# THE METABOLISM OF ELECTROLYTES IN PRESERVED BLOOD.

by

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T H E S I S

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2. Rev. Can. Biol 11,49,1952

"CELLS DO WORK TO MAINTAIN THEIR CHEMICAL IDENTITY".

H. Burr Steinbach.

TABLE OF CONTENTS

TABLE OF CONTENTS Cont'd

Page

# INTRODUCTION

## A. GENERAL:

The investigation to be described in this thesis forms part of a research project carried out in the Department of Biochemistry, McGill University on the problems of the preservation of human blood. This larger study was initiated early in 1940, under the guidance of Dr. O.F. Denstedt, and has been in progress since that time.

Although the use of preserved blood dates back to World War 1 when O.H. Robertson used specimens stored up to twenty-two days in the treatment of battle casualties, little attention was paid to the question until the Russians revived it in 1930. Preserved blood was used extensively during the Spanish insurrection. The most rapid advances in blood preservation, however, were made during the second World War, and a great deal was learned about the actual physical and chemical changes in blood during storage.

By 1945, however, it became apparent to those working in the field that any further substantial improvements in the methods of preservation would probably be made only through a more complete knowledge of the energy metabolism of the erythrocyte and the mechanism of loss of the viability of the cells during storage.

Among the greatest needs at the time was a practical laboratory test for determining the functional capacity, or viability of the preserved erythrocytes. Various laboratory methods, including the 'fragility' test, analyses for the loss of potassium, the degree of spontaneous hemolysis and others, were tried as a means of assessing the physical and chemical state of the erythrocytes, but have proven to be of limited value. The only reliable test of cell viability is still the laborious procedure of transfusing the preserved blood sample and following the survival, or conversely the rate of elimination, of the donor's cells in the recipients' circulation.

By 1945, the McGill group of Rochlin, Andreae, Osborn and Denstedt, having studied the electrolyte changes in the red cells during storage, became aware that the loss of potassium and the gain of sodium that invariably occurs is closely related to the depression of the metabolism of the cells in the cold. Furthermore, it became apparent that the widely held notion that the loss of potassium from the red cells was among the likely causes of the failure of cell viability was incorrect and that the escape of potassium actually is the effect, rather than the cause of the impaired metabolism. The emphasis of the research therefore shifted to the study of the enzymology and energy metabolism of the cell. A study of the electrolyte shifts during storage was of importance since it afforded valuable indications for reflecting metabolic changes. Harris (1940;1941a;1941b) had made the significant, but not widely

appreciated observation, that the cold-preserved red cells when returned to 37° C. are capable of recovering lost potassium, the degree diminishing with the duration of cold storage. Andreae (1946), in our laboratory, had suggested that the capacity of preserved cells to recover potassium at 37° C. might afford a basis for evaluating the viability of the cells.

The problem at this stage was taken up by the writer, with a view to relating the electrolyte behaviour of the preserved cells to other chemical changes as were being studied by several workers in our laboratory. Obviously, the action of the cells in regaining potassium against a high gradient of potassium in the cells and of expelling sodium against a similarly high gradient of sodium in the plasma, involves osmatic work and an expenditure of energy which is provided by the metabolic processes of the cells. The study being reported here was therefore initiated in 1948, with the view to further elucidate the chemical changes in preserved erythrocytes as related to their metabolic state, and to explore the possibility of assessing the efficacy of preservation by some of these metabolic interrelationships.

The uptake of potassium and the expulsion of sodium by the stored erythrocytes, against their concentration gradients, presented an intriguing problem in fundamental physiology, and an attempt to analyze the mechanism of this phenomenon was made. Of late, interest

in metabolically driven electrolyte shifts in all tissues has increased greatly. In the course of the writer's investigation it became evident that many of the interpretations in the literature concerning the maintenance of electrolyte gradients might be studied in blood. This tissue offers the advantage of providing intact and discrete cells, and can be studied reproducibly. These attributes are afforded by few other tissues. In the hope of deriving a maximum of profit from the present study, the writer proposes to refer to the movement of electrolytes in other tissues, and to attempt to compare these observations with those on the erythrocyte.

A cataloguing of the voluminous literature on the chemical and physical changes that occur in the erythrocyte during cold-storage would be superfluous in view of the availability of several excellent reviews on the subject. (Denstedt et al 1941: Maizels, 1943; Rapoport, 1947; Parpart et al. 1947; De Gowin, Hardin and Alsever,1949). The writer proposes, alternatively, to discuss fully the main electrolyte changes found during the storage of human blood in some of the more significant studies. An attempt will be made to make these findings comparable where possible, and to interpret the data presented in the light of the conditions of storage employed.

B. Sodium and Potassium in Stored Blood.

Living cells are characterized by their ability to maintain a higher internal concentration of potassium than the medium in which they are suspended. This phenomenon has been studied extensively for

many years, with the aid of a wide variety of experimental techniques. Since excellent recent reviews of the literature are available, no attempt to summarize it will be made here (Davson and Danielli,1943; Hober,1945; Krogh,1946; Conway,1947; Ussing,1949; Steinbach,1951).

In the human, the ratio of intracellular to extracellular potassium is about 23:1. The erythrocytes are typical, containing in health, about 100 milliequivalents of potassium per liter (390 milligrams per 100 milliliters), while the plasma contains approximately 4.5 milliequivalents per liter (18 milligrams per 100 milliliters). The reverse picture is seen in regard to sodium, which is confined mainly to the extracellular compartment, to the extent of about 140 milliequivalents per liter (325 milligrams per 100 milliliters), while only small amounts are found within the cells.

Until recent times it was thought that the cation distribution arose in some inexplicable manner during the active stage of cell development and was perpetuated in maturity by the cells becoming impermeable to at least one of the cations, sodium or potassium. The evidence at present favors the view that the ionic distribution is the result of a dynamic equilibrium which the living cell maintains with metabolic energy, and which gives way to a stable state only in metabolic failure and death.

That the cation content of the erythrocytes can be varied by the medium had been shown as early as 1924 (Ashby,1924; Kerr,1929; Ponder,1934), but because of the currently accepted theories, these

findings were either ignored or at best explained away. Kerr had even shown that within two hours the sodium and potassium concentration in sheep cells could be equalized by suspension in an isotonic NaCl -KCl mixture.

Blood storage studies finally showed that the erythrocyte membrane does permit the diffusion of cations, and much of the accumulated data based on the concept of impermeability must be reassessed.

Duliere (1931a,1931b) was the first to demonstrate the progressive leakage of potassium from the red blood cells upon removal from the circulation. He observed that the potassium content of the serum increased when left in contact with the clot. Furthermore, he succeeded in minimizing the escape of potassium into the serum by cooling the freshly drawn blood rapidly to $0^{\circ}$ C., allowing it to clot at that temperature and centrifuging in the cold. On the other hand, if the clotted blood was allowed to stand at room temperature, the potassium content of the serum was greatly increased, partly because of hemolysis, but mainly due to diffusion of potassium from the erythrocytes. When the whole clotted blood was allowed to stand for fourteen days, the potassium content of the serum had, in some cases, reached a level of 115 mg./100 ml. (30 m.eq/liter) This concentration approaches the potassium content of whole blood, and indicates that the diffusion of potassium from the cells and the clot into the serum had almost reached the point of equilibrium.

In Table I are given the serum potassium values obtained by Duliere, showing the diffusion rate as a function of time. This table has been compiled by the writer from data given in Duliere's publications.

TABLE I.

The effect of time on serum potassium of clotted blood

| DAYS | Serum $K^+$ | |
|---|---|---|
| $\frac{1}{4}$ | 23 mg./100 ml. | 5.9 m.eq./liter |
| 1 | 25 " " | 6.4 " " " |
| 2 | 31 " " | 8.0 " " " |
| 3 | 34 " " | 8.7 " " " |
| 4 | 37 " " | 9.5 " " " |
| 5 | 56 " " | 14.4 " " " |
| 8 | 75 " " | 19.2 " " " |

The data presented show that the increase in serum potassium was initially quite slow, and if graphically presented, would give an exponential type of curve. As shall be seen later, the mode of potassium exchange in clotted blood differs from that

in an unclotted sample. In the former, the potassium not only must diffuse through the cell membrane but also through the compact mass of the clot before reaching the serum.

In seeking a reason for the diminished sedimentation rate of erythrocytes stored in the cold, Jeanneney and Servantie (1938) observed progressive changes in the plasma potassium of unclotted whole blood. This was the first demonstration of the typical manner of potassium diffusion from the red blood cells in the cold. The increase in plasma potassium is most rapid during the first five days, then slows down as the concentration in the plasma and the cells approachs an equilibrium. In Table II is a compilation of data from several experiments of Jeanneney and Servantie that illustrates the increase in plasma potassium in cold-stored blood samples.

