in the study of blood preservation, as preservatives which allow rapid haemolysis will obviously be of little value.

Attempts to relate the loss of cell viability to a specific chemical change in the blood, red cells or plasma have so far proved abortive. DeGowin et al. (1939, 1940), Mainwaring et al. (1940), Loutit et al. (1943-1944) and others have found that the extent of the loss of potassium from red cells during storage bears no relation to the efficacy of the preservative used to retard the loss of cell viability. Although Aylward et al. (1940) have shown that

glucose decreases the rate of hydrolysis of organic acid-soluble phosphates in the red cells, preservation of the organic phosphates does not appear to be necessary for the maintenance of cell viability. Loutit (1943-44) has shown that while decreasing the pH of the preservative solutions to as low as pH 5 has no deleterious effect on cell preservation, it will increase the rate of liberation of inorganic phosphate. Maizels (1943-44) also has found that while diluents causing a rapid disappearance of easily hydrolysable phosphate (i.e. adenosine triphosphate) are associated with poor cell survival in vivo, persistence of this phosphate is not necessarily associated with good survival after transfusion.

According to Loutit et al. (1943-1944) the extent of glycolysis cannot be correlated with the capacity of the erythrocytes to survive in vivo after transfusion. However, it must be noted that these workers centrifuged the blood for one hour before analyses were carried out on the red cells and it is possible that a considerable amount of glycolysis might have occurred during this period.

On the whole, relatively few efforts have been made to find some in vitro method of evaluating the extent of corpuscular deterioration, and still fewer where both in vitro and in vivo studies have been conducted together. Furthermore, these studies have been by no means exhaustive in scope.

B. Studies on chemical changes in stored blood

On the other hand, there have been a number of studies on the nature and rate of autolytic changes in stored blood which have not been directly concerned with relating these to the loss of viability of the stored cells. Changes in plasma proteins, the stability of haemoglobin, the formation of ammonia, etc. have all formed the basis of one or more studies. However, by far the most extensive chemical studies on stored blood have been concerned with compounds associated with the glycolytic processes. Before proceeding to discuss glycolysis in stored blood, an outline of this metabolic process in blood studied under physiological conditions might be pertinent.

(a) Glycolysis in blood under physiological conditions

Glycolysis, which is defined as the enzymatic breakdown of glucose to lactic acid, has been reported to occur only in the formed elements of blood (Lépine, 1890; Rona and Döblin, 1911; von Noörden, 1912; Rona and Arnheim, 1913; MacLeod, 1913 and others), although Picado (1940) and Rick (1939) claim that it also occurs in plasma. According to Dische (1934, 1935, 1937, 1946), who is one of the leading workers in this field, glucose is phosphorylated in the presence of adenosine triphosphate and Mg to hexosephosphate, which in turn is converted to triose phosphate. The latter then undergoes the following reaction:

Triose phosphate + pyruvate → phosphoglycerate + lactate

This scheme is essentially similar to that elaborated by Meyerhof, Embden and others for muscle glycolysis, However, there is one respect in which muscle and red cell metabolism apparently differ, viz, that in most mammalian red cells only 2,3-diphosphoglycerate is found (Greenwald, 1925, Jost, 1927), while only the 1, 3 isomer has been demonstrated in muscle. Guest and Rapoport (1941, 1942) have suggested that in the red cells of animals which normally contain much 2,3-diphosphoglycerate (this usually accounts for about half of the acid-soluble phosphate), the labile 1,3 isomer is rapidly converted to the stable 2,3 form. There is, however, no direct evidence as yet that 2,3 diphosphoglycerate participates in the glycolytic cycle.

It has been suggested that glycolysis may have various important functions in the red cell. First, it is necessary to maintain the ester phosphates which Guest and Rapoport (1939, 1941a) believe are essential in the maintenance of ionic equilibria in the blood. It has been postulated also (Rapoport and Guest, 1939,1939b; Guest and Rapoport,1938) that these esters may serve as carriers of phosphorus to the tissues. Furthermore, there is considerable evidence that the glycolytic processes have an important role in the maintenance of the normal concentration gradients of cations between plasma and red cells (Danowski, 1941; Harris 1941).

(b) Factors influencing glycolysis in blood

A number of factors have been found to influence glycolysis in blood. The effect of temperature has long been appreciated. Arthus (1891), Bose et al. (1942) and others have observed a retardation of glycolysis at low temperatures.

The removal of glucose from blood is also decreased by lowering the pH, (Martland, 1925, 1929; Roche and Roche, 1927; Wurmser et al., 1942), and accelerated when alkali is added. Martland (1925) has reported that glycolysis practically ceases in blood with a pH below 7.29. Guest and Rapoport (1941) attribute this phenomenon to a decreased phosphorylation of glucose at a low pH.

During glycolysis, synthesis and hydrolysis of organic phosphates occur simultaneously, so that the inorganic phosphate content of the red cells remains low (Lawaczeck, 1924; Guest and Rapoport, 1941). At a low pH however, where glycolysis is inhibited, hydrolysis of organic acid-soluble phosphates predominates over synthesis so that there is a fall in the concentration of the organic phosphates and an accumulation of inorganic phosphate (Rapoport and Guest, 1939). It has been demonstrated that processes which accelerate glycolysis all cause an esterification (although it may be temporary) of inorganic phosphate, while processes which retard glycolysis all result in an immediate release of inorganic phosphate (Rona and Doeblin, 1911; Lawaczeck, 1924; Martland et al., 1924; Rona and Iwasaki, 1927; Engelhart and Braunstein, 1928; Roche

and Roche, 1929; and others).

Guest (1932) who summarizes much of the earlier work also demonstrated the retarding action of glucose on the dephosphorylation of organic acid-soluble phosphates in blood.

It has been generally stated that haemolysis leads to an inhibition of glycolysis (Rona and Doeblin, 1911; Rona and Arnheim, 1913; Barrenscheen and Braun, 1930). However, these workers have usually used water to haemolyze the cells. Edelmann (1912)and MacLeod (1913) have reported that laking blood with saponin does not destroy glycolytic powers, and Solomon et al. (1940) have observed that some breakdown of phosphoric esters does occur at 37°C. in a blood haemolysate produced by successive thawings and freezings.

Various chemical agents have been found to inhibit glycolysis in blood. Fluoride and monoiodoacetic acid have both been shown to retard glycolysis (Barrenscheen and Braun, 1930; Bueding and Wortis, 1940; Bueding and Goldfarb, 1941; Long, 1944; and others). The retarding action of oxalate has been observed by MacLeod,(1913); Long,(1944); Bueding and Goodhart, (1941) and others. Lohmann and Meyerhof, (1934) have observed that in muscle oxalate inhibits the dephosphorylation of phosphopyruvate. They also report that citrate in high concentrations inhibits both the dephosphorylation of phosphopyruvate and the conversion of phosphoglycerate to phosphopyruvate.

(c) Glycolysis and associated phenomena in stored blood

(i) Glucose - There are but a few reports in the literature on the behaviour of glucose in blood during storage. Denstedt et al. (1941) and Bick (1939) have shown that the glucose content of blood stored in citrate decreases during storage and that it disappears during the second or third week. The figures presented by DeGowin (1939) indicate that the glucose content of the plasma of citrated blood falls to zero during the second week of storage. In the plasma of blood stored in a glucose-containing preservative, a fall, often irregular, can be noted during thirty-five days of storage. A decrease in the glucose content of stored plasma has been reported by Bick (1939). Although Loutit et al. (1943-44) have stated that the pH of the stored blood does not have a marked effect on the removal of glucose, Thurmser et al. (1942) have found that at a low pH (6.0) glycolysis in blood stored for ten days is retarded.

(ii) Acid soluble phosphates - An increase in the inorganic phosphate content of blood during storage has been observed by Aylward et al. (1940); Maizels and Whittaker (1940); Maizels (1941b); Loutit (1943-1944) and others. Aylward et al. have found that the presence of glucose in the preservative retards the breakdown of organic acid-soluble phosphates (which occurs mainly in the red cells), and thus the increase in inorganic phosphate. This has been confirmed by Maizels (1941b, 1943-1944) and others. Maizels (1943-1944)

has demonstrated that the addition of sodium fluoride to the preservative results in a decreased breakdown of slowly hydrolysable phosphate and an increased breakdown of hydrolysable phosphate (adenosine triphosphate). The fall in hydrolysable phosphate can be attributed to the fact that, while this compound continues to be hydrolysed in the presence of sodium fluoride, formation of phosphopyruvate, which is required for the rephosphorylation of adenylic acid, is inhibited.

The effect of pH on the breakdown of organic acid-scluble phosphates has been studied by Maizels (1943-44), who showed that in stored blood at an alkaline reaction there was a decrease in inorganic phosphate during the first week of storage, while in acid systems, an increase occurred, indicating hydrolysis of organic phosphates. However, although there is a rapid decrease in total organic acid-scluble phosphate at a low pH, hydrolysable phosphate shows a better preservation at a slightly acid reaction. Maizels has suggested that this might be due to a decreased activity of adenosine triphosphatase in acid solutions. Loutit (1943-1944) has also demonstrated a rapid increase in inorganic phosphate in blood stored at a low pH.

In spite of the large increase in inorganic phosphate that has been found to occur mainly in the red cells, there is only slow diffusion of this substance into the plasma during storage at low temperatures (Maizels and Whittaker, 1940; Maizels, 1943-1944), although diffusion of inorganic

phosphate across the red cell membrane occurs very quickly at room temperature (Halpern, 1936). Similarly, when phosphate is added to the preservative solution, the increase in total acid-soluble phosphate in the red cell during storage is also slow. However, Maizels (1943-1944) finds that the ingress of phosphate is considerably more rapid than the egress in stored cells.

Aylward et al. (1940) have observed that the total acid-soluble phosphate content of blood remains constant (within the experimental error) although it decreases in the red cell; this indicates that there is no considerable liberation of inorganic phosphate from phospholipids in stored blood (see also Maizels and Whittaker, 1940.)

(iii) Potassium - Under physiological conditions, there is only a very slow exchange of potassium between the cells and the serum (Eisenman et al., 1940; Dean, 1941). The concentration of this cation in the red cells is about twenty times as great as in the plasma. During storage, however, there is a loss of potassium from the red cells, (Dulière, 1931a, 1931b; Jeanneney et al., 1938a, 1938b; DeGowin et al., 1939, 1940; Scudder et al., 1939, 1940, 1941; Denstedt, 1941; Muether and Andrews, 1941a, 1941b; Loutit, 1943-1944; Maizels, 1943-1944 and a number of others.) The rate of loss was found to be greatest during the first five to ten days or so of storage and then to become progressively slower.

Harris (1941) has shown that this loss does not represent a deterioration of the cell, as it starts immediately, and he attributes it to a reduction at the low temperature of storage of metabolic activities essential to the maintenance of a normal concentration gradient of potassium. In proof of this, he has shown that raising the temperature of the stored cells to 37°C. leads to an accumulation of the potassium by the cells. In this connection it may be stated that Downman et al. (1940) have found that the loss of potassium from the cells is greater when blood is stored at 2-4°C. than at room temperature. According to Harris, the metabolic activity required for the maintenance of a normal distribution of potassium between cells and plasma is not glycolysis per se, as depletion of glucose is not followed by an immediate loss of potassium. However, the accumulation of potassium by stored cells on incubation is greater and more prolonged if the cells are actively breaking down sugar.

DeGowin et al.,(1940); Scudder, (1939); Maizels, (1943-1944) and others have not found that the presence of glucose in the preservative retards the loss of cell potassium. Scudder et al., (1939) have stated that this loss is independent of the anticoagulant and takes place in blood stored under oil without admixture with a preservative.

Jeanneney and Servantie, (1939); Maizels, (1943-1944) and Loutit, (1943-1944) have shown that the rate of potassium leakage from the red cells decreases as the pH of the blood is lowered.

(iv) Sodium - The increase in sodium content of the red cells during storage which has been observed by Jeanneney, Servantie and Ringenbach, (1939); Maizels and Whittaker, (1940b); Scudder et al., (1941); Maizels,(1943-1944) and many others, has also been attributed by Harris to the reduction of the metabolic activities of cells stored at low temperatures. He states that the exchange of sodium and potassium during storage is not necessarily equivalent and depends to a large extent on the concentration of these ions in the extra-cellular medium. He also believes that the metabolic activities controlling sodium and potassium distribution are not necessarily the same.

(v) pH - A decrease in the pH of plasma and whole blood during storage has been reported by DeGowin, (1939); Maizels and Whittaker, (1940); Scudder and Smith, (1940); Maizels, (1943-1944) and others. Maizels and Whittaker state that the pH of stored blood is between 7.8 and 7.4 (sometimes more acid) after two weeks of storage in citrate, and 0.2 to 0.6 pH units lower in blood stored in citrate-glucose. Mollison and Young, (1942) found similar, although somewhat lower results. Their studies also indicate that the rate of decrease is most rapid during the first two to three weeks of storage. From the figures presented by DeGowin on the pH changes in the plasma of blood stored for thirty-five days, frequent fluctuations can be observed.

It has been suggested that the greater acidity of blood stored in citrate-glucose solutions may be due to the fact

that in the case of simple citrate solutions, the glucose present in the blood rapidly disappears so lactic acid formation is limited, whilst in the presence of additional glucose, glycolysis may proceed for longer intervals and thus more lactic acid be produced.

At the time this study was undertaken, there were a number of questions concerning the preservation of blood that required to be clarified. A few of these are summarized in the following sentences.

(a) What is the nature of the physical and chemical changes that occur in the red blood cells during storage?

(b) In what manner are these changes influenced by the conditions of storage?

(c) Do all the stored red cells undergo the same type and rate of change?

(d) To what change or changes in the cell contents or membrane can the loss of viability be attributed?

(e) Is there any chemical change in stored red cells that bears a quantitative relation to the loss of viability of the cells?

(f) Can the autolytic changes be reversed when the stored red cells are returned to a physiological environment?

The present study represents an attempt to find an answer to these questions. In view of the importance of glucose in cell preservation, autolytic changes in compounds associated with the glycolytic processes in the red cell have formed the main subject of investigation.

METHODS

A. Collection of the blood

The blood was collected from normal, human subjects under aseptic conditions. The mouth of the vessel in which the blood was received was covered with cellophane containing a hole just sufficient in diameter to allow insertion of the bleeding tube. In this way the blood was collected in a nearly closed system.

B. Preservative solutions

The composition of the preservatives and the proportion of blood to preservative used in these studies are given below.