TABLE II

The effect of time on plasma potassium of unclotted blood.

| Days | | Plasma K+ |
|---|---|---|
| 0 | 19 mg./100 ml | 5.0 m.eq./liter |
| 1 | 59 " " | 15.1 " " " |
| 2 | 74 " " | 19.0 " " " |
| 3 | 106 " " | 27.2 " " " |
| 4 | 110 " " | 28.2 " " " |
| 6 | 121 " " | 31.0 " " " |
| 7 | 132 " " | 34.0 " " " |
| 9 | 149 " " | 38.2 " " " |
| 11 | 145 " " | 37.2 " " " |

These authors suggested that the rate of diffusion of potassium from the red cells might be used to test the effect of different conditions of storage on the preservation of blood, and might afford an index of the quality of the blood for transfusion.

Continuing their studies, Jeanneny, Servantie and Rigenbach (1939) failed to find any change in the density of the plasma with the increase in potassium during storage. This observation led them to measure the plasma sodium content. They discovered that as potassium is lost from the cells, sodium enters them in exchange. They found, further, that the exchange is almost reciprocal, so that the sum of the concentrations of these two electrolytes in the cells or in the plasma remains nearly constant. Considering that they expressed the concentrations on a weight basis, as grams per liter, it is remarkable they noticed the reciprocal exchange. Obviously to obtain a true comparison of the movements of the potassium and sodium ions it is necessary to calculate the concentrations on a molar basis, such as milliequivalents per liter. The data shown in Table III have been recalculated to show the extent of reciprocal exchange of sodium and potassium when expressed on a weight basis and an equivalent basis.

TABLE III

Plasma sodium and potassium during cold-storage.

| | Grams per liter | | | Milliequivalents per liter | | |
|---|---|---|---|---|---|---|
| DAYS | $Na^+$ | $K^+$ | TOTAL | $Na^+$ | $K^+$ | TOTAL |
| 1 | 3.96 | 0.34 | 4.30 | 172 | 9 | 181 |
| 2 | 3.78 | 0.62 | 4.40 | 164 | 16 | 180 |
| 3 | 3.68 | 0.70 | 4.38 | 160 | 18 | 178 |

Had the blood storage been carried on for a longer period of time, to permit a still greater exchange of cations, the sum of these ions in the plasma would remain constant on an equivalent basis, but would increase on a weight basis, provided the exchange was truly reciprocal. However, from the trend indicated in the table, the exchange of potassium for sodium does not appear to be reciprocal, since the total base decreased by 3 milliequivalents. This means, of course, that the number of sodium ions that entered the cells exceeded the number of potassium ions that escaped.

Jeanneney et al (1939) showed, further, that the diffusion of potassium from the erythrocytes can be retarded when the $p^H$ of the citrated blood sample was lowered to $p^H$ 6.8 by the addition of citric acid. To achieve this they recommended the collecting of the blood into solutions of mono-, di-, and tri-sodium citrate prepared by mixing citric acid solution with the required amount of sodium bicarbonate. Their results, as set forth in Table IV show that the mono-sodium citrate retarded the rate of sodium and potassium diffusion markedly, and that the diffusion with di-sodium citrate was slower than with the tri-sodium citrate. On the premise that it is desirable in the preservation of blood for transfusion to minimize the loss of potassium from the cells, they were the first to recommend the use of an acidified medium for blood storage.

TABLE IV

The effect of $p^H$ on plasma Na⁄ and K⁄ during cold-storage

| | Monosodium Citrate | | Disodium Citrate | | Trisodium Citrate | |
|---|---|---|---|---|---|---|
| DAYS | K⁄ mg/100 ml | Na⁄ mg/100 ml | K⁄ | Na⁄ | K⁄ | Na⁄ |
| 3 | 29 | 381 | 34 | 396 | 44 | 417 |
| 5 | 44 | 367 | 62 | 378 | 81 | 387 |
| 10 | 49 | 361 | 70 | 368 | - | - |

Since sodium bicarbonate was used for the neutralization of the medium, it is evident that the initial sodium concentration in the blood varied with the degree of neutralization. This may have been a factor in the greater influx of sodium and efflux of potassium in the blood samples stored in the more completely neutralized media, in addition to the effect of the $p^H$ of the medium on the permeability of the erythrocyte membrane to these ions.

Scudder and co-workers (1939) undertook a study of the potassium changes in stored blood in order to determine the rate of potassium egress from erythrocytes when stored in different preservative media. When heparin was used as the anticoagulant, and the cells packed by centrifugation, the increase in plasma potassium during storage in the cold was comparable to that observed by Duliere (1931). However, when the blood was stored under liquid petroleum, without anticoagulant, they noted a very rapid initial increase in plasma potassium. The results obtained are shown in Table V.

TABLE V

The effect of packing the erythrocytes on plasma $K^+$ during cold-storage

| | Plasma $K^+$ - mg. per 100 ml. | |
|---|---|---|
| Days | Heparin and Centrifuged | Under Oil |
| 0 | 20 | 20 |
| 1 | 20 | 40 |
| 2 | 27 | 69 |
| 3 | 33 | 83 |
| 4 | 41 | 101 |

The findings do not show that heparin prevents the loss of potassium from the cells but the slow diffusion of this ion into the plasma is due to the fact that the cells were packed by centrifugation. The storage of blood under oil, without an anticoagulant, does not delay the loss of potassium from the erythrocytes.

Scudder and his associates (1939) further studied the effect of various blood preservative solutions that were recommended at that time, on the rate of loss of potassium from the eyrthrocytes during storage. Because of the significance of the results of these early studies, it is of interest to review the composition of the preservatives used. They are numbered for identification in Table VI, which follows.

1. Citrated blood: 17.5 ml. of 2.5% sodium citrate per 125 ml. of blood. The final concentration of sodium citrate in the sample was 0.31%. The dilution of the whole blood was 14%, and that of the plasma, approximately 25%.
2. Citrated blood: 15 ml. of 3.0% sodium citrate per 125 ml. of blood. The final concentration of sodium citrate was the same as in Mixture No. 1 above, but the dilution of the blood is slightly less. The anticoagulant, being more concentrated, would tend to delay swelling of the cells.
3. Russian citrate solution (Goodall et al, 1938):
   The blood was added to an equal volume of the anticoagulant-perservative mixture. This mixture contained 7 gm. sodium chloride, 5 gm. sodium citrate, 0.2 gm. potassium chloride,

0.004 gm. magnesium sulphate, and distilled water to make one liter of solution. The blood was diluted 100% and hence the plasma was diluted about 200%.

4. Russian citrate solution (as in Mixture No.3) used with double the volume of blood. In this mixture one part of the anticoagulant-preservative mixture was used with two parts of blood. The blood was thus diluted 50% and the plasma was diluted about 100%.

5. Rous-Turner solution: The blood was added to 5.4% glucose solution and 3.8% sodium citrate solution in the proportion of 3:5:2. This large volume of glucose and citrate diluted the blood more than three-fold, and the plasma by about six times.

6. Citrated blood with added adrenal cortical extract: To a blood-sodium citrate mixture (125:15) was added 10 ml. of an adrenal cortical extract. No description of this extract was given in the reports. The dilution of the blood was 20% and that of the plasma approximately 40%.

It should be pointed out that Scudder's procedure was to pipette portions of the plasma from the sedimented samples at intervals, without disturbing the cells. This method implies the assumption that the distribution of potassium throughout the plasma is uniform. Also the system of sampling progressively reduced the degree of dilution of

the blood, but no correction was made for this in their analyses.

Since the proportion of the diluent to blood differs so greatly with the various preservative mixtures, the values reported for the "plasma" potassium obviously are misleading in the implication concerning the rate of potassium loss from the cells. The data are therefore presented in Table VI as the percentage of potassium lost by the cells into the suspending medium. These have been calculated by the writer from the data of Scudder so as to be more easily interpretable.

TABLE VI

The effect of different preservatives on the loss of K+ from stored erythrocytes#

| Duration of Storage | % K+ Loss from Cells stored in 6 different media# | | | | | |
|---|---|---|---|---|---|---|
| Days | 1 | 2 | 3 | 4 | 5 | 6 |
| 7 | 19 | 19 | 23 | 26 | 19 | 20 |
| 14 | 33 | 29 | 41 | 38 | 37 | 34 |
| 21 | 44 | 41 | 60 | 50 | 44 | 52 |
| 30 | 49 | 45 | 66 | 58 | 53 | 63 |

# See text for description of preservatives used.

The rate of diffusion of potassium from the red cells in blood kept under oil, and in heparinized specimens in which the cells were permitted to settle and not sedimented by centrifugation, was essentially the same as that in the citrated samples. Small differences are attributable to the differences in the dilution brought about by different proportions of diluent and anticoagulant used. The slightly accelerated rate of escape of potassium in Mixture No. 1 as compared to Mixture No. 2 may be attributed partially to the hypotonicity of the sodium citrate in the former (2.5%), and also to the greater degree of dilution of the blood. This comment applies also to the two Russian preservatives, the larger dilution favoring potassium diffusion from the erythrocytes. The adrenal cortical extract appears to have had little effect, other than that of diluting the blood.