(a) Citrate:

Trisodium citrate: 3.2% solution

5 parts blood: 2.5 parts citrate solution

(b) Citrate-glucose (McGill):

Trisodium citrate: 3.2% solution

Glucose: 5.0% solution

McGill (1): 5 parts blood: 1 part citrate solution: 1.5 parts glucose solution

McGill (2): 5 parts blood: 2/3 parts citrate solution: 1 part glucose solution

(c) Disodium citrate-glucose (Loutit, Hollison and Young):

Disodium citrate: 2% solution

Glucose: 15% solution

42 parts blood: 10 parts citrate solution: 2 parts glucose solution. Disodium citrate is prepared by mixing 588.24 gm. of tri-sodium citrate with 210.11 gm. of citric acid.

(d) Citrate-glucose-sodium chloride (Alsever and Ainslie):

Trisodium citrate:	8.76 gm.
Glucose:	18.66 gm.
Sodium chloride:	4.18 gm.

Mix and add water to give one litre of solution: 1 part blood: 1 part solution.

(e) Citrate-glucose-phosphate (McGill):

Trisodium citrate:	3.2% solution
Glucose:	5.0% solution
Monobasic sodium phosphate:	1.72 gm.)
Sodium hydroxide:	0.46 gm.) dilute to 100 cc.

McGill (1): 5 parts blood: 0.75 parts citrate solution: 1 part glucose solution: 0.75 parts phosphate solution:

McGill (2): 5 parts blood: 1 part citrate solution: 1 part glucose solution: 0.5 parts phosphate solution:

(f) Citrate-glucose-phosphate (Muether):

Trisodium citrate:	0.42%
Glucose:	4.68%
Monobasic sodium phosphate:	0.1033%
Dibasic sodium phosphate:	0.1716%

C. Resuspension of the red cells

When the cells were to be resuspended, the blood was collected in citrate or citrate-glucose solution and centrifuged for thirty minutes. After removal of the plasma, the cells were made up to the original volume with the resuspending solution.

D. Solutions used for the resuspension of the red cells

(a) Citrate

(b) Citrate-glucose (McGill 1)
(c) Citrate-glucose (McGill 2)
(d) Disodium citrate-glucose (Loutit, Mollison and Young)
(e) Citrate-glucose-phosphate (McGill 1)
(f) Citrate-glucose-phosphate (McGill 2)
(g) Citrate-glucose-phosphate (Muether)

These solutions have the same composition as those described above.

(h) Sodium chloride:0.9% sodium chloride solution
(i) Sodium chloride-glucose:0.5 parts 0.9% sodium chloride solution: 2 parts 5% glucose solution
(j) Sodium chloride-citrate-glucose: 0.5 parts 0.9% sodium chloride solution: 1 part 3.2% trisodium citrate solution: 1 part 5% glucose solution
(k) Corn syrup: 10% corn syrup solution
(l) Citrate-corn syrup: 1 part 3.2% trisodium citrate solution: 1.5 parts 10% corn syrup solution

Except in the case of the disodium citrate-glucose preservative, the glucose solutions were autoclaved separately and added to the sterile citrate solutions prior to the collection of the blood. By this means, caramelization of the glucose during autoclaving was prevented. The pH of the disodium citrate-glucose preservative was sufficiently low so that the glucose and citrate solutions could be autoclaved together without extensive discoloration.

Citrate-glucose (McGill 1) was the preservative most frequently used in these studies, and unless specified, the term citrate-glucose will refer to this preservative in the subsequent pages.

E. Dispensing of the blood

Before the blood was dispensed, the cells were thoroughly resuspended by gently swirling the blood for three to five minutes. The blood was then dispensed into small serological

test tubes which were immediately stoppered, covered with sterile paper and placed in the refrigerator.

Throughout all the manipulations, sterile precautions were observed.

F. Storage of the blood samples

The blood was stored at 5°C. (±1°C). The tubes were inverted once each day to resuspend the cells. In the one case where the blood was kept in a flask, resuspension was effected by careful swirling of the sample.

G. Washing of the cells

At intervals, usually every third or fourth day, a tube of blood was centrifuged immediately after removal from the refrigerator, the plasma discarded, and the cells washed twice with ice-cold, hypertonic (12%) sucrose. The entire procedure took about 20 minutes to complete. The plasma-free cell residue was then made up to the original volume with distilled water, and chemical analysis of the haemolysate was carried out without delay.

To determine whether there was any loss of the cell constituents during washing, a preliminary study was carried out in which the cell glucose, lactic acid, pyruvic acid, total hydrazones, total acid-soluble phosphate and potassium were estimated by two methods: (a) the direct method whereby the analyses were carried out on the washed and haemolysed cells, and (b) the indirect method whereby the composition of the cell was estimated from data on whole blood, plasma and the haematocrit.

The red cell values obtained by the two methods are tabulated in Table I and with the exception of the total hydrazone values, show good agreement. In this way, it was possible to establish that no considerable loss of total acid-soluble phosphate, glucose, lactic acid, pyruvic acid or potassium occurred during the washing procedure. The cause of the increase in total hydrazones that was observed in washed cells is obscure and will be investigated.

TABLE I

A Comparison between the results of chemical analysis of various constituents of the red cell by two methods

		Direct (mg. percent)	Indirect (mg. percent)
(a) Total acid-soluble phosphate	(1)	13.5	13.4
	(2)	14.4	14.7
	(3)	13.3	13.3
(b) Glucose	(1)	28.8	27.1
	(2)	20.0	19.8
	(3)	55.8	57.8
(c) Lactic acid	(1)	20.0	21.2
	(2)	25.9	27.1
(d) Pyruvic acid		0.26	0.25
(e) Total hydrazones	(1)	2.83	0.72
	(2)	2.26	1.36
(f) Potassium	(1)	313	294
	(2)	413	432
	(3)	239	239

A loss of potassium from the red cells during suspension in a non-electrolyte medium has been reported by several investigators (Davson, 1939; Kurnick, 1941; and

others). To determine the rate of loss, the potassium content of the cell during resuspension in cold.hypertonic sucrose was followed for seventy-five minutes.

TABLE II

The potassium content of red cells during suspension in cold, hypertonic sucrose

Time (minutes)	Potassium (mg.per 100 cc.haemolysate)
15	104
45	100
75	91

When these values are plotted and the line connecting them is extrapolated back to zero time, it can be seen that the loss of potassium during the first fifteen minutes (which corresponds to the time required for washing of the cells) is less than 1 mg. percent.

As none of the substances studied was found to escape to to any appreciable extent from the red cells during the washing with cold, hypertonic sucrose, the direct method of analysis was used in this work. This method is simpler, involves fewer analyses than the indirect method and is hence less subject to error. Furthermore, this method can be applied to partially haemolysed blood, whereas the indirect can not. This latter advantage is of particular importance as it allowed a continuation of the studies on the chemical changes in stored blood past the time when haemolysis commenced.

H. Chemical methods

(a) Total acid-soluble phosphate - Total acid-soluble phosphate in the red cells was determined by the method of Warweg and Stearns (1933).

One cc. of 10N H_2SO_4 was added to 1 cc. of a 1:4 trichloroacetic acid filtrate in a large Pyrex test tube, and the solution digested to a pale amber colour. After permitting the specimen to cool slightly, a drop of 30 percent hydrogen peroxide was added and heating continued for several minutes to complete the oxidation. The tube was then cooled, and the contents diluted to 20 cc. with distilled water. The estimation of the phosphate content was carried out by the method of Fiske and Subbarow (1925), using an Evelyn colorimeter and allowing ten minutes for the colour to develop.

(b) Partition of the organic acid-soluble phosphates - The analytical procedure for the phosphate partition was based on the different rates of hydrolysis of the organic acid-soluble phosphates by mineral acids. This method was devised by Lohmann in 1928(1928) and first applied to blood by Bomskov(1932). While such a procedure does not allow an accurate estimation of the various phosphate fractions as no one of these is hydrolysed specifically during any of the more or less arbitrarily chosen periods, one can obtain a fair indication of the changes in the different fractions by such a method.

It has been shown that within seven minutes hydrolysis

in N HCl or H_2SO_4 at 100°C. two of the three phosphate groups of adenosine triphosphate are completely liberated as inorganic phosphate. On prolongation of this hydrolysis to one hundred minutes, Needham (1937) has found that the hexosediphosphate is converted to hexosemonophosphate with the liberation of one of the two phosphate groups as inorganic phosphate. For hydrolysis of both phosphate groups of commercial hexosediphosphate, Warweg and Stearns (1936) have shown that nine hours are required, but that hydrolysis proceeds most rapidly during the first two hours when about sixty per cent of the organic phosphate is released. Diphosphoglycerate has been shown to be very resistent to hydrolysis (Greenwald, 1925; Jost, 1927; Warweg and Stearns, 1936). Warweg and Stearns, using phosphoglycerate isolated from pig's blood by the method described by Greenwald for the isolation of 2,3 diphosphoglycerate, observed only a 4.8 percent hydrolysis of this compound within two hours of heating at 100°C. in N H_2SO_4. They also showed that hydrolysis of non-phosphoglycerate organic acid-soluble phosphates continues in blood filtrates for twelve hours.

The following procedure for the partition of organic acid-soluble phosphates was adopted in this work. One part of the haemolysate was added to four parts of cold 5% trichloroacetic acid, stirred and centrifuged. Two cc. of 2N HCl were added to each of the four 2 cc. aliquots of supernatant fluid and the tubes placed in boiling water. After thirty

seconds, they were tightly stoppered. Two of the tubes were removed after seven minutes hydrolysis, and the other two after one hundred minutes hydrolysis, and placed immediately in ice water. After cooling, 1 cc. of N HCl was added to each tube to bring the volume to 5 cc. The inorganic phosphate content was estimated by the method of Fiske and Subbarow (1925).

The phosphate liberated on seven minutes hydrolysis represents mainly two-thirds of the phosphate present in adenosinetriphosphate. This fraction will be designated as labile phosphate. The phosphate liberated between seven and one hundred minutes hydrolysis is derived mainly from one phosphate group of hexosediphosphate and will be referred to as HDP-phosphate. The remaining organic-acid soluble phosphate is composed mainly of diphosphoglycerate, but also contains hexosemonophosphate, some adenylic acid and possibly other compounds. This fraction will be referred to as stable phosphate in the subsequent pages.

Barrenschen and Braun (1930) have observed a rapid liberation of inorganic phosphate from easily-hydrolysable phosphate in haemolysed blood. It was important to determine in this study whether any considerable hydrolysis occurred during the five minute (or less) interval between the time when the cells were haemolysed and the time the proteins were precipitated. A comparison of the inorganic phosphate content of red cells as determined by both the direct method and indirect method (which does not involve haemolysis) is shown in Table III.

TABLE III

The extent of hydrolysis of organic acid-soluble phosphates in a blood haemolysate during five minutes

Inorganic phosphate (mg.percent)	
Direct method	Indirect method
2.14	2.08
6.22	6.12

It is apparent from these results that the extent of hydrolysis of organic acid-soluble phosphates during washing and haemolysis is very small.

(c) Glucose - Glucose was determined by Somogyi's modification (1937) of the method of Schaffer and Somogyi. The proteins of the haemolysate were precipitated using ten parts of copper sulphate (0.7 percent) to one part of haemolysate to four parts of sodium tungstate (10 percent).

(d) Non-fermentable reducing substances - Yeast cells were washed five times using four parts of water to one part of yeast. An aliquot of the protein-free filtrate from the haemolysate was added to the same volume of packed, washed yeast cells, and the latter were suspended by stirring. After standing for fifteen minutes at room temperature, the sample was centrifuged and the reducing substances in the supernatant fluid determined. A control determination of the non-fermentable reducing substances in yeast, using water instead of the filtrate, was carried out at the same time. The difference between the two values indicates the amount of non-fermentable reducing substances in the filtrate.

(e) Glycogen - To 1 cc. of haemolysate in a centrifuge tube was added a similar volume of 60 percent potassium hydroxide. The tube was placed in a boiling water bath until the contents were clear (about fifteen minutes were required). It was then removed, cooled, and the glycogen in the protein-free solution precipitated with 90 percent ethanol. After standing overnight in the refrigerator, the precipitate was washed twice with 60 percent, and once with 90 percent, ethanol. After removing most of the alcohol by aspiration, the remaining traces were dissipated by placing the tube in hot water for several minutes. Two cc. of N HCl were blown on the precipitate, and the glycogen hydrolysed in a boiling water bath for three hours. After the hydrolysate was cooled and neutralized to phenol red, it was filtered, and the glucose content of the filtrate determined.

(f) Lactic acid - Lactic acid was determined by the Koenmann modification (1940) of the method of Miller and Muntz.

(g) Pyruvic acid, total keto acids and "Total hydrazones" - These substances were determined by the method of Friedemann and Haugen (1943). The total hydrazones are the substances which react with 1,4-dinitrophenylhydrazine within five minutes under the conditions of the procedure. By subtracting the values obtained for pyruvic acid from the total hydrazones, an estimation of the concentration of reactive, neutral, keto compounds in blood (aldehydes, ketones, trioses) can be obtained.

(h) Potassium - Potassium was estimated by Wood's colorimetric adaptation (1942) of the method of Harris.

(i) Survival of erythrocytes after transfusion - Red cell survival time after transfusion was followed by a modification of the serological method of Ashby (1919) using A,B, and O blood types, and by the method of Wiener and Schaefer (1939) using the M and N blood types. These studies were carried out by Dr. Dorothy Osborne.

I. Calculation of analytical results

For purpose of uniformity, all the analytical values in this study were calculated to a base of 10 grams of haemoglobin per 100 cc. Immediately after the cells were washed and haemolysed, a haemoglobin estimation was carried out. If, for example, the haemoglobin content of the specimen was found to be x grams per 100 cc., the analytical values for potassium, glucose, etc. were multiplied by the factor $\frac{10}{x}$ so that the values obtained in all analyses could be compared. In this way, such factors as the number of cells in the sample and the degree of swelling or haemolysis undergone by the cells during storage are taken into account. It is well established from previous studies in this laboratory that the haemoglobin in red cells stored at 5°C. remains stable for longer then ten weeks (Denstedt et al., 1941).

Plasma and whole blood values were also recalculated on this basis. In the case of plasma, the analytical values were multiplied by the same factor as was used for the whole blood.

It has been found in these studies that a haemoglobin value

of 10 grams per 100 cc. of blood corresponds to a haematocrit value of about 35 - 37 percent, or in other words, to a red cell volume of about one-third of the total volume of the sample. The analytical values, although determined as the concentration per 100 cc. of haemolysate, and not per 100 cc. of red cells, will be referred to in the following pages as cell values for the sake of simplicity.