It is noteworthy that despite the relatively large dilution in the case of the Rous-Turner blood mixture (No.5), the egress of potassium was not excessive, but is comparable to the behaviour in Mixtures No. 1,2 and 7, which represent a comparatively low dilution of the blood. The Rous-Turner mixture is the only glucose-containing solution that has been reported on by Scudder and his associates.

De Gowin, Harris and Plass (1939) have made an extensive study of the loss of potassium from the erythrocytes during storage in the cold. They investigated the behaviour of blood when preserved under the following conditions:

1. Twenty-three parts of blood were added to two parts of 3.2% sodium citrate, and the mixture stored at $2^{o}$ - $5^{o}$ C.
2. The blood was added to 5.4% glucose solution and 3.2% sodium citrate in the proportion of 10:13:2. This is a modification of the original Rous-Turner mixture, in which the amount of citrate has been greatly diminished, and the blood diluted less.
3. The blood was diluted to the same extent as in No. 2, but using a solution of heparin instead of sodium citrate, as the anticoagulant. Thus, the blood mixture contained 10 parts of blood and 15 parts of 5.4% glucose solution containing 6 mg. heparin.
4. The blood was stored in a mixture identical to No. 2, but instead of having free access to the air through a sterile gauze cover, the blood was kept under 'anaerobic' conditions by filling the bottles completely and stoppering them.
5. The blood was diluted to the same extent as in mixtures No. 2,3 and 4 but 0.95% sodium chloride solution was added in place of the glucose solution. The mixture comprised ten parts of blood, thirteen of 0.95% sodium chloride and two of 3.2% sodium citrate solution.

6. Twenty-one parts of blood were added to two of 5.4% glucose and two of 3.2% sodium citrate solution. This mixture represents a much smaller dilution of the blood than with the preservative mixtures No. 2,3,4 and 5.

De Gowin has reported his values for "plasma" potassium as measured directly on the supernatant of the preserved specimens after sedimentation of the cells. Since the degree of dilution of the plasma in mixtures No. 2,3,4 and 5 is the same (about four times), they are directly comparable. This is not so in mixture No. 1, where the original plasma has been diluted only 1.2 times, or with mixture No.6 where the plasma was diluted 1.4 times. The writer has re-calculated De Gowin's data as given in Table VII to correct for the dilution by the preservative mixture and to make the results directly comparable in different blood mixtures.

TABLE VII

The effect of different preservatives on the loss of K+ from stored erythrocytes (De Gowin et al,1939)*

| Duration of Storage in Days | Plasma K+ (mg./100 ml.) in 6 different media* | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| 0 | 19 | 44 | 64 | 52 | 48 | 18 |
| 5 | 184 | 148 | 140 | 220 | 276 | 161 |
| 10 | 215 | 216 | 200 | 256 | 348 | 206 |
| 15 | 232 | 252 | 240 | 264 | 388 | 231 |
| 20 | | 300 | 252 | 260 | | 243 |
| 25 | | 308 | 260 | 384 | | 242 |
| 30 | | 312 | 324 | [illegible] | | 250 |
| 35 | | | | 308 | | |
| 35 | | 316 | | | | |

*See text for description of preservatives used.

Since no figures are available for the changes in the cell volume during storage, it is not possible to calculate cellular potassium values. However, the values in the above table are directly referable to the concentration of potassium in the erythrocytes.

It is apparent from the recalculated data that potassium ions migrate into the plasma at about the same rate in the various preservative mixtures, with the exception of mixture No.5. The presence of a large volume of sodium chloride would seem to have a definitely deleterious effect on the retention of potassium by the cells. This is probably attributable to the presence of an excess of chloride ions, since the sodium ion concentration seems less critical. Thus, in mixture No. 3 no additional sodium salts have been added but potassium diffusion is unaltered. Isotonic saline differs from isotonic glucose in its effect on red cells in that it does not penetrate the cells, whereas glucose does. The data presented (mixture No.2) shows that the addition of glucose does not retard the loss of potassium from the erythrocytes in highly diluted blood. The total amount of potassium lost from the cells is greatest with mixtures No. 2-5 inclusive, due to the large dilution of the blood.

De Gowin and co-workers (1940) further investigated the problem of the loss of potassium from stored eyrthrocytes under various conditions of preservation to ascertain whether the concentration of potassium in the plasma might serve as an index of corpuscular deterioration. That is, could plasma potassium concentration serve

as a basis for evaluating the physiological state of the erythrocytes? The results are closely similar to those reported in their previous study (De Gowin et al,1939). They found that diffusion equilibrium, that is, the point where the concentration of potassium in the plasma equals that in the red cells, is reached between the tenth and fifteenth day in the blood specimens that are only slightly diluted. On the other hand, in extensively diluted specimens where the volume of extracellular fluid has been greatly increased, the diffusion of potassium from the cells continues until the third or fourth week of storage. A considerable variation in the rate of potassium loss was observed from one individual's blood to another, and it is unfortunate that De Gowin did not attempt to compare the effect of different preservatives on the potassium loss from the blood of the same individual.

De Gowin and his associates concluded that the addition of various amounts of sodium, chloride, glucose, citrate or heparin did not affect the rate of diffusion of potassium from the cells to a significant degree, but the rate of hemolysis did vary significantly. The writer cannot agree with that view, as it seems apparent that an excess of chloride ions in the preservative medium tends to accelerate potassium loss as well as hemolysis.

Studies on the rate of increase of free hemoglobin in the

plasma during the storage of blood have definitely established that the progressive increase in the plasma potassium is not due to the breakdown of the red cells.

The sealing of the storage container, thereby retaining the carbon dioxide content, or the storage of the blood under an atmosphere of carbon dioxide or nitrogen was found to have no noticeable effect on the rate of potassium diffusion from the cells.

De Gowin studied some specimens of blood during storage at room temperature ( 20° C.), and reported that although the rate of hemolysis was more rapid with time, the rate of potassium diffusion apparently was relatively unaffected. We now know that the rate of loss of potassium is less at room temperature than in the cold, but the increased hemolysis was probably responsible for offsetting this.

Dowmman, Oliver and Young (1940) also investigated extensively the partition of potassium in blood during storage in the cold. They collected the blood into the standard solution recommended at the time by the Medical Research Council (Great Britain). Two parts of blood were mixed with one part of the anticoagulant, which contained 1.05 gm. of sodium citrate and 0.85 gm. of sodium chloride per 100 ml. of solution. The mixtures were stored at 2° - 4° C. The "plasma", which in these samples were actually half plasma and half diluent, contained initially 10 mg. of potassium per 100 ml. This level was observed to increase rapidly during the first week of storage, after which time the rate of increase diminished and finally reached

a plateau, presumably when an equilibrium between cellular and plasma potassium was established. At this point, the potassium concentration per volume of water in the plasma equals the concentration of potassium in the cell water, which in turn is the same as the concentration in the whole blood on the basis of water content. The data presented by Downman and associates show "plateau" values for plasma potassium ranging from 80 to 130mg./100 ml., uncorrected for the plasma dilution. The authors suggest that their lower values possibly do not represent a true equilibrium state, but no values for the potassium in the whole blood are given to substantiate this. Since the hematocrit values are not given, there is ground, in the writer's opinion for suspecting that some of the law "equilibrium" values for the plasma potassium can be attributed to a low cell count. Low cellular potassium values similarly would result in a low equilibrium point for the plasma potassium; however, it is highly unlikely for a healthy donor to have a low concentration of potassium in his red cells. The writer offers these arguments only to emphasize the necessity of knowing what the cell volume and cellular potassium concentration are, before one attributes the results to differences in permeability of the red cells.

In further experiments, Downman et al (1940) tested the effect of dilution of the blood on the rate of escape of potassium from the red cells by comparing the behaviour of blood admixed with crystalline sodium citrate and that in samples collected into 3% sodium citrate in the proportion of 9 parts of blood to 1 part of

anticoagulant solution. It is unfortunate that these investigators did not attempt to compare the effect of dilution on individual blood samples. Since the rate of increase of plasma potassium was found to be so variable when stored with the standard M.R.C. solution, it is impossible to compare the influence of the different anticoagulants and dilutions on the escape of potassium from the red cells in different blood specimens, except perhaps by carrying out a statistical analysis of the results.

Downman states that the use of a smaller proportion of diluent did not significantly alter the rate of diffusion of potassium from the red cells during storage in the cold. He based this opinion on the concentration of potassium in the supernatant "plasma", without having corrected the values for the dilution with the added preservative. For example, if the red cells had comprised 50 percent of the volume of the original blood prior to dilution, they would make up only one-third of the total volume after dilution with one-half volume of anticoagulant solution. The proportion of "plasma" in the sample is therefore doubled, and values of 80 mg. and 130 mg. of potassium per 100 ml. of the diluted plasma would be equivalent to 160 mg. and 260 mg. per 100 ml. respectively in the plasma before dilution. During the first week of storage the plasma potassium in an undiluted blood sample may increase to about 130 mg. per 100 ml. It is apparent therefore that dilution of the blood does in fact hasten the escape of potassium from the erythrocytes.