RESULTS

The results of these studies are presented in the following tables. In each experiment, aliquots of blood obtained from one donor were stored in different preservatives. The symbols used for the preservatives in the tables are as follows:

Symbol	Preservative
C	citrate
CG(1)	citrate glucose (McGill 1)
CG(2)	citrate glucose (McGill 2)
CGP(1)	citrate glucose phosphate (McGill 1)
CGP(2)	citrate glucose phosphate (McGill 2)
CGP(M)	citrate glucose phosphate (Muether)
CGN(A&A)	citrate glucose sodium chloride (Alsever and Ainslie)
L	disodium citrate glucose (Loutit)
	Solutions for resuspension of cells
N	sodium chloride
NG	sodium chloride glucose
NCG	sodium chloride glucose citrate
Cs	corn syrup
CsC	corn syrup citrate

In some cases, cells were resuspended in the preservatives.

A. Inorganic phosphate

An increase in the inorganic phosphate content of red cells during storage was observed in all experiments (Table IV). When cells were preserved in the absence of glucose or in Loutit's or Alsever and Ainslie's solutions, a rapid rise in inorganic phosphate was always apparent within the first nine days (and usually within the first week) of storage.

In several cases, e.g., experiment A (citrate), this increase continued for about three weeks when the inorganic phosphate made up 80 to 85 percent of the total acid-soluble phosphate in the red cells, after which a fall in concentration occurred. This fall was found to be due to the diffusion of the inorganic phosphate out of the cells. When cells were stored in other phosphate-free preservatives, the increase in inorganic phosphate was not marked until the tenth day or later, after which the rate of increase was accelerated (although it was always less rapid than in the cases previously described). Cells stored in any phosphate-containing preservative showed a steady increase from less than one milligram percent at the time the blood was collected to about ten to seventeen mg. percent after four weeks of storage.

TABLE IV

The effect of various preservatives on the behaviour of inorganic phosphate in red cells during storage

INORGANIC PHOSPHATE
(as milligrams of phosphorus per 100 cc. of haemolysate)

Experiment	A			B					C	
Preservative	C	CG (1)	CGP (1)	C	CG (1)	CGP (1)	L	CGP (M)	CG (1)	CGP (1)
Days of storage										
0	0.21	0.21	1.66	-	-	-	-	-	-	-
1	0.21	0.25	0.96	-	-	-	-	-	0.20	1.95
2	-	-	-	0.38	0.46	1.19	1.65	3.78	0.28	1.95
3	0.40	0.27	1.65	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	-	-	-	1.62	0.34	1.72	2.41	3.91	-	-
6	-	-	-	-	-	-	-	-	0.55	2.08
7	1.62	0.55	2.39	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	0.87	3.26
10	4.78	0.86	3.36	5.16	1.08	3.33	4.47	4.94	-	-
11	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	2.14	5.42
14	6.12	1.73	6.66	8.64	1.52	5.97	5.55	6.81	-	-
15	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	2.98	9.49
17	5.95	2.51	5.39	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-
19	-	-	-	10.6	3.06	9.30	6.99	8.55	-	-
20	10.4	6.35	12.9	-	-	-	-	-	6.22	12.5
21	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-
23	9.5	7.23	13.8	-	4.37	11.9	9.81	9.98	7.11	13.3
24	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-
26	-	-	-	10.5	6.64	15.3	6.97	11.7	7.84	16.4
27	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	8.64	17.4
30	-	-	-	8.67	7.13	15.4	8.03	12.0	-	-
31	8.1	7.49	16.2	-	-	-	-	-	-	-

TABLE IV (Cont'd.)

The effect of various preservatives on the behaviour of inorganic phosphate in red cells during storage

INORGANIC PHOSPHATE
(as milligrams of phosphorus per 100 cc. of haemolysate)

Experiment	D											
Preservative	C	CG (1)	CG (2)	CGP (1)	CGP (2)	L	CGP (M)	CGN (A&A)	CGP[x] (M)	N[x]	CGN[x] (A&A)	Cs[x]
Days of storage												
0	-	0.38	-	0.72	0.44	-	-	0.38	-	0.38	0.30	-
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
3	0.24	-	0.31	0.94	0.64	0.48	1.42	-	5.37	1.13	0.64	0.28
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
6	3.80	-	-	-	-	-	-	0.86	5.44	-	1.41	-
7	-	-	-	-	-	-	-	-	-	-	-	-
8	-	0.42	0.44	1.88	1.16	0.84	2.76	-	-	-	-	-
9	5.36	-	-	-	-	-	-	1.67	-	-	2.03	-
10	-	0.51	-	2.55	1.82	1.25	3.48	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-
13	8.58	-	-	-	-	-	-	3.35	7.91	9.99	4.09	2.50
14	-	1.06	1.14	4.72	3.48	2.52	5.14	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	4.79	8.40	10.2	4.48	3.68
17	-	2.34	1.85	7.27	5.26	5.41	6.89	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-	-	-
20	10.4	-	-	-	-	-	-	6.08	9.90	9.94	6.65	5.45
21	-	2.81	4.51	11.4	9.73	7.41	8.13	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	-	-	-	-
27	-	6.41	-	14.3	-	8.07	10.8	7.37	11.6	-	7.16	6.77
28	-	-	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-	-	-
31	-	-	-	-	-	-	-	-	-	-	-	-

x Resuspended cells.

B. Total acid-soluble phosphate

From the results presented in Table V it can be seen that in most experiments, a decrease in the total acid-soluble phosphate content of the red cells was apparent after a month of storage in a phosphate-free preservative. In some cases, this fall was preceded by a rise in the total acid-soluble phosphate during the first two weeks, e.g. experiment A (citrate); in others the total phosphate was maintained at a fairly constant level for about the first three weeks of storage, e.g. experiment D (Alsever and Ainslie). In still others, e.g. experiment B (Loutit) a fall was encountered after the first few days and finally in some cases, no appreciable change in concentration was observed after a month of storage, e.g., experiment C (citrate-glucose). The extent of the decrease in total acid-soluble phosphate in the red cells varied from blood to blood. Thus in experiment A (citrate-glucose) there was about a fifteen percent loss during thirty-one days of storage, while in experiment C (citrate-glucose) only a very small change in the total acid-soluble phosphate content of the red cells was observed after twenty-nine days. There was always a greater loss of total phosphate from cells preserved in Loutit's solution than from cells in ordinary citrate-glucose solution. With cells preserved in other solutions, the relative loss in different preservatives was not constant.

A progressive increase in total acid-soluble phosphate during storage was always observed in cells preserved in any phosphate-containing solution. The extent of the increase varied from about 55 to 122 percent with the greater increases in concentration generally being observed in resuspended cells.

TABLE V

The effect of various preservatives on the behaviour of total acid-soluble phosphate in the red cells during storage

TOTAL ACID-SOLUBLE PHOSPHATE
(as milligrams of phosphorus per 100 cc. of haemolysate)

Experiment	A			B					C	
Preservative	C	CG (1)	CGP (1)	C	CG (1)	CGP (1)	L	CGP (M)	CG (1)	CGP (1)
Days of storage										
0	12.8	12.6	14.3	13.9	13.7	14.8	13.1	18.5	-	-
1	13.4	14.5	15.4	-	-	-	-	-	13.5	15.1
2	-	-	-	13.4	13.4	16.3	13.9	20.4	13.9	15.1
3	13.0	12.3	17.5	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	-	-	-	14.6	-	16.4	11.3	23.4	-	-
6	-	-	-	-	-	-	-	-	13.5	16.8
7	13.9	14.2	19.0	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	13.8	18.8
10	14.2	14.2	17.3	12.9	12.5	15.5	11.9	20.4	-	-
11	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	14.4	19.9
14	13.35	13.6	20.5	13.4	12.3	18.7	11.7	27.4	-	-
15	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	13.7	20.0
17	12.3	12.9	18.4	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-
19	-	-	-	14.5	13.6	19.5	12.1	27.1	-	-
20	12.6	12.9	21.8	-	-	-	-	-	13.3	21.8
21	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-
23	11.96	11.8	20.7	-	13.0	19.2	11.4	29.7	13.3	20.6
24	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-
26	-	-	-	12.4	13.4	23.0	11.4	33.3	13.4	22.8
27	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	13.2	22.5
30	-	-	-	11.7	12.4	21.7	10.6	27.2	-	-
31	9.74	10.6	21.5	-	-	-	-	-	-	-

TABLE V (Cont'd.)

The effect of various preservatives on the behaviour of total acid-soluble phosphate in the red cells during storage

TOTAL ACID-SOLUBLE PHOSPHATE
(as milligrams of phosphorus per 100 cc. of haemolysate)

Experiment	D											
Preservative	C	CG (1)	CG (2)	CGP (1)	CGP (2)	L	CGP (M)	CGN (A&A)	CGP^{x} (M)	N^{x}	CGN^{x} (A&A)	Cs^{x}
Days of storage												
0	13.0	13.9	13.7	-	-	-	18.4	13.6	20.6	14.8	13.0	14.2
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
3	13.0	13.9	13.4	-	-	-	-	13.6	20.4	14.6	12.8	13.9
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
6	13.0	-	-	-	-	-	-	13.6	26.0	15.1	12.0	14.2
7	-	-	-	-	-	-	-	-	-	-	-	-
8	-	13.9	13.6	16.6	15.4	12.7	21.6	-	-	-	-	-
9	12.6	-	-	-	-	-	-	13.8	28.4	14.8	-	-
10	-	13.9	-	17.8	15.9	12.1	21.0	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-
13	12.8	-	-	-	-	-	-	13.1	27.8	12.8	12.1	13.4
14	-	13.9	13.7	17.4	16.4	13.0	24.1	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	12.7	28.4	12.2	12.0	12.4
17	-	13.9	-	18.0	17.1	12.6	26.5	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-	-	-
20	12.7	-	-	-	-	-	-	13.0	31.6	11.8	21.1	12.1
21	-	13.5	-	21.1	17.3	13.1	27.7	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	12.5	27.7	13.3	32.1	11.3	-	-
25	-	-	-	-	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	-	-	-	-
27	-	13.5	-	20.8	-	11.7	29.1	12.1	32.1	-	11.6	13.0
28	-	-	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-	-	-
31	-	-	-	-	-	-	-	-	-	-	-	-

x Resuspended cells.

TABLE V (Cont'd.)

The effect of various preservatives on the behaviour of total acid-soluble phosphate in the red cells during storage

TOTAL ACID-SOLUBLE PHOSPHATE
(as milligrams of phosphorus per 100 cc. of haemolysate)

Experiment	E									F			
Preservative / Days of storage	G	CG (1)	CGP (1)	CGP (M)	N	NG	NGC	CsC	Cs	CG (1)	NCG	CGN (A&A)	CGP (M)
0	14.6	13.6	17.0	16.5	14.0	13.4	13.4	13.6	13.7	13.3	12.8	12.2	16.0
1	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-
3	13.6	13.9	20.5	18.4	14.2	14.0	13.7	-	14.1	-	-	-	-
4	-	-	-	-	-	-	-	-	-	13.8	14.1	12.1	18.4
5	-	-	-	-	-	-	-	-	-	-	-	-	-
6	13.8	13.6	22.0	18.0	13.9	13.2	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-
9	13.5	14.2	24.3	22.4	14.2	13.4	13.7	14.1	14.1	-	-	-	-
10	-	-	-	-	-	-	-	-	-	13.6	12.9	13.3	25.4
11	-	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-
13	14.1	14.2	25.0	22.5	13.7	-	14.2	13.6	12.7	-	-	-	-
14	-	-	-	-	-	-	-	-	-	12.5	14.1	12.9	25.4
15	-	-	-	-	-	-	-	-	-	-	-	-	-
16	12.8	15.5	26.8	26.0	13.3	13.8	14.0	13.3	12.6	-	-	-	-
17	-	-	-	-	-	-	-	-	-	13.6	14.2	12.8	26.5
18	-	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-	-	-	-
20	12.4	12.8	29.6	-	12.4	-	13.1	12.9	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-	-
24	13.3	-	20.5	24.8	12.8	13.2	14.0	12.2	12.2	-	-	-	-
25	-	-	-	-	-	-	-	-	-	13.2	13.5	13.0	28.6
26	-	-	-	-	-	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-	-	-	-
29	14.6	-	-	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-	-	-	-
31	-	-	-	-	-	-	-	-	-	-	-	-	-
32	-	-	-	-	-	-	-	-	-	-	-	-	-
33	-	-	-	-	-	-	-	-	-	-	-	-	-
34	-	-	-	-	-	-	-	-	-	-	-	-	-
35	-	-	-	-	-	-	-	-	-	-	-	-	-
36	-	-	-	-	-	-	-	-	-	-	-	-	-
37	-	11.7	30.8	23.6	13.0	-	12.0	10.9	10.4	-	-	-	-

C. Labile phosphate

The labile phosphate is derived mainly from the two pyrophosphate groups of adenosine triphosphate. During storage it tended to decrease slowly and irregularly (Table VI) except in cells stored in Muether's solution. The fall in labile phosphate was found to proceed more rapidly in cells preserved in the absence of glucose, although in experiment A (citrate) a temporary increase in this fraction was observed between the tenth and fourteenth days of storage, and during this period the labile phosphate content exceeded that found in cells in citrate-glucose solution. The results with Muether's solution indicate a synthesis of labile phosphate during storage. In experiment B the synthesis of labile phosphate in cells in Muether's solution continued during the first ten days of storage; in experiment D, an increase in labile phosphate was apparent until the twentieth day. Occasionally in these studies, an increase in concentration was found towards the end of storage, e.g., experiment B (Loutit). Cells stored at a low pH (i.e. cells in Loutit's solution) maintained their labile phosphate content as well as cells stored in less acid solutions such as citrate-glucose (McGill 1).

TABLE VI

The effect of various preservatives on the behaviour of labile organic acid-scluble phosphates in red cells during storage

LABILE ORGANIC ACID-SOLUBLE PHOSPHATE
(as milligrams of phosphorus per 100 cc. of haemolysate)

Experiment	A			B					C	
Preservative	C	CG (1)	CGP (1)	C	CG (1)	CGP (1)	L	CGP (M)	CG (1)	CGP (1)
Days of storage										
0	2.53	2.36	0.80	-	-	-	-	-	-	-
1	2.70	2.25	3.22	-	-	-	-	-	2.60	2.52
2	-	-	-	2.17	2.06	2.75	2.65	3.34	2.10	-
3	2.09	2.09	2.38	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	-	-	-	1.52	2.24	2.76	2.63	4.06	-	-
6	-	-	-	-	-	-	-	-	1.88	2.58
7	1.57	2.23	3.12	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	1.67	2.32
10	2.20	2.12	3.01	0.81	2.70	2.46	2.36	4.27	-	-
11	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	0.90	1.47
14	3.73	1.73	2.54	0.37	1.21	1.40	1.84	4.11	-	-
15	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	1.60	1.82
17	2.14	0.57	3.71	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-
19	-	-	-	0.40	1.10	2.00	1.19	4.03	-	-
20	0.56	1.16	1.73	-	-	-	-	-	0.67	1.93
21	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-
23	0.54	0.81	1.67	-	1.12	1.03	0.31	4.21	0.50	1.25
24	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-
26	-	-	-	0.31	0.84	0.61	2.16	3.84	0.92	1.82
27	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	0.72	0.50
30	-	-	-	0.56	1.06	1.71	1.90	3.30	-	-
31	1.05	1.76	0.86	-	-	-	-	-	-	-

TABLE VI (Cont'd.)

The effect of various preservatives on the behaviour of labile organic acid-soluble phosphates in red cells during storage

LABILE ORGANIC ACID-SOLUBLE PHOSPHATE
(as milligrams of phosphorus per 100 cc. of haemolysate)

Experiment	D											
Preservative	C	CG (1)	CG (2)	CGP (1)	CGP (2)	L	CGP (M)	CGN (A&A)	CGP^{x} (M)	N^{x}	CGN^{x} (A & A)	Cs^{x}
Days of storage												
0	2.49	2.42	1.52	2.87	2.93	1.61	2.93	2.46	2.95	2.25	2.44	2.08
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	3.09	3.10	-	-	2.62	3.93	2.13	1.87	2.36
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
6	2.12	-	-	-	-	-	-	2.10	4.80	1.40	-	3.50
7	-	-	-	-	-	-	-	-	-	-	-	-
8	-	1.98	-	2.50	2.60	2.30	3.90	-	-	-	-	-
9	-	-	-	-	-	-	-	1.84	4.57	1.20	1.23	0.54
10	-	2.03	2.06	2.10	2.46	1.57	4.07	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-
13	0.72	1.81	-	1.85	1.75	-	2.67	1.53	3.80	0.62	1.54	1.17
14	-	-	-	-	-	1.44	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-	-
16	0.88	1.38	-	-	-	-	-	1.81	4.62	0.85	1.33	1.19
17	-	-	1.89	1.95	1.40	1.24	3.67	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	1.66	4.90	-	1.80	0.91
21	-	1.12	1.46	0.65	0.48	1.22	3.60	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-
24	0.05	-	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	-	-	-	-
27	-	0.60	-	1.06	-	0.99	3.15	1.43	3.50	-	1.29	1.25
28	-	-	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-	-	-
31	-	-	-	-	-	-	-	-	-	-	-	-

x Resuspended cells.

D. Hexosediphosphate

The inorganic phosphate liberated between seven and one hundred minutes hydrolysis at 100°C. in N HCl is derived mainly from one phosphate group of hexosediphosphate. For the sake of simplicity, this phosphate will be referred to as hexosediphosphate. This fraction showed a tendency to decrease during storage except in cells stored in Muether's solution in which an appreciable synthesis occurred during the early periods of storage (Table VII). An increase in hexosediphosphate was also observed in cells in citrate-glucose-phosphate, and occasionally in other preservatives during the first few days of storage, but in no case was this synthesis as prolonged or as extensive as that observed in cells stored in Muether's solution. The most rapid fall in concentration was generally found in cells stored in the absence of glucose and in Loutit's solution. Usually, although not always, the hexosephosphate and labile phosphate tended to show a roughly parallel behaviour during storage.

TABLE VII

The effect of various preservatives on the behaviour of hexosediphosphate in red cells during storage

HEXOSEDIPHOSPHATE
(as milligrams of phosphorus per 100 cc. of haemolysate)

Experiment	A			B					C	
Preservative	C	CG (1)	CGP (1)	C	CG (1)	CGP (1)	L	CGP (M)	CG (1)	CGP (1)
Days of storage										
0	2.94	2.66	4.50	-	-	-	-	-	-	-
1	2.66	3.79	3.43	-	-	-	-	-	1.80	1.62
2	-	-	-	2.98	2.97	4.25	1.94	5.58	3.21	-
3	2.93	3.80	3.45	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	-	-	-	1.99	2.55	4.00	1.10	5.21	-	-
6	-	-	-	-	-	-	-	-	3.39	3.41
7	2.33	3.36	3.21	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	2.29	2.93
10	1.19	2.84	2.60	1.11	2.56	2.55	1.92	4.51	-	-
11	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	1.77	2.40
14	0.73	1.79	1.85	1.09	1.82	2.08	0.66	-	-	-
15	-	-	-	-	-	-	-	-	2.04	1.59
16	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-
19	-	-	-	0.88	1.77	1.83	1.81	4.37	-	-
20	1.65	1.40	2.28	-	-	-	-	-	1.31	1.80
21	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-
23	0.43	0.91	-	1.63	1.26	1.83	1.76	4.05	1.36	1.15
24	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-
26	-	-	-	1.70	2.35	1.00	1.80	4.90	1.91	1.64
27	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	1.12	1.50
30	-	-	-	0.98	1.30	2.57	1.70	4.13	-	-
31	-	0.93	0.72	-	-	-	-	-	-	-

TABLE VII (Cont'd.)

The effect of various preservatives on the behaviour of hexosediphosphate in red cells during storage

HEXOSEDIPHOSPHATE
(as milligrams of phosphorus per 100 cc. of haemolysate)

Experiment	D											
Preservative	C	CG (1)	CG (2)	CGP (1)	CGP (2)	L	CGP (M)	CGN (A&A)	CGP^{x} (M)	N^{x}	CGN^{x} (A&A)	Cs^{x}
Days of storage												
0	2.38	3.60	2.28	3.26	3.37	2.57	3.50	2.53	1.32	2.99	3.08	3.85
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	3.95	3.87	1.98	-	3.24	-	-	2.87	2.63
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
6	2.83	-	-	-	-	-	-	2.30	0.56	1.80	-	3.10
7	-	-	-	-	-	-	-	-	-	-	-	-
8	-	3.30	3.09	3.50	3.70	1.70	5.0	-	-	-	-	-
9	-	-	-	-	-	-	-	2.0	-	-	1.54	1.11
10	-	3.26	3.60	3.60	3.20	1.98	5.15	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-
13	1.82	-	-	-	-	-	-	1.70	6.54	1.21	1.63	1.74
14	-	2.77	-	2.64	3.12	1.55	7.38	-	-	-	-	-
15	1.19	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	5.56	1.09	1.39	1.82
17	-	-	3.61	1.26	1.85	1.27	5.0	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	--	-	-	-	-	-	-
20	-	-	-	-	-	-	-	1.57	5.50	-	1.28	1.20
21	-	1.84	-	1.50	1.70	0.87	3.9	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-
24	1.19	-	1.53	-	-	0.61	4.52	1.21	-	0.66	-	-
25	-	-	-	-	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	-	-	-	-
27	-	2.33	-	1.85	-	0.99	4.28	1.53	4.40	-	1.09	1.60
28	-	-	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-	-	-
31	-	-	-	-	-	-	-	-	-	-	-	-

x Resuspended cells.

E. Stable phosphate

The stable phosphate is derived mainly from diphosphoglycerate. The results which are presented in Table VIII indicate that the behaviour of stable phosphate in red cells during storage is dependent upon the preservative used. In cells preserved in the absence of glucose, the stable phosphate was observed to fall abruptly sometime during the first ten days of storage (in all cases except one, this fall was observed during the first week). In cells stored in Loutit's or Alsever and Ainslie's solution an early fall was also apparent. When cells were stored in citrate-glucose (McGill 1) or citrate-glucose-phosphate (McGill 1) solutions this fall did not occur until the third or fourth week of storage. Often a small increase in stable phosphate was observed during the first few days. It can be observed from Table VIII that in experiments A and B where the citrate preservative was used, the stable phosphate completely disappeared from the cells by the twentieth and twenty-sixth days of storage respectively, and then reappeared to the extent of about one and one-half milligrams per 100 cc. of haemolysate. In cells stored in citrate-glucose or citrate-glucose-phosphate (McGill 1) solutions there was approximately a seventy percent decrease in stable phosphate in the red cell after about a month of storage (except in experiment D, where only about a fifty percent fall occurred). Cells preserved in Alsever and Ainslie's and Loutit's solutions showed about a ten percent greater loss during this period.

The addition of phosphate to the preservative resulted in an increase in stable phosphate. This increase was of short duration in cells preserved in citrate-glucose-phosphate (McGill 1) solution, and a fall was observed at the same time as in cells in citrate-glucose. When the cells were preserved in Muether's solutions, however, this rise in stable phosphate was more prolonged. In experiment E, for instance, an increase in stable phosphate during the first twenty-six days of storage was observed, after which a fall was noted.

Studies on resuspended cells revealed similar results for cells resuspended in Muether's solution, but less consistent results in the other cases. In experiment E, for example, the values for stable phosphate in cells resuspended in phosphate-free solutions show a downward trend from the beginning of storage, while in experiment F, the cells resuspended in citrate-glucose solution showed no decrease in stable phosphate content within a period of twenty-five days of storage.

TABLE VIII

The effect of various preservatives on the behaviour of stable organic acid-soluble phosphate in red cells during storage

STABLE ORGANIC ACID-SOLUBLE PHOSPHATE
(as milligrams of phosphorus per 100 cc. of haemolysate)

Experiment	A			B					C	
Preservative	C	CG (1)	CGP (1)	C	CG (1)	CGP (1)	L	CGP (M)	CG (1)	CGP (1)
Days of storage										
0	7.1	7.4	6.3	9.2	8.5	8.4	6.5	8.0	-	-
1	7.8	8.2	7.8	-	-	-	-	-	8.8	8.7
2	-	-	-	7.9	7.9	8.1	7.8	7.7	8.3	-
3	7.6	6.1	9.0	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	-	-	-	9.5	-	8.8	5.2	10.2	-	-
6	-	-	-	-	-	-	-	-	8.1	8.7
7	8.4	8.1	10.3	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	8.9	10.2
10	6.1	8.4	8.3	4.8	7.3	-	3.2	-	-	-
11	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	8.9	10.6
14	2.6	8.4	9.5	3.3	7.7	9.2	3.7	9.5	-	-
15	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	7.6	7.1
17	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-
19	-	-	-	2.7	7.7	6.7	2.1	10.2	-	-
20	0.0	4.0	4.9	-	-	-	-	-	5.0	5.6
21	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-
23	1.4	2.8	-	-	6.2	4.4	.57	11.4	4.3	4.9
24	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-
26	-	-	-	0.0	3.6	6.0	-	12.8	2.6	2.9
27	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	2.6	2.8
30	-	-	-	1.5	2.9	2.0	1.0	7.8	-	-
31	-	0.48	2.1	-	-	-	-	-	-	-

TABLE VIII (Cont'd.)

The effect of various preservatives on the behaviour of stable organic acid-soluble phosphate in red cells during storage

STABLE ORGANIC ACID-SOLUBLE PHOSPHATE
(as milligrams of phosphorus per 100 cc. of haemolysate)

Experiment	D											
Preservative	C	CG (1)	CG (2)	CGP (1)	CGP (2)	L	CGP (M)	CGN (A&A)	CGP^{x} (M)	N^{x}	CGN^{x} (A&A)	Cs^{x}
Days of storage												
0	7.7	7.5	-	-	-	9.2	9.6	8.2	10.3	9.2	7.2	7.8
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
3	7.8	8.2	-	-	-	7.1	-	-	10.0	9.2	8.4	8.3
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
6	6.6	-	-	-	-	-	-	8.3	10.2	4.5	7.9	7.4
7	-	-	-	-	-	-	-	-	-	-	-	-
8	-	8.2	7.6	8.7	7.9	8.5	9.9	-	-	-	-	-
9	4.7	-	-	-	-	-	-	8.3	11.3	4.9	5.3	8.5
10	-	8.0	7.5	8.8	8.4	7.3	8.3	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-
13	2.1	-	-	-	-	-	-	6.5	9.5	0.9	4.8	5.7
14	-	8.0	-	8.2	8.0	6.4	8.0	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	8.0	1.6	4.3	4.5
17	-	8.4	7.1	7.4	8.5	4.6	10.9	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-	-	-
20	1.1	-	-	-	-	-	-	3.7	11.3	-	2.4	-
21	-	7.7	-	7.6	5.4	3.7	12.1	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-
24	-	-	5.9	-	-	3.2	11.2	3.7	-	1.1	-	-
25	-	-	-	-	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	-	-	-	-
27	4.1	-	3.6	-	-	1.6	10.1	1.6	12.3	-	2.1	2.5
28	-	-	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-	-	-
31	-	-	-	-	-	-	-	-	-	-	-	-

x Resuspended cells.

TABLE VIII (Cont'd.)

The effect of various preservatives on the behaviour of stable organic acid-soluble phosphate in red cells during storage

STABLE ORGANIC ACID-SOLUBLE PHOSPHATE
(as milligrams of phosphorus per 100 cc. of haemolysate)

Experiment	E									F			
Preservative	G[x]	CG[x] (1)	CGF[x] (1)	CGP[x] (M)	N[x]	NG[x]	NGC[x]	CsC[x]	Cs[x]	CG[x] (1)	NCG[x]	CGN[x] (A&A)	CGP[x] (M)
Days of storage													
0	-	8.2	7.6	8.1	8.7	8.4	8.3	7.8	8.2	7.9	7.6	7.3	7.7
1	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-
3	7.3	7.5	7.6	7.4	7.8	7.7	7.2	-	7.9	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	8.1	7.8	7.1
5	-	-	-	-	-	-	-	-	-	-	-	-	-
6	7.6	7.7	8.7	7.8	6.9	7.1	6.9	7.3	6.8	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-
9	6.1	7.0	8.0	9.0	5.2	6.0	6.8	7.1	6.8	-	-	-	-
10	-	-	-	-	-	-	-	-	-	8.5	7.4	7.7	9.5
11	-	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-
13	7.0	7.0	7.2	8.0	2.7	-	6.6	6.4	6.5	-	-	-	-
14	-	-	-	-	-	-	-	-	-	7.4	8.6	5.3	9.8
15	-	-	-	-	-	-	-	-	-	-	-	-	-
16	5.4	6.2	6.4	9.1	-	6.3	6.3	5.9	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	8.4	8.3	3.9	10.7
18	-	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-	-	-	-
20	4.1	4.8	8.1	-	0.9	-	-	5.8	5.0	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-	-
24	4.7	4.6	12.2	8.6	1.2	-	6.0	3.4	4.3	-	-	-	-
25	-	-	-	-	-	-	-	-	-	8.6	6.1	1.7	9.5
26	-	-	-	-	-	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-	-	-	-
31	-	-	-	-	-	-	-	-	-	-	-	-	-

x Resuspended cells.