In an experiment in which the blood was mixed with heparin (2.5 ml. containing 10 mg. per 150 ml. blood), the rate of diffusion

of potassium from the red cells was retarded, reaching a value of about 100 mg. per 100 ml. plasma by the end of the first week. Since the authors report only one experiment with heparinized blood, the writer is reluctant to agree that the apparent retardation of the potassium diffusion was attributable to the influence of heparin.

Downman and co-workers also have reported that the storage of blood in the fully oxygenated state, and under an oxygen tension of two atmospheres was found to have no effect on the rate of loss of potassium from the erythrocytes.

Of greater interest and significance are the observations of these workers regarding the effect of temperature on the egress of potassium from the red blood cells during storage. The most rapid loss apparently occurred at 38° C., the plasma potassium concentration having increased to 110 mg. per 100 ml. at the end of the second day of storage, and had reached the probable equilibrium point of 140-150 mg. per 100 ml. by the sixth day. No samples were analyzed prior to the second day of storage.

The rate of loss of potassium from the red cells in blood samples kept at room temperature was significantly less than that which occurred at 2° - 4° C. The rate of increase in the plasma potassium concentration during the first week of storage was found to be practically linear. The concentration was only 40-50 mg. per 100 ml. at the 6th day and 65-75 mg. per 100 ml. at the 12th day.

This raises the question, is the membrane of the erythrocytes more permeable to potassium at 38° C. and near 0° C. than at room temperature ? If Downman had analyzed more frequently, he would have found, in the blood stored at 38° C., that the concentration of cellular potassium actually remains normal for up to ten hours, while glycolysis proceeds. After the cells become depleted of glucose, however, they deteriorate and lose potassium very rapidly.

The relatively slow loss of potassium from the red cells when kept at room temperature, as reported by Downman, is difficult to explain. At 20° -25° C. glycolysis is sufficiently active to offset a loss of potassium for several days, whereas this is not so at 2-4° C. At room temperature the blood glucose should be used up in glycolysis in two or three days, and the cells then should be expected to lose potassium rapidly as a consequence of the failure in the metabolic activity. The relatively slow diffusion of potassium from the erythrocytes at room temperature has not yet been satisfactorily explained and the problem should be further investigated.

Maizels and Whittaker (1940b) emphasized the striking increase in the sodium content of the erythrocytes during cold-storage. Fresh human erythrocytes contain about 25 mg. of sodium per 100 ml., or about 11 milliequivalents per liter. When blood is stored in the cold the red cells take up sodium from the plasma

(Jeanneny, Servantie and Rigenbach,1939). Maizels and Whittaker used two anticoagulant solutions: one, which may be designated "A" was the standard M.R.C. solution devised by Harington and Miles (1939) and containing 1.05 gm. sodium citrate and 0.85 gm. sodium chloride per 100 ml.; the other mixture, "B", contained the same amount of sodium citrate as in "A", but only 0.45 gm. sodium chloride per 100 ml. Solution "B" was found to be isotonic with the contents of the blood cells, while "A" obviously is hypertonic. One part of either of these solutions was used with two parts of blood. Mixture "A", because of its greater content of sodium favours a greater rate of increase in the rate of sodium uptake by the red cells. Thus, after storage for 16 days in solution "B", with lower sodium content, the concentration of sodium in the red cells was about 150 mg. per 100 ml. (65 m.eq./liter), while in the hypertonic anticoagulant solution "A" it had increased to about 170 mg. per 100 ml. (74 m.eq./L) It would seem that the sodium concentration in the external medium, or more correctly, the external-to-internal sodium gradient influences, to a certain extent, the rate and magnitude of the entrance of sodium into the cell.

Miazels and Whittaker also studied the influence of acidification of the citrate medium with dilute hydrochloric acid on the entry of sodium into the red cells during storage. They confirmed the earlier observations of Jeanneny, Servantie and Rigenbach (1939) on the loss of potassium and gain of sodium by the red cells during storage in acidified media. The rate of spontaneous hemolysis during storage likewise was reduced by acidification of the citrate medium with hydrochloric acid, as used by Maizels, or by citric acid, as

first used by Jeanneny and his associates, and by citric acid with added glucose as used by Bushby and associates (1940). Loutit and associates (1943) popularized the use of the acidified diluent in Great Britain, while buffered media have been employed to this end by Meuther and Andrews (1941), Denstedt and co-workers (1943) and Parpart and co-workers (1947).

The influence of the lower $p^H$ of the medium on the sodium intake of the red cells is noticeable after one week in the cold, for when kept in a non-acidified medium they contain from 180-207 mg. sodium per 100 ml. (78-90 m.eq./liter), while those in the acidified medium contain only 82-149 mg. per 100 ml. (36-65 m. eq./liter). To explain the retardation of sodium uptake, Maizels suggested that a lower $p^H$ brings the cell membrane nearer the iso-electric point of the stroma protien ( $p^H$ 5) and thus reduces its permeability to positively charged ions ( Mond,1928, Jorpes,1932, Maizels,1935). Bushby (1940), on the other hand suggested that the retardation of glycolysis in acidified blood may be the factor responsible for the dminished alteration of the electrolyte composition in the cells.

In investigating the effect of various sugars and polysaccharides on the influx of sodium into the cells, Maizels and Whittaker found that both glucose and dextrin tend to retard electrolyte changes in the cells as was shown by the relatively small degree of sodium permeation, decreased swelling and decreased hemolysis. The effect of dextrin is understandable since it is readily hydrolyzed

by the amylases in the blood plasma and may yield glucose. A glucose concentration of 1000 mg. per 100 ml. of blood appeared to be the most efficient level in retarding hemolysis; lower concentrations of 300 or 100 mg. per 100 ml. were less effective. Fructose and sucrose were found to be relatively ineffective in favouring the preservation of the cells compared to glucose.

Aylward, Mainwaring and Wilkinson (1940a) tested the effect of storing the blood samples in the cold under various gases, with various anticoagulants and in the presence and the absence of glucose, on the diffusion of potassium from the erythrocytes. Storage of the blood under an atmosphere of air, oxygen, carbon dioxide, or under reduced pressures caused very little difference in potassium exchanges between the cells and the external medium. Storage in a variety of preservative solutions on the other hand, gave rise to very considerable differences in the rate of potassium loss from the red cells in a given period of storage. Six hundred milliliters of blood were mixed with each of the following solutions:

1.) 25 ml. 8.72% anhydrous sodium citrate.

2.) 9 ml. (45 mg.) heparin

3.) 25 ml. of a solution containing 8.72 gm. anhydrous sodium citrate and 25 gm. glucose per 100 ml. The behaviour of the potassium in the three blood mixtures is indicated in Table VIIIbelow.

TABLE VIII

The effect of different preservatives on the loss of $K^+$ from stored erythrocytes. (Aylward et al, 1940a)*

| Duration of Storage in Days | Plasma $K^+$ (mg./100 ml.) in 3 different media* | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| 0 | 21.2 | 21.7 | 21.9 |
| 1 | 27.9 | 33.7 | 38.7 |
| 2 | 39.9 | 48.7 | 53.6 |
| 3 | 58.2 | 65.0 | 62.1 |
| 5 | 88.0 | 79.7 | 77.9 |
| 9 | 92.4 | - | 97.6 |
| 16 | 129.0 | 131.0 | 115.0 |
| 23 | 154.0 | 159.0 | 129.0 |
| 30 | 170.0 | - | 145.0 |

* See text for description of preservatives used.

Alward et al. agree with previous investigations that the egress of potassium from the cells during the first few days of storage is not due to hemolysis, but suggest that the greatly diminshed rate of loss of potassium after two weeks of storage in the presence of added glucose is due to the stabilizing effect of glucose on the cells and the prevention of hemolysis.

In a later study, Aylward, Mainwaring and Wilkinson (1940b) investigated the effect of dilution of the blood with

solutions of sodium chloride, glucose or dextrin on the rate of loss of potassium from the erythrocytes during cold-storage. The compositions of the mixtures used were as follows:

1. 600 ml. blood and 25 ml. 8.72% sodium citrate solution.
2. 600 ml. blood and 9 ml. heparin solution.
3. 600 ml. blood and 25 ml. of a solution containing 8.72 gm. sodium citrate and 24 gm. glucose per 100 ml.
4. 200 ml. blood, 100 ml. 2.1% sodium citrate and 100 ml. 1.7% sodium chloride.
5. 200 ml. blood, 100 ml. 2.1% sodium citrate and 100 ml. of a solution containing 1.7 gm sodium chloride and 6 gm. glucose.
6. 200 ml. blood, 100 ml. 2.1% sodium citrate and 100 ml. of a solution containing 1.7 gm. sodium chloride and 18 gm. glucose.
7. 200 ml. blood, 100 ml. 2.1% sodium citrate and 100 ml. of a solution containing 1.7 gm. sodium chloride and 12 gm. dextrin.
8. 420 ml. blood, 70 ml. of a solution containing 2.5 gm. sodium citrate and 0.85 gm. sodium chloride per 100 ml. and 10 ml. 50% glucose solution.