F. Potassium

The results presented in Table IX show that in all cases there was a loss of potassium from the red cells during storage. The rate of loss was most rapid during the first two weeks and then decreased. After the third week, there was little change in the potassium content of the cells. The extent of the loss varied with the preservative used, being greatest in cells stored in citrate solution, and least in cells in Muether's and Loutit's solutions.

TABLE IX

The potassium content of red cells during storage

POTASSIUM
(millimoles per litre of haemolysate)

Experiment	A			B					C	
Preservative	C	CG	CGP (1)	C	CG	CGP (1)	L	CGP (M)	CG	CGP (1)
Days of storage										
0	117	104	109	109	119	114	110	114	-	-
1	97	98	109	-	-	-	-	-	117	118
2	-	-	-	82	88	82	100	114	105	94
3	90	83	96	-	-	-	-	-	-	-
5	-	-	-	64	-	64	105	108	-	-
6	-	-	-	-	-	-	-	-	80	88
7	78	84	93	-	-	-	-	-	-	-
9	7	-	-	0	-	-	-	-	67	-
10	68	77	89	42	85	57	85	102	-	-
14	62	73	79	41	45	62	86	91	-	-
16	-	-	-	-	-	-	-	-	61	67
17	40	75	83	-	-	-	-	-	-	-
19	-	-	-	40	42	58	79	82	-	-
20	45	73	82	-	-	-	-	-	-	54
23	21	-	68	-	40	-	69	80	57	72
26	-	-	-	32	41	51	68	82	-	-
30	-	-	-	30	41	50	63	80	-	-
31	21	62	73	-	-	-	-	-	-	-

G. Glucose

From Table X it is obvious that the glucose content of the red cell underwent marked fluctuations during storage. In Figure 1, where the values are presented graphically, the periodic nature of the fluctuations is apparent. In general, there was a fall in cell glucose during the first few days of storage, followed by an increase which reached a maximum value sometime between the fifth and tenth days. A rapid fall and subsequent rise in cell glucose was then apparent, with a second peak being attained between the twentieth and twenty-sixth days of storage. In many cases the second peak was higher than the first. There was an indication of another rise in several instances which suggested that a third peak might have been observed if the studies had been prolonged. Figure 2, in which the peak values are plotted against the time of storage, summarizes these time relations in graphical form. In several experiments, a rapid increase in cell glucose was found during the first day of storage.

There were only a few cases where the fluctuations in the glucose content of the red cell did not conform to the pattern outlined above. In cells stored in citrate alone, the first peak was found on the second or third day of storage.

In experiment A (citrate) and Experiment E, although a second rise in cell glucose was found, this was prolonged to the end of the experimental period. In experiments B (Muether) and G (citrate-glucose), no preliminary fall in

glucose was observed. In the latter case, an additional peak was found on day 19, which was not noted in any other experiment.

Fluctuations in the glucose content of cells were also observed in experiment J where the blood was stored in a flask and samples removed aseptically at intervals for analysis. This indicates that the fluctuations cannot be attributed to a difference in behaviour of glucose in blood stored in individual tubes.

The magnitude of the fluctuations can be seen to bear a relation to the concentration of glucose in the red cell, both being greatest in cells stored in Muether's solution, and smallest in cells stored in citrate.

The results of studies on whole blood and plasma (Table X) indicate that the fluctuations were not limited to the red cells. In one experiment (K) where the glucose content of whole blood preserved in citrate was followed, it can be noted that after the glucose had completely disappeared for a period of more than one week, it suddenly reappeared in appreciable amounts.

To compare the relative magnitude of the fluctuations observed in whole blood and plasma with those found in an equal volume of red cells, it is necessary to multiply the "cell glucose" values by three, as these were obtained from a haemolysate containing about one-third cell matter. When this was done in experiments G and H where the glucose content of whole blood, plasma and red cells were determined simultaneously, it was seen that the changes in glucose concentration are comparable in extent, although somewhat smaller in the cells than in the plasma.

TABLE X

The effect of various preservatives on the behaviour of glucose in whole blood, plasma, and red cells during storage

GLUCOSE
(as millimoles per litre of plasma, whole blood or haemolysate)

Experiment	A[x]			B[x]					C[x]	
Preservative	C	CG	CGP (1)	C	CG	CGP (1)	L	CGP (M)	CG	CGP (1)
Days of storage										
0	0.34	1.38	0.69	0.11	7.32	3.44	2.32	10.7	-	-
1	0.00	0.61	0.11	-	-	-	-	-	5.16	3.16
2	-	-	-	0.27	4.44	2.45	1.44	14.7	4.94	2.11
3	0.59	0.48	1.00	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	-	-	-	0.11	5.06	3.45	1.22	21.2	-	-
6	-	-	-	-	-	-	-	-	3.97	2.58
7	0.19	0.43	1.33	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	7.78	3.38
10	0.09	2.22	2.40	0.00	3.50	2.45	1.58	21.5	-	-
11	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	7.66	2.58
14	0.14	0.33	0.43	0.00	3.10	1.07	1.14	13.8	-	-
15	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	5.17	2.66
17	0.00	1.02	0.73	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-
19	-	-	-	0.00	2.84	1.50	0.54	19.3	-	-
20	0.00	2.66	0.85	-	-	-	-	-	6.39	3.72
21	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-
23	0.09	1.25	0.81	0.98	2.74	1.61	0.00	16.0	9.11	6.33
24	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-
26	-	-	-	0.00	6.50	3.82	1.33	25.9	-	-
27	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	9.00	2.67
30	-	-	-	0.00	4.10	2.67	1.30	13.9	-	-
31	0.19	3.00	1.39	-	-	-	-	-	-	-
32	-	-	-	-	-	-	-	-	-	-
33	-	-	-	-	-	-	-	-	-	-
34	-	-	-	-	-	-	-	-	-	-
35	-	-	-	-	-	-	-	-	-	-

x Red cells.

TABLE X (Cont'd.)

The effect of various preservatives on the behaviour of glucose in whole blood, plasma, and red cells during storage

GLUCOSE
(millimoles per litre of plasma, whole blood or haemolysate)

Experiment	G^{x}	H^{x}	I^{x}	J^{x}	G^{xx}	G^{xxx}	H^{xx}	H^{xxx}	I^{xx}
Preservative	CG	CG	CG	CG	CG	CG	CG	CG	C
Days of storage									
0	-	-	2.54	7.37	-	-	-	-	-
1	9.75	11.16	8.00	9.62	56.1	61.4	61.3	71.2	-
2	-	-	4.55	6.25	-	-	-	-	3.11
3	-	-	-	-	-	-	-	-	-
4	-	4.18	-	5.34	-	-	39.9	40.8	-
5	-	-	7.00	-	-	-	-	-	-
6	12.35	-	6.78	-	45.6	48.4	-	-	-
7	11.28	-	6.22	10.36	49.5	32.2	-	-	-
8	-	8.73	5.82	-	-	-	52.7	61.1	0.0
9	7.84	-	5.48	9.92	46.5	56.1	-	-	-
10	-	-	-	6.83	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-
12	-	4.40	5.37	-	-	-	34.2	41.7	0.0
13	8.15	-	5.09	-	46.1	51.0	-	-	-
14	-	-	4.73	6.78	-	-	-	-	-
15	-	3.83	-	-	-	-	42.1	26.7	-
16	8.89	-	5.08	7.81	40.9	48.2	-	-	-
17	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-
19	-	6.19	-	-	-	-	44.8	49.5	-
20	9.73	-	4.93	-	41.3	48.0	-	-	-
21	-	-	5.15	10.90	-	-	-	-	2.11
22	-	-	6.23	-	-	-	-	-	-
23	7.56	6.73	4.97	11.35	31.6	36.1	49.4	49.9	-
24	-	-	-	-	-	-	-	-	-
25	-	-	-	9.89	-	-	-	-	1.01
26	5.78	-	6.06	-	38.3	44.1	-	-	-
27	-	7.65	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-
29	-	8.95	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-
31	-	-	-	-	-	-	-	-	-
32	-	-	-	-	-	-	-	-	-
33	-	12.30	-	-	-	-	-	-	-
34	-	-	-	-	-	-	-	-	-
35	6.36	-	-	-	27.0	27.0	-	-	-

x Red cells xx Whole blood xxx Plasma

Figure 1.

Fluctuations in the glucose content of red cells during storage in various preservatives[x]

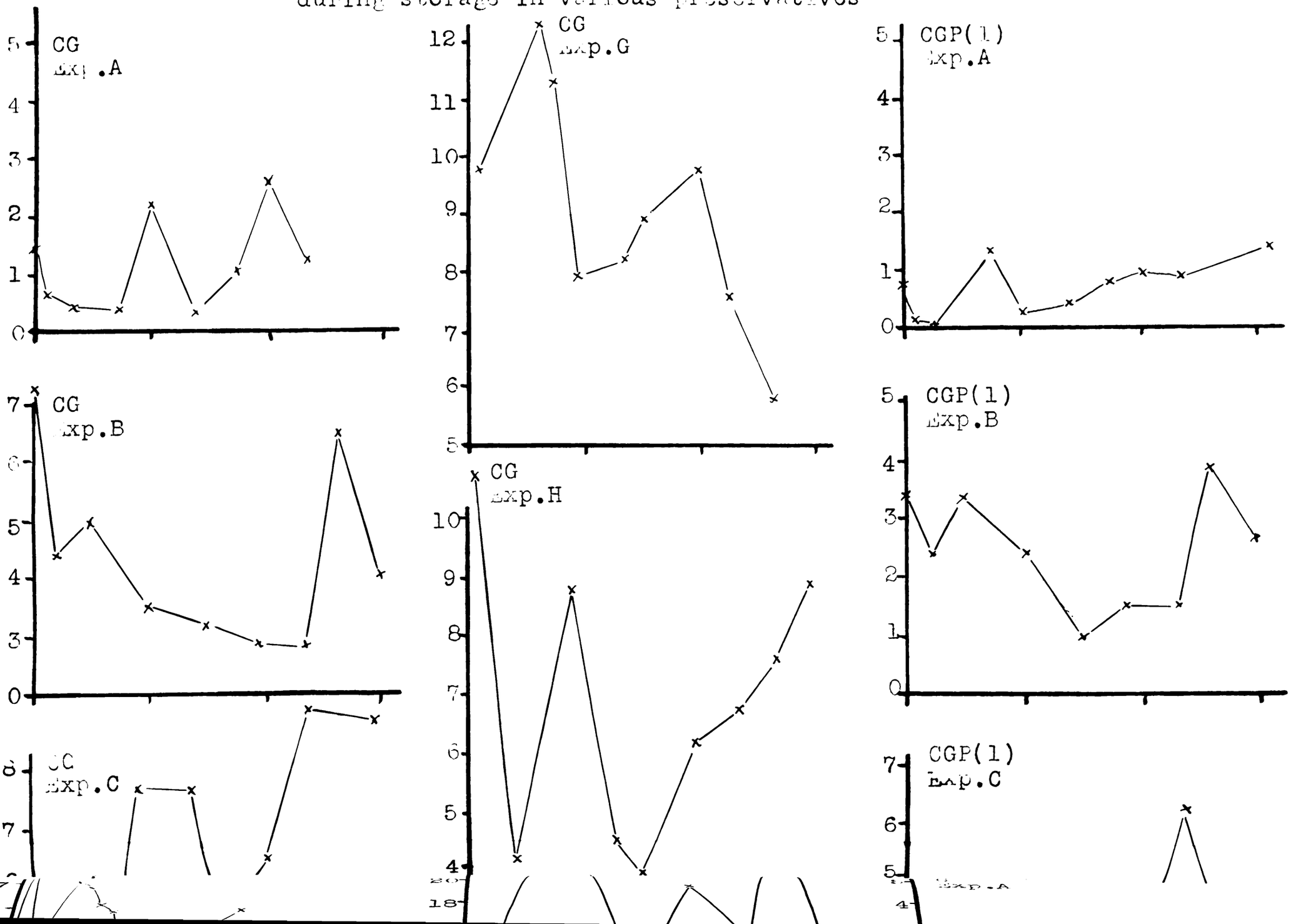
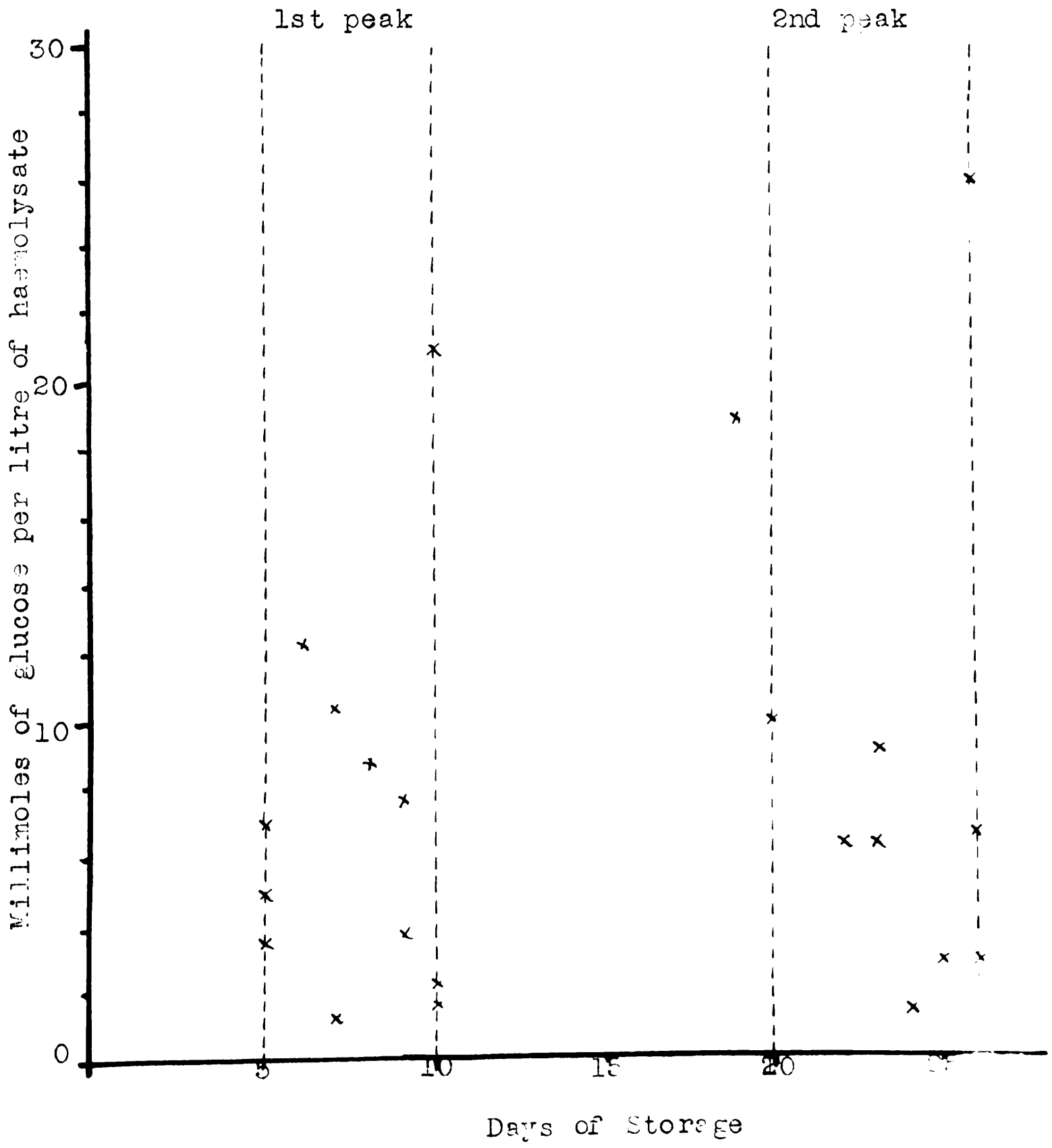


Figure 2.

Summary of the peak values obtained during each fluctuation in the glucose concentration of red cells stored in glucose-containing preservatives.



H. Non-fermentable reducing substances

The non-fermentable reducing substances in red cells were followed during storage in experiment G. The results which are presented in Table XI indicate that there were slight fluctuations in these substances up to the thirteenth day of storage, after which they disappeared from the cells.

TABLE XI

The non-fermentable reducing substances in red cells during storage. The results are expressed as millimoles of glucose equivalents per litre of haemolysate.

Experiment	Non-fermentable reducing substances G
Preservative	CG
Days of storage	
1	0.18
6	0.01
13	0.18
16	0.00
20	0.00
23	0.00
26	0.00

I. Glycogen

The results presented in Table XII show a fall in the glycogen content of red cells during storage, and a disappearance of this compound from the cells by the tenth day.

TABLE XII

The glycogen content of red cells during storage. The results are expressed as millimoles of glucose equivalents per litre of haemolysate.

	Glycogen
Experiment	I
Preservative	CG
Days of storage	
0	0.47
1	0.54
2	0.28
4	0.35
7	0.31
10	0.00

J. Lactic acid

The lactic acid content of red cells was followed in experiment J. The results presented in Table XIII indicate a gradual rise in concentration until the fourteenth day. There was then little change for the next few days, but another increase was noted by the twenty-first day.

TABLE XIII

The lactic acid content of red cells during storage

	Lactic acid (millimoles per litre of haemolysate)
Experiment	J
Preservative	CG
Days of storage	
0	1.57
1	1.32
2	2.27
4	2.41
7	3.05
10	3.50
14	4.22
16	4.01
18	4.15
21	5.42
23	5.17

K. Pyruvic acid, total keto acids and total hydrazones

From the results presented in Table XIV it is evident that pyruvic acid, total keto acids and total hydrazones showed an almost parallel behaviour during storage. In both experiments, they all fell during the first few days of storage, and then remained at a fairly constant level for about two weeks, after which they increased progressively for the remainder of the experimental period. The rate of increase was most rapid during the first few days of the rise. The total keto acid values (expressed as pyruvic acid) were approximately 0.03 to 0.07 mM/l higher than those found for pyruvic acid for the first two to three weeks of storage; after this the difference between the two values decreased, indicating that keto acids, other than pyruvic acid, disappeared from the red cell. In experiment I, the total hydrazone values (expressed as pyruvic acid) were 0.2 to 0.4 mM/l higher than the pyruvic acid values from the first to the nineteenth day of storage, and 0.5 to 0.6 mM/l higher for the rest of the study. In experiment J, the difference between the total hydrazones and pyruvic acid values increased from 0.28 mM/l on the day the blood was collected to 0.67 mM/l on the twenty-third day of storage. These results indicate an increase in the neutral, reactive keto compounds (aldehydes, ketones, trioses) during storage.

Friedemann and Haugen (1943) have reported that they

find only 0.12 to 0.69 mg. percent (i.e. 0.01 to 0.08 mM/l) difference between the values for pyruvic acid and total hydrazones (expressed as pyruvic acid) in blood. In the present studies it was found that the reason for the much larger difference between these two values in our studies is due to the washing procedure. The total hydrazone values estimated by the indirect method, which does not involve washing of the cells, are much lower. This was not realized until the completion of these studies.

TABLE XIV

The pyruvic acid, total keto acid and total hydrazone content of red cells during storage. The values are all expressed as millimoles of pyruvic acid per litre of haemolysate.

	Pyruvic acid		Total keto acids		Total hydrazones	
Experiment	I	J	I	J	I	J
Preservative	CG	CG	CG	CG	CG	CG
Days of storage						
0	0.12	0.16	-	-	0.79	0.44
1	0.03	0.05	-	0.09	0.38	0.52
4	-	0.03	-	0.06	-	0.30
5	0.05	-	0.10	-	0.43	-
7	0.03	0.03	0.07	-	0.41	-
8	0.03	-	0.07	-	0.33	-
9	0.06	-	0.09	-	0.43	-
10	-	0.06	-	0.13	-	0.61
12	-	-	0.07	-	0.33	-
13	0.05	-	0.08	-	0.29	-
14	0.06	0.07	0.09	0.12	0.39	0.65
16	0.16	0.21	0.16	0.24	0.47	0.69
18	-	0.32	-	0.37	-	-
19	0.51	-	-	-	0.81	-
20	0.51	-	-	-	1.04	-
21	0.52	0.50	0.54	0.51	1.11	1.07
22	-	-	0.51	-	-	-
23	0.55	0.52	-	0.54	1.07	1.19
26	0.60	-	0.60	-	1.15	-

L. Survival of erthrocytes after transfusion

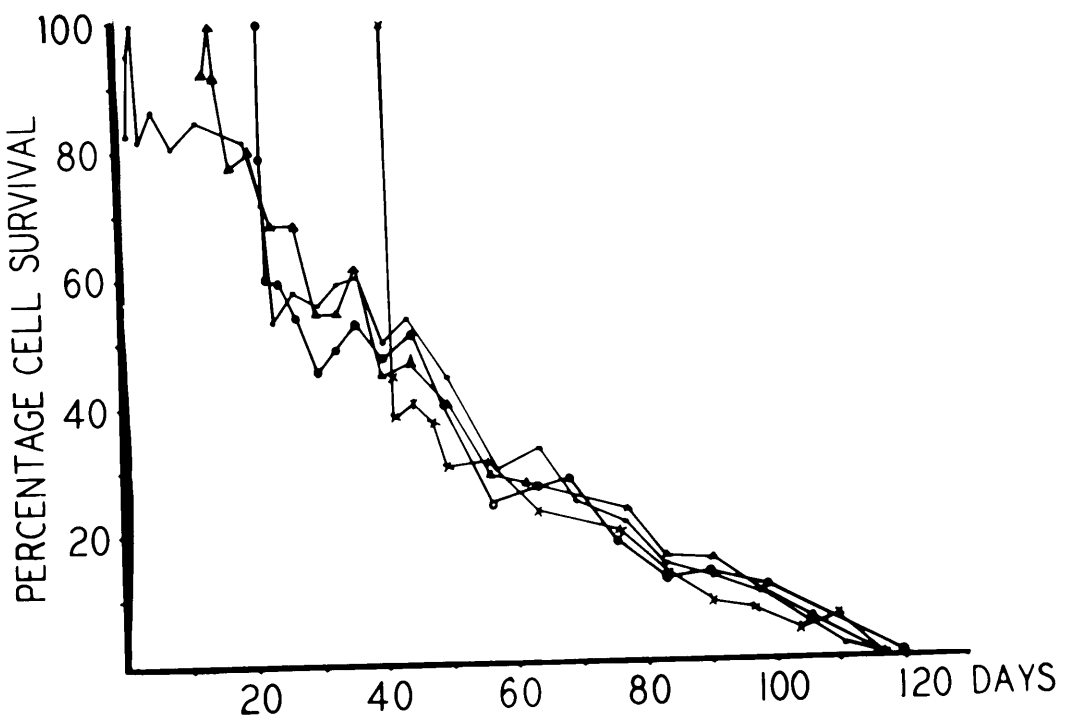
The results of the cell survival studies are presented graphically in Figures 3, 4 and 5. In each experiment, blood taken from one donor was divided into two to four aliquots and transfused after various intervals of storage. The blood was collected into citrate-glucose, Loutit's and Muether's solutions respectively; the disappearance of cells from the recipient's circulation was followed in all cases. The stable phosphate content of the red cells was determined at various intervals during storage on small samples of the blood stored in individual tubes. An analysis of the stable phosphate content of the blood kept in the flasks was carried out just prior to transfusion in most of the experiments. The results of the studies on the stable phosphate are presented with results of the cell survival studies in Figures 3, 4 and 5.

From these figures, it is clear that there was little difference between the in vivo survival of the cells stored in citrate-glucose, Loutit's and Muether's solution. Furthermore, it can be noted that the disappearance of the transfused cells proceeded at the rate of about 0.9 percent per day. Finally, the rate of loss of viability of the stored cells was also about 0.9 percent per day, as can be seen from the fact that the proportion of cells which are eliminated from the recipient's circulation within a few hours after transfusion corresponded to slightly less than the number of days that they were stored.

The stable phosphate content of cells stored in Muether's solution showed a very rapid increase during the first two weeks of storage, then a gradual fall until about the fortieth day, after which it decreased abruptly. It can be noticed that even after the fortieth day of storage, the stable phosphate content of the cells was appreciably higher than the initial concentration. In cells in citrate-glucose solution, the stable phosphate started to decrease after about a week of storage and gradually fell to about thirty-five percent of the original value by the thirty-fifth day, after which a more rapid rate of decrease was observed. In no other case did the stable phosphate of cells stored in citrate-glucose solution start to fall as soon after storage. A fall in stable phosphate was apparent from the beginning of storage in cells in Loutit's solution, and was more precipitous than in cells stored in citrate-glucose, reaching about twenty percent of the initial concentration by the twenty-seventh day of storage. However, an increase in concentration then was apparent.

Figure 3.

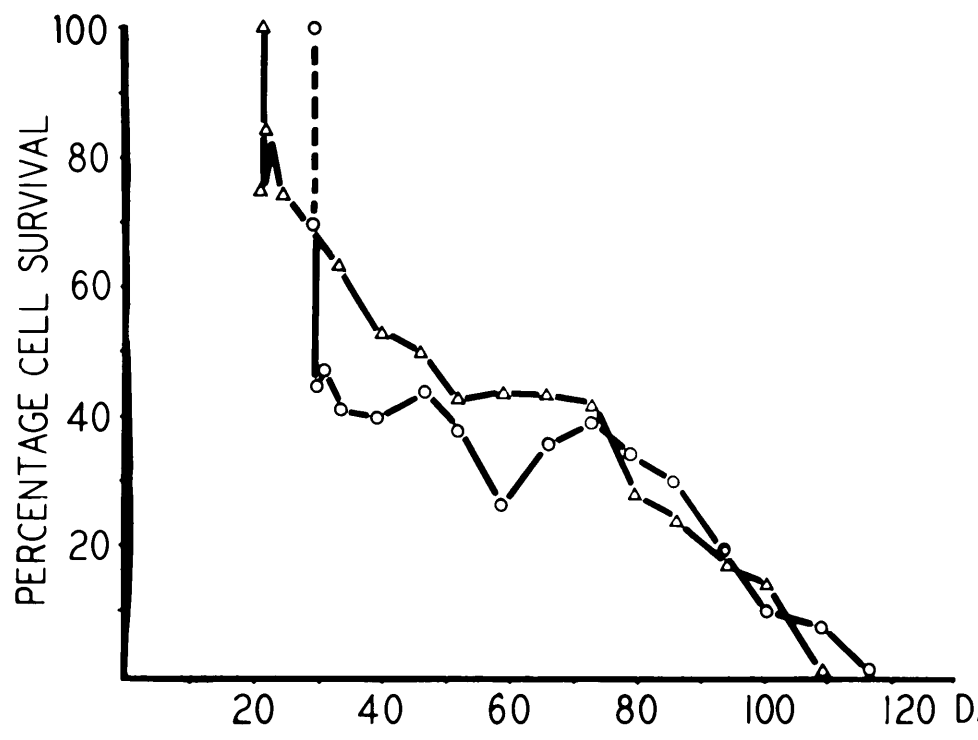
The percentage survival of red cells transfused after storage for 1, 13, 21 and 40 days respectively in citrate-glucose solution



The stable P content of the red cells (expressed as milligrams of phosphorus per 100 cc. of haemolysate) was 6.7, 5.9, 4.9, 2.3 and 0.4 on days 0, 8, 15, 29, 35 and 42 respectively of storage.

Figure 4.

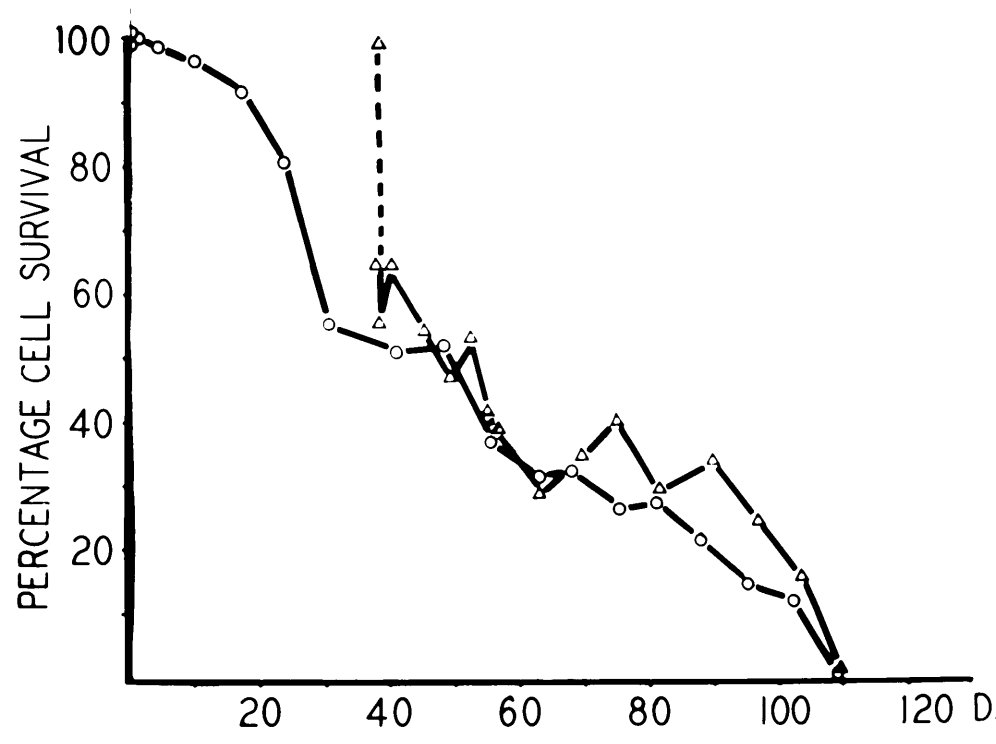
The percentage survival of red cells transfused after storage for 22 and 28 days respectively in Loutit's solution



The stable phosphorus content of the red cells (expressed as milligrams of phosphorus per 100 cc. of haemolysate) was 7.6, 5.9, 1.4, 1.4 and 2.2 on days 0, 8, 21, 27 and 31 of storage.