The increase in the plasma potassium concentration during storage of the blood mixture at 4° C. is indicated in Table IX, which has been compiled from the data given by Aylward et al.

TABLE IX

The effect of different preservatives on the loss of $K^+$ from stored erythrocytes (Aylward et al., 1940b)*

| Duration of Storage in Days | Plasma $K^+$ (mg./100 ml.) in 8 different media* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 0 | 21 | 22 | 22 | 13 | 14 | 15 | 20 | - |
| 2-4 | 58 | 65 | - | - | - | - | - | 56 |
| 14-16 | 128 | 131 | 115 | 51 | 56 | 63 | 61 | 82 |

*See text for description of preservatives used.

In order to compare the effect of different preservatives and dilutions on the increase in plasma potassium, it is necessary to apply a correction to the plasma potassium concentrations found, to allow for the dilution of the plasma. Aylward and his associates have intended to do this, but in the case of mixtures 4,5,6 and 7, have ignored the fact that by diluting the blood with an equal volume of diluent the volume of the plasma was not doubled but tripled (assuming a hematocrit value of 50% for undiluted blood). This is illustrated in Table X, in which the first column gives the uncorrected values for the concentration of potassium in the plasma on the 14-16th day of storage; the second, the values as corrected by Aylward, and the third, the values as corrected by the writer.

TABLE X

Plasma $K^+$ after 14-16 days of cold-storage in different preservatives (Aylward et al., 1940b)*

| Blood Mixture* | Concentration of $K^+$ in the Plasma (mg./100 ml.) | | |
|---|---|---|---|
| | Uncorrected | Corrected Partially | Corrected |
| 1 | 128 | 139 | 139 |
| 2 | 131 | 135 | 135 |
| 3 | 115 | 124 | 124 |
| 4 | 51 | 97 | 153 |
| 5 | 56 | 107 | 169 |
| 6 | 63 | 120 | 188 |
| 7 | 61 | 116 | 182 |
| 8 | 82 | 104 | 112 |

* See text for description of preservatives used.

The corrected values in column 3 are the direct reciprocal of the potassium concentration in the red cells, and show the true magnitude of potassium egress from the erythrocytes during storage in the various media. From Aylward's corrected values, it would appear that dilution of the blood delays the rate of potassium loss, whereas the writer's corrections show that dilution of the blood actually accelerates the rate. Additional difficulty arises in the interpretation of the results because of the wide differences in the tonicity of the various diluents used. All the solutions, with

the possible exception of mixture 2 containing heparin, were hypertonic and may be expected to cause shrinkage of the erythrocytes. This would produce an increase in the volume of the plasma, and dilute the plasma contents further, the degree depending on the degree of cell shrinkage, but this was not determined.

Scudder and Smith (1940), and Scudder, Smith, Tuthill and Drew (1940) have advocated the storage of blood under an atmosphere of carbon dioxide, since their results showed that this procedure delayed the escape of potassium from, and the entry of sodium into, the cells. Table XI illustrates this effect of carbon dioxide.

TABLE XI

The effect of storing blood under an atmosphere of carbon dioxide on the cation changes in the plasma

| Duration of Storage in Days | Plasma $K^+$ in m.eq./l | | Plasma $Na^+$ in m.eq/l. | |
|---|---|---|---|---|
| | Air | $CO_2$ | Air | $CO_2$ |
| 0 | 4.5 | 4.5 | 156 | 161 |
| 1 | 8.0 | 6.5 | 156 | 161 |
| 5 | 14.5 | 9.0 | 149 | 159 |
| 8 | 19.0 | 13.0 | 145 | 155 |
| 15 | 23.0 | 16.0 | 121 | 133 |

The blood specimens were collected into a 3.5% solution of sodium citrate in the proportion of 9 parts of blood to 1 part of the anticoagulant, and stored at 4° C. After 15 days, 27% of the potassium had diffused out of the red cells in the

specimen collected and stored under air while the potassium loss amounted to 10% in the sample collected and stored under $CO_2$. By storing blood under $CO_2$, the decomposition of the plasma bicarbonate with a resultant elevation of the $p^H$ is repressed. The retardation of changes in the electrolyte composition of the cells may be attributed to the influence of the lower $p^H$ under these conditions of storage. However, Downman et al (1940) state that they observed no protective effect when they stored blood under oil. Furthermore, Aylward et al (1940a) did not observe this effect when they stored blood under $CO_2$, but they offer no data to substantiate their statement.

In a very comprehensive investigation, Maizels (1943) studied the effect of various preservative mixtures on the changes in electrolyte distribution in stored blood. The changes in cell volume and the content of total base in the cells after storage of blood in the cold for six and twelve days are described in Table XII, which has been compiled from Maizels' data. The blood-preservative mixtures employed were as follows:

1. Citrated blood as described above.
2. Citrated blood, plus added glucose to bring the glucose concentration to 650 mg. per 100 ml.
3. Citrated blood, plus added glucose to bring the glucose concentration to 2300 mg. per 100 ml.
4. Rous-Turner solution (1916) as described above.

5. Acid-citrate-dextrose (ACD) solution of Loutit, Mollison and Young (1943), containing glucose in a concentration of 600 mg. per 100 ml.
6. Strongly acid-citrate-dextrose solution, the composition being the same as that of mixture No. 2, except that 5 ml. of N/10 HCl was added per 25 ml. of blood.
7. Sucrose solution of Wilbrandt (1940).

Table XII

The effect of different preservatives on the cation changes in stored erythrocytes (Maizels, 1943)*

| Preserved Sample | Duration of Storage in Days | Cell Volume as percent of original | Cation content of cells in milliequivalents/ liter | | |
|---|---|---|---|---|---|
| | | | $K^+$ | $Na^+$ | $K^+$ & $Na^+$ |
| Original blood | 0 | 100 | 103 | 12.2 | 115 |
| 1 | 6 | 103 | 69.6 | 52.8 | 122 |
| | 12 | 119 | 56.0 | 93.1 | 149 |
| 2 | 6 | 102 | 68.2 | 51.8 | 120 |
| | 12 | 107 | 56.2 | 69.2 | 125 |
| 3 | 6 | 99 | 64.9 | 48.6 | 113 |
| | 12 | 103 | 56.2 | 65.0 | 121 |
| 4 | 6 | 110 | 58.2 | 33.6 | 92 |
| | 12 | 109 | 39.3 | 43.2 | 82 |
| 5. | 6 | 106 | 74.0 | 40.0 | 114 |
| | 12 | 109 | 61.8 | 53.8 | 116 |
| 6 | 6 | 111 | 86.1 | 25.2 | 111 |
| | 12 | 115 | 73.6 | 40.2 | 114 |
| 7 | 6 | 89 | 47.2 | 51.2 | 98 |
| | 12 | 93 | 35.3 | 68.0 | 103 |

* See text for description of preservatives used.

It should be noted that unlike the procedure followed by the majority of investigators in following the changes in electrolyte content of the red cells by periodic analysis of the plasma, the values for sodium and potassium in the above table were obtained by analysis of the erythrocytes themselves. There is no necessity, therefore, to make corrections for the dilution of the sample with the preservative solutions. Thus, a basic conclusion arrived at from indirect evidence earlier in this review is confirmed, namely that glucose itself does not check the escape of potassium from the red cells during storage, although the entry of sodium apparently is delayed. The increase in the total cell base is somewhat diminished and the degree of swelling is decreased. A lowered $p^H$ of the preservative medium delayed both the egress of potassium and the ingress of sodium. It is noteworthy that in the acidified medium the total base content of the cells remains unchanged, but despite this, the cells undergo a progressive swelling during storage. The relatively lower $p^H$ of solution No. 6 is more effective than the standard acidified solution of Loutit et al. (1943) in retarding the cation shifts between the cells and the plasma. The effect of diluting the blood with a large volume of solution of low potassium content is evident in the case of the Rous-Turner and the Wilbrandt solutions. The increase in the sodium content of the cells is relatively small in the solutions because the diluents are themselves relatively low in sodium content. Thus, a decrease in the cation content of the cells occurs when either of these solutions is employed, but this does not necessarily entail a shrinkage in cell volume. It is apparent that other factors

in addition to the alteration of the concentration of sodium and potassium in the cells contribute to changes in the volume, or water content of the erythrocytes during cold-storage.

Maizels concluded that none of the changes in the inorganic elements of the blood can serve as a basis for evaluating the physiological condition of the red cells in preserved specimens, inasmuch as there is little correspondence between the magnitude of these changes and cell "viability" as measured by the capacity of the preserved cells to survive in the circulation after transfusion.

Loutit, Mollison and Young (1943) studied further the effect of $p^H$ and more specifically, the effect of acidification of the blood specimen on the rate of diffusion of potassium from the erythrocytes into the plasma. By adding citric acid to the anticoagulant solution, the $p^H$ of the blood mixture was reduced to 7.15. In a neutral anticoagulant the $p^H$ of the blood mixture is about 7.4. This slight degree of acidification was definitely effective in retarding the diffusion of potassium from the erythrocytes, compared to the rate of escape in blood stored with neutral anticoagulants. Table XIII illustrates the changes in the plasma potassium concentration in the three following blood mixtures:

1. Medical Research Council solution (Harington and Miles,1939), as described above.
2. Rous and Turner solution (1916) as described above.
3. Acid-citrate-dextrose solution (Loutit et al,1943), as described above.

TABLE XIII.

The effect of different preservatives on the loss of $K^+$ from stored erythrocytes (Loutit et al. 1943)*

| Duration of Storage in Days | Plasma $K^+$ (mg./100 ml.) in 3 different media* | | |
|---|---|---|---|
| | 1 (MRC) | 2 (R-T) | 3 (ACD) |
| 0 | 14 | 18 | 15 |
| 7 | 98 | 96 | 76 |
| 14 | 121 | 131 | 96 |
| 21 | 146 | 149 | 121 |
| 28 | 150 | 157 | 141 |

* See text for description of preservatives used.

One might suppose that if the natural levels of sodium and potassium in the blood could be maintained during storage, or the degree of disturbance be kept to a low degree, the survival of the erythrocytes after transfusion might be extended. The experimental evidence does not support this hypothesis, for although the rate of loss of potassium from the red cells in the acidified solutions has been reduced, the fact remains that the preservation of cell viability also is very satisfactory in highly diluted samples such as the mixtures of Rous and Turner, and De Gowin, which favor a relatively rapid escape of potassium from the erythrocytes.

Rapoport (1947a) has studied the exchange of sodium and potassium in the red cells during storage in an endeavour to compare the relative preservative efficiency of a simple citrated blood mixture, a citrated blood mixture with enough added glucose to raise the level to 600 mg. per 100 ml., and a citrate-glucose mixture acidified with citric acid according to the method of Loutit and Mollison. In the first mentioned specimen, 10 ml. of 3.2% sodium citrate solution were mixed with 100 ml. of blood. The second specimen received 15 ml. of 2.13% sodium citrate and 5% glucose solution per 100 ml. of blood. The third blood mixture consisted of 25 ml. of a solution containing 1.33 gm of sodium citrate, 0.47 gm of citric acid and 3.0 gm. of glucose per 100 ml., plus 100 ml. of blood.

The results recorded with respect to the diffusion of potassium are typical; the concentration in the plasma increases rapidly initially, then the rate gradually diminishes until diffusion equilibrium is reached. In the simple citrated blood the halfway value towards equilibrium (about 78 mg. per 100 ml.-20 m. eq. per liter) is reached in about 8 days. Complete equilibrium, that is, when extracellular and intracellular potassium concentrations are equal, is reached in about 30 days, when the plasma potassium level reaches 156 mg. per 100 ml. (40 m.eq. per liter). In contrast, half the equilibrium concentration is reached in about 12 days in the citrate-glucose mixture, and in about 23 days in the acidified blood mixture. Complete equilibrium is reached in about 40 days in the former mixture and in about 60 days in the latter.

The increase in the plasma potassium concentration during storage reflects the progressive decrease in the concentration in the red cells, but these quantities show equivalence only when the plasma potassium values are corrected for the dilution of the plasma by the preservative solution added. Rapoport has not corrected for this, creating the illusory impression that the presence of glucose in the preservative solution delays the loss of potassium from the erythrocytes. However, the corrected values show that glucose actually does not retard this process compared to that in the simple citrated specimen. If anything, the extra dilution represented in the glucose-containing medium accelerates the rate of escape of potassium. The retarding effect of the lower $p^H$ of the acidified medium, on the other hand, is a real one, although it is not as marked as it would appear from the uncorrected plasma values.

The shift of sodium from the plasma to the cells appears to be reciprocal to that of the potassium from the cells into the plasma in all the preservative mixtures investigated by Rapoport. A comparison of the three preservatives with regard to the rate and magnitude of the transfer of sodium leads to essentially the same conclusions as arrived at for potassium in the preceding paragraph.

In a further study, Rapoport (1947b) compared the rate of escape of potassium from the red cells in blood stored with four different mixtures, the McGill II solution ( Denstedt et al. 1944), the ACD solution (Loutit et al. 1943), De Gowin's

solution (1939) and Alsever's solution (1941). The McGill II solution consisted of two parts of 3.2% sodium citrate solution, 1 part of 0.3 M sodium phosphate buffer at $p^H$ 7.35, and 2 parts of 5.4% glucose solution, per 10 parts of blood. The ACD solution consisted of 25 ml. of a solution containing 1.33 gm. of sodium citrate, 0.47 gm. of citric acid and 3.0 gm. of glucose per 100 ml. of blood. De Gowin's mixture consisted of 2 parts 3.2% of sodium citrate solution and 13 parts of 5.4% glucose per 10 parts of blood. Alsever's mixture as modified and prepared by the Baxter Laboratories and used in Rapoport's study, contained 0.80 gm. of sodium citrate, 0.0075 gm. of citric acid, 0.42 gm. of sodium chloride and 2.05 gm. of glucose per 100 ml., and mixed with an equal volume of blood.

The rate of loss of potassium from the erythrocytes during storage was lowest in the ACD solution, although this is not apparent from the plasma potassium values, due to differences in the dilution of the plasma with the various preservatives. The relatively small degree of dilution in the case of the ACD solution would, in itself, favour the retention of potassium by the erythrocytes. The two-fold dilution of the plasma in Denstedt's mixture favours a relatively greater rate of loss of potassium, while the four-fold dilution of the plasma with De Gowin's mixture, which is low in electrolyte, favours a rapid loss of potassium during storage. Blood preserved in Alsever's solution, at $4^o$ C.,

apparently behaves as in the neutral solutions with regard to potassium loss from the red cells.

Rapoport also studied the exchange of potassium in blood stored with Alsever's solution at 25° C., in an endeavour to test the claim that satisfactory blood preservation could be achieved with this solution without the use of refrigeration. The rate of escape of potassium from the red cells was precipitous, the concentration in the cells reaching equilibrium with that in the plasma within ten days. The effect of storage at 25° C. on the functional state of the cells was, to quote Rapoport, "catastrophic".

Parpart and co-workers (1947b) made a careful study of the effect of temperature, $p^H$ and glucose concentration on the rate of potassium diffusion from the erythrocytes. The influence of temperature on the loss of potassium is indicated in Table XIV. These data were obtained with a preserved sample containing nine parts of blood, one part of 3.25% sodium citrate solution and two parts of 5.4% glucose solution. The values are expressed as the percentage of the original cell content of potassium that had escaped into the plasma during storage at the various temperatures indicated.

TABLE XIV

The effect of temperature on the loss of $K^+$ from stored erythrocytes

Parpart et al., (1947b)

| Duration of Storage in Days | Percent of $K^+$ lost from the erythrocyte stored at various temperatures | | | | |
|---|---|---|---|---|---|
| | 0° | 4° | 10° | 12° | 18° |
| 15 | 65 | 50 | 40 | 35 | 30 |
| 36 | 90 | 60 | 50 | 65 | - |

It is significant that the magnitude of the potassium loss after 15 days of storage was greater the lower the temperature of storage. However, for a storage period of 36 days 10° C. would appear to be the optimum temperature so far as potassium retention by the red cells is concerned.

In investigating the effect of $p^H$ on the rate of loss of potassium from the erythrocytes, Parpart used phosphate buffers to maintain a constant hydrogen ion concentration. Table XV has been compiled to illustrate this effect.

TABLE XV

The effect of $p^H$ on the loss of $K^+$ from stored erythrocytes.

| Duration of Storage in Days | Percent of $K^+$ lost from the erythrocytes stored at various $p^{Hs}$ | | | | | |
|---|---|---|---|---|---|---|
| | 5.7 | 6.1 | 6.5 | 6.7 | 7.0 | 7.5 |
| 7 | 68 | 38 | 21 | 0-5 | 5 | 20 |
| 14 | 80 | 55 | 35 | 25 | 25 | 80 |
| 28 | 100 | 95 | 70 | 65 | 80 | - |

These data indicate that the rate of loss of potassium from preserved erythrocytes is minimal in specimens of $p^H$ 6.7 -7.0, and that the rate of loss is increased at higher or lower $p^H$ than these limits.