Figure 4

The percentage survival of red cells transfused after storage for 1 and 39 days respectively in Huether's solution



The stable phosphorus content of the red cells (expressed as milligrams of phosphorus per 100 cc. haemolysate) was 8.8, 17.4, 11.6, 10.5 and 6.9 on days 3, 12, 27, 38 and 54 respectively of storage.

DISCUSSION

Chemical changes in red cells during storage

Of all the compounds studied, glucose showed the most spectacular and unexpected behaviour during storage. It can be seen that there were periodic fluctuations in the glucose concentration in red cells during storage, and that in almost every case where the blood was stored in a glucose-containing preservative, the cell glucose, after an initial fall, rose to a peak between the fifth and tenth days and then fell again. After several days, another rise was observed and a maximum value was attained between the nineteenth and twenty-sixth days. These second peaks were sometimes higher than the values observed after the first twenty-four hours of storage, when diffusion of glucose into the red cells from the plasma had ceased.

The magnitude of the fluctuations in glucose concentraion appears to depend to some extent on the concentration of glucose in the cells on the first day of storage, being smallest in cells with a low glucose content (e.g. in cells in citrate solution) and greatest in cells with a very high glucose content (e.g. cells stored in Huether's solution).

These fluctuations in glucose cannot be attributed simply to experimental errors in analysis. Although a casual inspection of the graphs may give the impression that the fluctuations

are sharply discontinuous, it will be observed on closer examination that the points on many of the curves (especially in experiments I and J, where analyses were carried out at one or two day intervals during most of the study) indicate a smooth course of rise and fall. More important still, the fluctuations occurred at about the same time in nearly all of the fourteen experiments.

While the increase in cell glucose during the first few hours after the collection of blood can be explained by the diffusion of glucose into the cells from the external medium, the reason for the subsequent fluctuations is obscure. As there was no evidence of any significant increase in the amount of non-fermentable reducing substances in the red cell during storage, the fluctuations in reducing substances appear to represent real changes in glucose[x]. The precursor of the synthesised glucose, which appeared at the two regular and predictable intervals during storage still remains unidentified. The glucose cannot be derived from the glycogen in the red cells to any extent as this compound was found only in small concentration and, in the one case where it was studied, it was found to disappear after the tenth day of storage. Nor can it be derived from hexosephosphates in the cells as these were never present in sufficient quantities to

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x However, Castellani and Taylor (see Clinical Biochemistry by Cantarow and Trumper, 3rd edition, page 60) have found that baker's yeast usually consists of one or two species of saccharomyces with a contaminating gram-positive bacillus and that this "yeast" will ferment glucose, levulose, galactose, sucrose, maltose and often lactose..

explain the large increases in glucose. Again, no relationship could be demonstrated between the changes in any of the other phosphorylated compounds or in lactic acid and the increase in glucose.

The possibility that the fluctuations in glucose might be due to a transitory diffusion of glucose into the cell from the plasma, and out again, has not been ignored. Attempts to settle the point, however, by carrying out simultaneous analyses on the cells, whole blood and plasma were complicated by the high concentration of glucose in the preservative media which made the estimation of small changes in the glucose content of plasma liable to large analytical error. It is necessary to repeat these studies using a preservative with a minimal content of glucose. The analytical data obtained, however, do not point to the diffusion of glucose into the red cell as being the cause of the fluctuations, particularly as these fluctuations were also observed in stored whole blood.

That the increase in glucose could result from the liberation of "protein sugar" or from hydrolysis of some glycosidic linkage is another suggestion that comes to mind. But, if this was so, one would not expect the magnitude of the periodic increases to show any relation to the concentration of glucose in the red cell. A similar objection can be raised to the explanation that the increases are due to some kind of reaction of citrate on the cell surface (citrate does not enter the red cell to any extent during the first month of storage).

It is possible also that the glucose which enters the cells during the first day of storage is converted to some as yet unidentified compound which is subsequently broken down at intervals during storage with the liberation of the glucose.

It is equally difficult to explain the rapid disappearance of glucose after the periods of synthesis. As it can be seen from the graphs, the fall is often as precipitous as the rise is steep. No corrresponding change could be demonstrated in the lactic acid concentration in the one experiment in which it was followed. Nor did the changes in the phosphorylated compounds, pyruvic acid, total keto acids, or reactive, neutral, keto compounds show any relationship to the fall in glucose concentration. It might be mentioned in this connection that Dische (1946) has reported an increase in triose-phosphate and hexosediphosphate in citrated blood kept at 4°C. for twenty-four hours. On subsequent incubation for thirty minutes at 40°C. the ester phosphate was found to break down to lactic acid. Although an increase in hexosediphosphate was occasionally found during the first few days of storage in the present studies, it was never large enough to account for more than a small part of the glucose disappearing.

Only two reports have been found in the literature concerning fluctuations in the glucose concentration of plasma and whole blood. Kleiner and Halpern (1933) observed that hyperglycemic blood, when kept at room temperature, frequently showed marked fluctuations in glucose content when studied

at fifteen minute intervals over a period of three hours. This phenomenon was observed in blood collected in citrate, fluoride, oxalate or hirudin. The fluctuations in many of the curves were found to range from 30 to 75 mg. percent. The range and type of fluctuations found by these workers in hyperglycemic blood kept at room temperature for three hours are similar to those observed in this work over a period of thirty days in blood stored at 5°C. Kleiner and Halpern could not explain this phenomenon and their results could not be confirmed by Neuwirth(1934).

Literature on the behaviour of glucose in stored blood is scanty. As far as the author is aware, there are only two reports where the glucose concentration in blood was followed at intervals during storage. From the figures which Bick (1939) and Densted et al.(1941) present there is no indication of any increase in the glucose content of the stored blood. Previous studies conducted in this laboratory by other workers, however, have revealed fluctuations in the glucose concentration of whole blood during storage similar to those observed by the writer. The results of DeGowin's studies on glucose content of the plasma of stored blood indicate a fall in concentration during thirty-five days, with occasional evidence of an increase.

The behaviour of the organic acid-soluble phosphates in red cells during storage has been reported by several previous workers. The present study, while confirming these reports,

was more extensive than the earlier investigations and has revealed several points of interest which have not as yet been recorded in the literature.

From the results it can be seen that there was a fall in the concentration of stable phosphate in red cells during storage and that the time when this fall commenced was fairly characteristic of the preservative used. The early decrease observed in cells stored in a glucose-free preservative can be ascribed to the rapid depletion of glucose which is required for the synthesis of stable phosphates, so that the balance between synthesis and hydrolysis is not maintained and the concentration of stable phosphate falls.

The equally early fall observed in cells stored in Loutit's solution is explainable on the basis that the relatively low pH of blood preserved in this solution inhibits the formation of organic phosphates from glucose. Guest and Rapoport (1941) believe that the rate of hydrolysis of stable phosphate is less affected by a low pH than is the phosphorylation of glucose.

The fall of stable phosphate in cells preserved in other glucose-containing preservatives has been ascribed by Maizels to an accumulation of metabolic products which "change the reaction of the erythrocytes and so impart a certain momentum to phosphate breakdown". However, again it is more likely that the change of reaction results in a decreased utilization of glucose rather than in an increased rate of hydrolysis of organic phosphates.

According to Martland (1925) the disappearance of glucose from blood is greatly retarded, and the increase in inorganic phosphate is accelerated at a pH below 7.3. Therefore it would be expected that a retardation of glycolysis and a fall in stable phosphate should be observed at the time when the pH of the stored red cells falls to 7.3 or lower. The pH of the stored cells was not followed in the present studies. Mollison et al. (1942) have found that the pH of blood in citrate-glucose solution falls to below 7.3 by the fifth day or earlier of storage.

These workers measured the changes in pH in blood by means of a glass electrode. Maizels (1943-1944) asserts that such a method only measures the plasma pH, and that an exaggerated idea of the changes in the reaction of blood is obtained if one attempts to judge whole blood pH from the plasma pH alone, since part of the fall in plasma pH is due to the presence of the non-penetrating anion citrate and is accompanied by a corresponding tendency for the cell pH to rise. This tendency is, however, offset by an increase in acidity due to the production of lactic acid. It is possible, therefore, that in the present studies the pH of the cells stored in citrate-glucose solution did not fall to below 7.3 until the second or third week of storage, i.e. the time when stable phosphate began to fall.

It is not possible to establish from the studies on cell glucose whether or not glycolysis was inhibited at the time when the decrease in stable phosphate became apparent. In one case where lactic acid concentration was followed, it

was observed that the rate of increase was most rapid during the first two weeks of storage, and that during the subsequent period of study, when stable phosphate showed a marked decrease, the rate of production of lactic acid was retarded. In this connection it may be mentioned that the results presented by Mollison and Young (1942) indicate a decreased rate of fall in the pH of blood after the second or third week of storage. It is possible that the lactic acid which appeared during the later stages of storage was derived almost wholly from the breakdown of intermediary compounds involved in glycolysis, and that little or no phosphorylation of glucose took place.

However, before any conclusions can be drawn regarding the extent of glycolysis in blood during storage, more experimental data are required.

The synthesis of stable phosphate in cells stored in Muether's solution is more difficult to explain. The initial pH of blood in Muether's solution is slightly lower than blood in citrate-glucose-phosphate (McGill 1) and yet cells stored in the former solution showed not only a much greater increase in stable phosphate but also a more prolonged maintenance of this substance at a high level than was found in cells stored in the McGill preservative. The synthesis and maintenance of stable phosphate and other organic acid-soluble phosphates in cells stored in Muether's solution cannot be explained entirely by the high concentration of inorganic phosphate in the red cells since an equally high concentration was observed

in cells preserved in the McGill solution; possibly the exceedingly high concentration of glucose in cells in Muether's solution may be partly responsible for this behaviour. Maizels (1943-1944) has found that an increase in organic acid-soluble phosphate (other than labile phosphate) can be accomplished by storing cells in alkaline citrate-glucose solutions and that an even greater synthesis will occur if alkaline citrate-glucose-phosphate solution is used. It should be noted, however, that cells in Muether's solution showed an increase in all the phosphate fractions studied, including labile phosphate.

Rapoport (1939) on the other hand, has found no evidence of any synthesis of diphosphoglycerate in blood during incubation for six hours in the presence of added glucose and phosphate. However, the concentration of glucose and phosphate in his experiments was not as high as in blood stored in Muether's solution.

In one experiment where the behaviour of pyruvic acid was followed in cells preserved in citrate-glucose solutions, a sudden increase in the concentration of this compound was observed about the fourteenth day of storage. As it previously had been found that the stable phosphate content of cells stored in citrate-glucose usually begins to decrease at about this time, a relationship between the breakdown of stable phosphate and the accumulation of pyruvic acid was suspected. A second experiment in which stable phosphate and pyruvic acid were followed simultaneously lent further support to this

suggestion, in that the decrease in stable phosphate was observed to coincide with the rise in pyruvic acid. Calculating the stable phosphate in terms of diphosphoglycerate, it was found that during the period of most marked change in concentration, there was a decrease of 0.93 mM/l in diphosphoglycerate and an increase of 0.46 mM/l in pyruvic acid. During this period, lactic acid formation was continuing, hence one would not necessarily expect to find a quantitative relation between the changes in pyruvic acid and diphosphoglycerate. Furthermore, the method for determining stable phosphate is not specific for diphosphoglycerate. These results, however, do suggest that in the red cells, 2, 3-diphosphoglycerate may be broken down by way of pyruvic acid. Up to the present, the breakdown products of 2, 3-diphosphoglycerate have been a matter of speculation. Braunstein (1934) has found that diphosphoglycerate, in contrast to monophosphoglycerate, is broken down but slowly in blood haemolysate and that pyruvic acid is not formed in the process. Rapoport and Guest (1939) suggest that since the disappearance of diphosphoglycerate is not stopped by the addition of iodoacetic acid, it is independent of the triose-phosphate-pyruvate reaction postulated by Dische. However, these workers did not follow the behaviour of pyruvic acid in their studies. If they had, it is possible that they would have observed, during the breakdown of diphosphoglycerate, an accumulation of pyruvic acid, which, as it has been shown in the present studies, does not inhibit the disappearance of diphosphoglycerate.

According to Dische, pyruvic acid in blood is disposed of by the following reaction:

Triose phosphate + pyruvate → diphosphoglycerate + lactate. It is possible that in the present studies there was a simultaneous fall in both triose phosphate and stable phosphate during storage, and that as the concentration of triose phosphate fell, the rate of reduction of pyruvic acid became slower than the rate of formation, so that an increase in concentration resulted.

In these studies, the triose phosphate content of red cells was not followed during storage. The total reactive neutral keto compounds which include trioses showed a small increase during storage. However, this does not preclude a fall in trioses masked by an increase in some other keto compound.

An increase in the pyruvic acid content of shed blood has been observed by a few workers. Bueding and Goodhart (1941) have found in heparinised or defibrinated (but not oxalated) blood a transitory fall in the concentration of pyruvic acid followed by an increase, so that after four hours at room temperature, the concentration of pyruvic acid was greater than was found at the beginning of the study. These workers suggest that as the amount of available trioses in blood decrease, the rate of removal of pyruvic acid also decreases. Long (1944) has also observed that after a preliminary fall in the pyruvic acid content of citrated blood kept at room temperature, there was a subsequent rise and that the concentration after twenty-four hours exceeded the

initial value. This rise continued for about one week. Von Euler et al. (1940) have reported a tenfold increase in pyruvic acid content of rat's blood maintained at 30°C. for twelve hours, after which a fall in concentration occurred. The concentration of lactic acid was also greatest about the twelfth hour, while the sugar decreased slowly during the experiment.