In addition to the above experiments with phosphate buffers, Parpart also tested the effect of other buffers on the retention of potassium by the red cells during storage. It appears that phosphate ions exert a specifically beneficial effect on potassium retention,

quite apart from its participation in the buffer activity. Parpart found that a glycyl-glycine buffer also was exceptionally effective in stabilizing the preserved erythrocytes. A maleic acid buffer, and to a lesser degree, a maleic-succinate buffer was detrimental to the preservation of the erythrocytes, even at the optimum $p^H$. The effect is undoubtedly due to the ability of maleic acid to completely inhibit the glycolytic activity of the erythrocytes during storage.

The beneficial effect of added glucose in the preservative mixture on the retention of potassium by the red blood cells during storage is slight. This action is most pronounced with a glucose concentration of 500 mg. per 100 ml.;higher concentrations up to 6000 mg. per 100 ml. exert little benefit and in fact appear to accelerate the potassium egress in older blood, compared to that at lower glucose levels. The rate of loss, however, is not as great as when no glucose is incorporated in the medium.

C. Glycolysis and Phosphorolysis in Stored Blood.

The process by which glucose or glycogen is converted to lactic acid is commonly referred to as "glycolysis", involving an integrated series of enzyme catalyzed reactions. The enzymatic process in the erythrocytes is closely similar to, if not identical with that in muscle, as formulated by Meyerhof, Embden, Parnas and others. The nature of the glycolytic process in blood has been extensively reviewed by several authors (Guest,1932; Guest and Rapoport,1941; Andreae,1946; Woodford,1948; Kwiencinska,1948).

That the glycolytic activity in blood is confined exclusively to the formed elements has been amply demonstrated (Rona and Doblin,1911; MacLeod,1913; Maizels,1941; etc.) Bird (1947) has estimated that the contribution of the erythrocytes to the total glycolytic activity of the blood is about eight times that of the leucocytes. The glycolytic activity of the whole blood varies with the blood of different individuals, but most bloods are capable of causing 10-25 milligrams of glucose to disappear per 100 ml. of blood per hour at 37° C. (MacLeod,1913; Schmitz and Glover, 1927; Guest,1932; Bose and De,1942).

The present day view of the course of glycolysis and of the intermediates involved in the process in muscle has been recently reviewed by the author (1952). Glycolysis in blood proceeds in much the same manner as in muscle. In the erythrocyte glucose is the primary substrate since glycogen is present only in traces. To be utilized, glucose must first be phosphorylated at the expense of a

high-energy phosphate bond of adenosine triphosphate (ATP). This is effected by the enzyme hexokinase and the glucose-6-phosphate thus formed is isomerized to fructose-6-phosphate, which is phosphorylated again at the expense of ATP to give fructo furanose -1,6-diphosphate. So far 23,000 calories have been lost by the breakdown of two molecules of ATP, while the two energy-poor phosphate bonds of fructose diphosphate amount to a gain of 4,000 calories (Meyerhof and Green,1949). The net loss to the system is therefore about 19,000 calories. These initial "priming" reactions, resulting in a gain of free energy, seem to be essential for the energy of the hexose molecule to become biologically available.

Cleavage of fructose diphosphate gives two molecules of "triose phosphate", an equilibrium mixture of dihydroxyacetone phosphate (95%) and 3-phospho-glyceraldehyde (5%), followed by a simultaneous phosphorylation and dehydrogenation of the latter in the presence of diphosphopyridine nucleotide (DPN) catalyzed by the enzyme 3-phosphoglyceraldehyde dehydrogenase. The mechanism of this unique reaction was clarified by Meyerhof and Junowicz-Kocholaty (1943). The 3-phosphato-glycerol phosphate thus formed contains a bond of even higher energy than those in ATP, and in the presence of a specific phosphokinase (Bucher,1947) transfers this to ADP, forming 3-phosphoglyceric acid and ATP. The 3-phosphoglyceric acid isomerizes, with 2,3 - diphosphoglyceric acid functioning as the co-enzyme of the mutase (Cori et al. 1949), to give 2-phosphoglyceric acid. This compound is dehydrated

by enolase with a decrease in free energy of only about 600 calories (Meyerhof and Oesper,1949), giving rise to pyruvic acid enol phosphate. Here again we see the augmentation of the energy level of the phosphate bond, making possible the formation of another molecule of ATP, and trapping the energy of glycolysis in a transferable form.

It is thus apparent that during the oxidation of one molecule of glucose to two molecules of pyruvic acid, the energy of two 11,500 calorie bonds has been used up, while four 16,000 calorie bonds have been generated. These appear eventually as four 11,500 calorie bonds in ATP. However, the greater part (about 80%) of the energy of the glucose molecule is still present in the two molecules of pyruvate.

In the absence of oxygen, when the co-enzymes of the hydrogen transport system are in the reduced state, or in tissues such as erythrocytes with a weak or incomplete respiratory system, the pyruvic acid is reduced to lactic acid by the DPN. $H_2$ formed during the oxidation of 3-phosphoglyceraldehyde.

Several of the phosphorylated intermediates and co-enzymes of glycolysis are present in more than trace or catalytic amounts in the erythrocytes. These compounds are generally designated together as the "organic acid-soluble phosphorus compounds "(OASP), and their amounts are referred to by their phosphorus content. Guest and Rapoport (1938) divide these compounds into three main

classes according to their resistance to acid hydrolysis. (1) adenosinetriphosphate phosphorus ( 9-12 mg. P. per 100 ml. of red cells), (2) Hexose phosphate phosphorus (15 mg. P. per 100 ml. of red cells) and (3) diphosphoglyceric acid phosphorus (25-30 mg. P. per 100 ml. of red cells). Fresh blood contains, in addition, 1-3 mg. P as inorganic phosphorus and about 15 mg. P as lipid phosphorus per 100 ml.

Greenwald (1925) showed that human blood cells contain a relatively large amount of an acid-stable diphosphoglyceric acid. He identified this as being the 2,3 isomer, which Cori and associates (1949) have shown to be present in catalytic amounts in other tissues where it serves merely as the co-enzyme of the mutase which catalyzes the interconversion of the 2- and the 3-phosphoglyceric acids. The acid resistant 2,3-diphospho ester, which contains about one-half of the organic acid soluble phosphorus of the blood is derived from the acid labile 1,3- diphospho ester. In the transfer of the phosphate from the first to the second carbon atom in the molecule the high energy bond is lost. This reaction in the erythrocyte has been investigated by Rapoport and Luebering (1950,1952), who have suggested that 3-phosphoglyceric acid has a catalytic role. The reaction is essentially irreversible because of the considerable decrease in the free energy associated with the transfer of the phosphate group from the carboxyl to the secondary alcohol group.

That the 2,3-isomer of diphosphoglyceric acid can be metabolized in blood, however, has been well established. Instead

of phosphorylating a mole of adenylic acid as does 1,3-diphosphoglyceric acid, inorganic phosphate is liberated yielding the 2-or3-phosphoglyceric acid. The actual mechanism of this reaction is not yet understood. The phosphoglyceric acid is metabolized normally, eventually yielding pyruvic acid and providing a phosphate for the phosphorylation of ADP to ATP in the process. Since the large amount of 2,3-diphosphoglycerate in the erythrocyte is already in the oxidized state, it cannot reduce DPN during its enzymatic breakdown, and consequently the pyruvic acid derived from it does not undergo reduction to lactic acid.

The rate of utilization of glucose in blood at body temperature becomes slower when the $p^H$ is lowered, and accelerated when the $p^H$ is raised above the physiological level (Martland et al., 1924,1925; Roche and Roche,1927; Warmser et al, 1942). Guest and Rapoport (1941) have attributed this phenomen to the effect of $p^H$ on the rate of phosphorylation of glucose.

During normal glycolysis the rate of entry of glucose into the scheme balances the rate of breakdown of the phosphorylated intermediates, and the inorganic phosphate remains at a constant low level. If the entry of glucose is in any way retarded, and the phosphate esters continue to be metabolized normally, the amount of organic acid-soluble P compounds will be decreased,

and inorganic P increased (Rapoport and Guest,1939a 1939b). Similarly if the rate of phosphorylation of glucose exceeds the rate of the other glycolytic reactions, there will be an increase in the phosphorylated intermediate esters, and a decrease in inorganic phosphate (Rona and Doblin,1911; Lawaczeck,1924; Martland et al., 1924; Rona and Iwasaki,1927; Engelhart and Braunstein,1928; Roche and Roche,1929, etc.) This phenomenon can occur not only at body temperature, but as Dische (1946) has shown, fructose diphosphate and triose phosphate can accumulate in citrated human blood when kept at 4° C. for 24 hours. Incubation of the blood subsequently at 40° C. results in the complete breakdown of these intermediates to lactic and pyruvic acid.