The present studies showed no evidence of an increase in any keto acids other than pyruvic acid during storage. There was, however, an indication of a small increase in total reactive, neutral keto compounds (aldehydes, ketones, trioses). The identity of the keto compounds which increased is not known.

Before concluding this section, mention should be made of the effect of pH on the chemical composition of the red cells during storage. This effect can be observed from a comparison of the chemical changes found in cells stored in ordinary citrate-glucose solution with those found in cells in Loutit's solution. (The initial pH of blood collected in the former preservative is about 7.4 and in the latter, about 7.1.) In cells stored at the lower pH, a greater loss of total acid-soluble phosphate and a smaller loss of potassium was observed than in cells preserved in ordinary citrate-glucose solution. Maizels (1943-1944) and many others have previously demonstrated that the diffusion of potassium from the red cells is decreased in acid solutions.

The effect of pH on the distribution of phosphate between red cells and a potassium phosphate solution has been studied by Maizels and Hampson (1927). These workers found that on lowering the pH of the resuspending phosphate solution from 8.1 to 5.35 there was a progressive increase in the inorganic phosphate concentration of the cells. These workers to not appear to have appreciated the fact, however, that lowering the pH of the cells will also result in an increase in inorganic phosphate due to the decreased utilization of this phosphate which is constantly produced by hydrolysis of the organic acid-soluble phosphates. It can be noticed from Maizels and Hampson's results that there was an increase in the inorganic phosphate content of both cells and resuspending solution when the pH was lowered from about 8.0 to 5.5, which indicates that the increase in cell phosphate did not result from an inward diffusion of this substance.

The increased loss of phosphate from cells in the more acid solution (Loutit's) cannot be attributed, to any extent, to the greater concentration of inorganic phosphate in these cells, as an equally high or higher concentration was observed in citrated cells, which maintained their inorganic phosphate content much better than the cells stored in Loutit's solution. It is tentatively suggested that the lower the pH of the stored blood, the greater the loss of phosphate from the red cells.

The increase in total acid-soluble phosphate observed

occasionally in red cells during the earlier stages of storage both in the present studies and by Maizels (1943-1944) may be related to the pH of the blood. This cannot be decided from the experimental results on hand.

The effect of pH on the stable phosphate content of red cells has already been discussed. However, in spite of more rapid disappearance of stable phosphate at a low pH, the concentration of labile phosphate in cells stored in Loutit's solution was maintained as well as it was in cells preserved in less acidic solutions. This has been previously noted by Maizels, who attributed this phenomenon to a decreased activity of adenosine triphosphatase in an acid medium. Guest and Rapoport (1939) have also reported that the decrease in organic acid-soluble phosphates in blood occurs mainly at the expense of diphosphoglycerate and that the labile phosphate is less affected.

Chemical changes in the red cells in relation to cell survival

During the course of the first three experiments it was observed that the stable organic phosphate content of cells preserved in citrate solutions started to decrease abruptly at about the end of the first week of storage, while in citrate-glucose preservatives, this fall did not occur until the third week. Furthermore, while the stable phosphate completely, although temporarily, disappeared from cells in citrate by the twentieth to twenty-sixth days of storage,

this was never observed in cells stored in citrate-glucose for thirty-one days.

It was believed at the time that these studies were initiated (although later disproved) that preserved cells would remain as viable as fresh cells if stored in citrate for not more than one week, or in citrate-glucose for two to three weeks. With more prolonged storage, a rapid deterioration of the cells was stated to occur. The rate of deterioration was found to be greater in the citrated cells than in cells stored in citrate-glucose; thus citrated cells transfused after three weeks of storage were found to be virtually all eliminated from the circulation within a few hours, while cells stored in citrate-glucose could be detected for at least three months in the circulation after transfusion.

The results of the first three chemical studies suggested, therefore, that there might be a relationship between the stable phosphate content of the stored cells and their viability.

Further studies revealed that, depending upon the nature of the preservative used, the stable phosphate in the red cells showed one of the following types of behaviour:
(a) a rapid fall starting sometime within the first ten days of storage. This was observed in cells stored in all glucose-free preservatives and in Loutit's solution and in two of the three cases where cells were preserved in Alsever and Ainslie's diluent; (b) maintenance of the stable phosphate content of the cells for two or three weeks. This was usually

observed in cells stored in glucose-containing preservatives other than Loutit's, Alsever and Ainslie's and Muether's solutions, (c)a marked increase in stable phosphate during the first few weeks of storage, and the maintenance of a concentration exceeding that found initially for at least a month. This was observed only in cells preserved in Muether's solution.

If the concentration of stable phosphate in the red cells is a factor in determining their viability, one would expect that cells stored in Muether's solution should show a markedly superior survival after transfusion to cells preserved in Loutit's solution for the same length of time. This was not substantiated by the results of transfusion and cells survival studies. It was found, for instance, that despite the fact that cells in Muether's solution showed a thirty percent increase in stable phosphate during four weeks of storage and those in Loutit's solution showed a sixty percent decrease during the same time, the in vivo survival of cells stored in both Loutit's and Muether's solutions was practically the same. It is clear, therefore, that the concentration of stable phosphate in the red cells cannot be related to the viability of the cells.

These cell survival studies (and others conducted in this laboratory) besides showing that the nature and extent of the autolytic changes may greatly differ in cells stored in different preservatives without influencing the viability

of the cells, also indicated that stored blood undergoes a progressive deterioration from the moment it is removed from the body, so that the concept that blood can be stored for several weeks and remain clinically equivalent to fresh blood must be regarded as fallacious. Judging from the number of donor's cells which disappear from the recipient's circulation within a few hours after transfusion (i.e. the cells that have lost their viability), about 0.8 to 1 percent of the cells in blood preserved in any glucose-containing solution become non-viable per day. It is interesting that this is the rate of discard of red cells that normally occurs in the body. In preservative mixtures not containing glucose, this rate of cell deterioration during storage is greatly accelerated.

None of the chemical changes studied in stored blood were found to show any constant quantitative relationship to the extent of cellular deterioration, i.e. none of the substances underwent a constant change at the rate of about one percent per day. It was concluded, therefore, that neither the concentration of potassium, glucose, lactic acid, pyruvic acid, etc., or the extent of hydrolysis of any of the organic acid-soluble phosphates in the red cells can be used as a guide to the viability of the stored cells.

The mechanism whereby glucose prolongs cell viability during storage is not fully understood. The most evident protective action of glucose is the retardation of haemolysis. Maizels et al. (1940) have suggested that glucose in the

medium in some way increases the extensibility of the cell membrane so that the cells are capable of swelling to a much greater extent than they are in the absence of glucose. However, the retardation of haemolysis cannot be the only or even main function of glucose, for it has been shown that the degree of haemolysis of blood bears no relation to the capacity of the remaining intact cells to survive after transfusion (Mollison and Young 1941). In this present study also it was observed that while cells preserved in Muether's solution showed little evidence of haemolysis when stored for fifty days, and cells in citrate-glucose underwent an appreciable amount of haemolysis within this time, there was little difference in the ability of either of the solutions to preserve the viability of the red cells.

Maizels et al. (1940) have suggested the glucose exerts a beneficial action by retarding the loss of semi-permeability of the cell membrane to cations. DeGowin et al.(1939) others have not found that the addition of glucose to the preservative prevents the loss of potassium from stored cells, although Harris (1941a) has shown that there is a decreased loss when cells are preserved in the presence of glucose. The results of the present studies also indicate that the loss of potassium is somewhat greater in cells stored in the absence of glucose, but that the least change occurred in cells in Loutit's and Muether's solutions (the two most acid solutions). It is possible that the smaller loss of potassium from cells stored in glucose-containing

preservatives can be attributed more to the lower pH of the blood stored in these solutions than to the presence of glucose, per se.

It has been postulated that glucose acts by retarding the decrease in organic acid-soluble phosphate compounds. Loutit (1943-1944) and others have shown, however, that a rapid breakdown of organic acid-soluble phosphate does not necessarily result in an impaired viability of the stored cells. Maizels (1943-1944) has noted that an accelerated fall in labile phosphate is always associated with poor in vivo cell survival. In the present study it was found that the only change during storage which was peculiar to cells stored in poor preservatives was a rapid fall in the labile phosphate concentration to a low level. In other preservatives, this fall was more gradual. On the other hand, maintenance of the labile phosphate at a high level (e.g. in cells stored in Muether's solution) was not found to be essential to good preservation. Furthermore, in some cases, the labile phosphate content of cells after a month of storage in glucose-containing preservatives was as low as that observed in citrated cells stored for about three weeks. Therefore, it does not appear that the beneficial action of glucose on cell preservation can be wholly ascribed to a retardation of the rate of disappearance of labile phosphate from the red cells.

It is possible that the loss of viability of red cells during storage is not due to any specific chemical or physical change but is the result of the sum total of the autolytic changes which take place. In the present studies, glucose has been found to lead to a general retardation of all autolytic processes in the red cells during storage. It may be that by this retardation glucose maintains the cell functions for a longer period in a state where they can resume normal activities when the cells are returned to the circulation and thus bring about a reversal of autolytic changes that have taken place during storage. Haizels et al. (1940) have shown that, with respect to sodium and potassium, such a reversal does occur; they found that very rapidly after stored cells are transfused, the cells which survive lose sodium and accumulate potassium, thus tending to revert to their original state. It is conceivable that in the absence of glucose, the autolytic processes advance rapidly to a state where the cells become functionless and are therefore unable to reverse the autolytic changes when transfused. One would, however, expect that cells stored in Huether's solution (which retards all the autolytic processes studied to a much greater extent than any other preservative) should survive for much longer periods than they do.

That a progressive loss of metabolic activity does occur during storage has been demonstrated by Harris (1941 a,b)

who found that in citrated cells stored at 4°C., both the glycolytic powers and the ability to recover lost potassium on incubation at 37°C. decrease rapidly, and that by the twentieth day of storage, both of these properties are almost completely absent. It has been shown that red cells preserved in citrate for twenty days are speedily eliminated from the circulation after transfusion. It seems possible, therefore, that there might be a quantitative relationship between the viability of the preserved cells and their ability to carry out glycolysis or to accumulate potassium. The writer has carried out one experiment which suggests that such a concept is plausible. In the experiment, blood samples were subject to incubation at 37°C. for twenty-one hours after five and twenty-six days of storage respectively and the potassium content of the cells was determined both before and after incubation. It was found that the cells incubated after twenty-six days of storage took up only about one-third as much potassium as did the cells of the five day old sample. As it has been previously stated, about 0.9 percent of the cells have been found to deteriorate per day of storage; therefore in the above experiment one might expect about a 19 percent cell deterioration between the fifth and twenty-sixth days of storage. Although the agreement between the loss of ability of the cells to recover potassium and the expected amount of cell deterioration is not very close in this experiment, one should allow for the fact that the

metabolic products which accumulate during storage tend to interfere with glycolysis and the accumulation of potassium. Probably a more correct estimate of the capacity of stored cells to take up potassium on incubation could be obtained by washing the cells and then resuspending them in compatible plasma prior to incubation.

It is suggested that the degree to which stored cells can reverse autolytic processes when returned to a physiological environment may provide the basis for an in vitro method for ascertaining the viability of these cells. If such a method could be developed it would undoubtedly be of great use in research on the improvement of blood preservation.

SUMMARY

1. Chemical changes in compounds associated with glycolysis were investigated in red cells stored in different preservatives at 5°C. for about one month.

2. The glucose concentration of the red cells underwent marked fluctuations during storage. After the first day there was usually a fall in concentration followed by a rise which reached a maximum value between the second and tenth days of storage. A second fall and a subsequent increase then occurred with another peak being found between the nineteenth and twenty-sixth days. These fluctuations were observed in nearly all instances, regardless of the preservative used. The glucose which appeared periodically was not derived to any extent from the glycogen or hexose-phosphates present in the red cells, nor did it appear to be the result of a transitory diffusion of glucose into the red cells from the plasma. The fluctuations in glucose concentration also appeared to occur independently of the glycolytic processes. The concentration of the non-fermentable reducing substances in the red cells remained at a low level during the first two weeks of storage and subsequently disappeared.

3. The glycogen content of the red cells decreased during storage and disappeared from the cells after the tenth day.

4. The rate of increase of lactic acid concentration was most rapid during the first two weeks of storage in cells stored in citrate-glucose solution and then decreased.

SUMMARY (Cont'd.)

5. Pyruvic acid, total keto acids and total hydrazones decreased during the first few days of storage and remained at a low level until about the end of the second week, after which a steady increase in concentration occurred. There was no increase in any keto acids other than pyruvic acid. The increase in total hydrazones was greater than could be accounted for by the increase in pyruvic acid, indicating a rise in the concentration of neutral reactive keto compounds.

6. A fall in stable phosphate (which is derived mainly from 2, 3-diphosphoglycerate) was found to be accompanied by an accumulation of pyruvic acid. These results suggested that 2, 3-diphosphoglycerate is an intermediary in the formation of pyruvic acid in red cells.

7. The fall in stable phosphate took place more rapidly in cells preserved in the absence of glucose or in Loutit's solution than in other solutions. In cells stored in Muether's solution, there was a marked synthesis of stable phosphate.

8. Hexosediphosphate and labile phosphate decreased slowly and irregularly during storage. The rate of decrease was most rapid in cells preserved in glucose-free solutions. In cells preserved in Muether's solution, there was an extensive synthesis of these phosphate fractions which persisted for several weeks, after which a fall became apparent.

9. The increase in the inorganic phosphate content of the cells resulted from the breakdown of organic acid-soluble phosphate compounds in cells stored in phosphate-free preser-

SUMMARY (Cont'd.)

vatives. The rate of increase in inorganic phosphate was most rapid during the time when the breakdown of stable phosphate was most marked.

10. The total acid-soluble phosphate content of red cells tended to show a decrease after a month of storage, except in cells stored in a phosphate-containing preservative, in which a progressive rise in concentration occurred.

11. The chemical changes observed in all of the compounds with the exception of glucose are in harmony with the scheme of glycolysis in blood as elaborated by Dische and others.

12. None of the chemical changes in the red cells were observed to bear a quantitative relation to the loss of cell viability which proceeded at the rate of 0.8 to 1 percent of the red cells per day of storage.

13. It was suggested that the degree to which the autolytic changes take place in the red cells during storage can be reversed when the cells are returned to a physiological environment may be quantitatively related to the viability of the cells.

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