Most of the fundamental investigational work on the interrelationships between the metabolism of glucose, the phosphate esters and inorganic phosphate during the cold-storage of human blood has been done in the Department of Biochemistry, McGill University, under the supervision of Dr. O. F. Denstedt (Andreae, 1946; Woodford,1948; Kwiecinska,1948).

Although the rate of glycolysis becomes progressively slower with decrease in the temperature (Tolstoi,1924; Bose and De,1942), the overall operation of the system at 4° C. is practically identical with that at 37° C. ( Kwiecinska,1948). From a perusal of the available data it appears that the rate

of glucose disappearance and lactic acid formation is 15-25 times greater at 37° C. than at 4° C. When the supply of glucose becomes depleted, lactic acid production practically ceases, while pyruvic acid accumulates rapidly as the phosphorylated esters, consisting mainly of diphosphoglyceric acid, continue to be metabolized. This observation is in accord with the earlier findings of Guest (1932) that the organic acid-soluble phosphate esters in the red cells remain unaltered for 10-20 hours when blood is incubated at 37° C. At this time the supply of glucose usually is exhausted, and a sharp increase occurs in the inorganic phosphate content of the blood. Glycolysis can be maintained for a longer time by adding glucose to the blood and thus affording a continued production of the intermediate phosphate esters at 37° C., and at lower temperatures (Maizels,1941; Andreae, 1946).

Several investigators have determined the rate and extent of glycolysis during the cold-storage of citrated whole blood (De Gowin et al.,1939; Bick,1939; Belk et al, 1939; Gwynn and Alsever,1939; Rosenthal et al,1941; Denstedt et al., 1941). Their findings are in general agreement and show that during the first three days of storage the rate of disappearance of glucose amounts to 15-25 mg. per 100 ml. of blood per 24 hours. From the third day on, glycolysis slows down, but the normal glucose content of the blood is usually exhausted within ten days. The difficulty in determining small amounts of glucose in the presence of other

reducing substances has misled some investigators into believing that glucose is present even after two or three weeks of storage in the cold.

Concurrently with the disappearance of glucose from the blood, a slow increase in the inorganic phosphate content usually occurs, even during the early and more active phase of glycolysis. Occasionally there is a slight decrease in inorganic phosphate, indicating that the rate of phosphorylation exceeds that of dephosphorylation, or, in other words, the rate of formation of the phosphate esters exceeds the rate of their breakdown. The rapid accumulation of inorganic phosphate that occurs after the sugar has been depleted signifies the continued breakdown of the phosphorylated intermediates.

The addition of glucose to the preserved blood sample results in the maintenance of the glycolytic activity for a longer period and the retarding of the breakdown of the phosphate esters. The glycolytic activity, which is quite pronounced in the initial period of storage, diminishes progressively, and eventually fails about the fifth week, despite the presence of and ample supply of glucose (De Gowin et al., 1939: Bick,1939;etc). While the glycolytic activity remains relatively active, the concentration of the organic acid-soluble P compounds remains fairly constant, and the increase in inorganic P is relatively

slight. However, during the second week of storage the diminishing rate of phosphorylation of glucose fails to balance the rate of metabolism of the diphosphoglyceric acid, resulting in a rapid breakdown of this compound, and a correspondingly marked increase in the concentration of inorganic phosphate in the blood. The supply of diphosphoglyceric acid in the erythrocytes gives rise to pyruvic acid, which is not reduced to lactic acid. The pyruvic acid concentration therefore tends to rise in preserved blood during the second week of storage (Andreae,1946).

While it has long been known that acidification of blood decreases the rate of glycolysis in the erythrocytes at higher temperatures, there has been some difference of opinion among certain authorities regarding the effect of lowering the $p^H$ on the glycolytic activity of preserved blood during cold-storage. Maizels and Whittaker (1940) acidified citrated blood with dilute hydrochloric acid and noted an apparently improved preservation. No glucose was added to the blood sample, but at the end of one month of storage at $4^o$ C. the glucose content of the sample was determined. The results led these investigators to believe that the improved preservation that had been effected by having acidified the blood was not attributable to the retarding of the glycolytic activity by the lower $p^H$, since the reducing "sugar" content of the acidified, the neutral and the

slightly alkaline blood specimens was about the same. They failed to realize that the glucose is depleted within a few days during storage, and that what they were measuring was a residual non-fermentable reducing substance which is un-effected by acidification.

Loutit, Mollison and Young (1943) studied the glycolytic activity of blood preserved in various media including the Medical Research Council (MRC) preservative then in use in Great Britain, the Rous-Turner mixture, and with their own acidified type of preservative mixture (ACD). All the solutions contained glucose. Periodically during the storage of the blood, the glucose concentration of both the plasma and the cells was determined. Some of their results are tabulated in Table XVI.

TABLE XVI.

The disappearance of glucose from blood over a period of 28 days at 4° C. (Loutit, et al 1943).

| | Glucose in mg./100 ml. in 3 preservative media* | | | | | |
|---|---|---|---|---|---|---|
| | MRC | | R-T | | ACD | |
| | Plasma | Cells | Plasma | Cells | Plasma | Cells |
| Initial Concentration | 700-740 | 540-560 | 2850 | 2280 | 700 | 550 |
| Net Decrease in 28 days | 80- 90 | 140 | 110 | 160 | 60-110 | 150 |

* See text for description of preservative used.

These results give the impression that the rate of glycolysis is not altered by the lower $p^H$ of the acidified, ACD mixture, but evidently Loutit neglected to take into account the much larger volume of diluent in the M.R.C. and Rous-Turner blood mixtures than in the ACD mixture. While the decrease in glucose in terms of concentration was practically the same in all the mixtures, the absolute amount of glucose metabolized in the acidified blood was obviously less than in the neutral mixtures.

Loutit concluded from the above data that glycolysis had taken place both in the cells and the plasma, since glucose apparently had been used up at different rates in the two compartments. We know now that the cell-free plasma possesses no glycolytic capacity. Two facts are noteworthy from these results, however.

1) Added glucose is partitioned equally between the cellular and the plasma water up to levels of at least 3000 mg. per 100 ml. The ratio of cellular to plasma water in human blood is $\frac{70\%}{93\%}$, or 0.75, which closely approximates the ratio of the cellular to the plasma glucose concentrations.
2) The rate of disappearance of glucose from the cells exceeds that in the plasma. This could easily be an apparent difference due to swelling of the cells, with a dilution of the cell glucose content, and a corresponding shrinkage of the extracellular phase, with an increase in the plasma glucose concentration. The relatively greater rate of fall in the

glucose concentration of the cells might also arise from a decrease in the amount of non-fermentable reducing substances which are confined within the cells. From a theoretical point of view, it is also quite possible that the metabolic mechanism that admits glucose into the cell interior ( Le Fevre, 1947; 1948; Wilbrandt, 1947,1950) undergoes a progressive failure during cold storage, and that glucose consequently is not brought into the cells as rapidly as it is used up.

Rapoport (1947a) found that blood containing about 600 mg. of added glucose per 100 ml. utilized about 200 mg. of glucose per 100 ml. during storage in the cold. With neutral preservative media, this amount of glucose was metabolized in 30 days, when the process ceased, whereas with acidified preservative media the process continued for 50 days. These observations that acidification lowers the rate, but not the overall extent of glycolysis in blood at 4° C. have been confirmed by the McGill group, and are analagous to the findings of Martland concerning the glycolytic behaviour of blood at 37° C. Similarly, as Guest and Rapoport (1941) pointed out, acidification of the blood inhibits the phosphorylation of glucose at 37° C.,it is reasonable to suppose that it does so at 4° C. as well. Despite the decreased rate of entry of glucose into the glycolytic process, the metabolism of the organic acid-soluble phosphate esters

continues at an unaltered rate, resulting in a rapid decrease in the concentration of these compounds and a corresponding increase in inorganic phosphate. These changes proceed from the beginning of storage of acidified blood (Maizels,1943; Loutit et al.,1943).

Maizels (1943), Rapoport (1947a,b,d) and the McGill Group have studed the behaviour of the "easily hydrolyzable" phosphorus compounds in the blood during storage in the cold. This fraction reflects the state of the labile phosphate groups of adenosine triphosphate (ATP), which is of particular interest since the compound is not only required for the phosphorylation of glucose, but serves as a store of high-energy phosphate bonds, and indicates the overall metabolic condition of the cells. The addition of glucose to blood, by sustaining glycolysis, helps maintain the amount of ATP during storage. Whereas the ATP has usually disappeared within 10-15 days from citrated blood in the cold, it is preserved for 25-30 days when glucose has been added, and for 45-50 days when acid as well as glucose are in the preservative medium. Maizels has attributed the effect of acidification on the conservation of ATP to inhibition of an adenosine triphosphatase. However, since the phosphorylation of glucose by ATP is retarded by a lowering of the $p^H$ of the blood, it is more reasonable to attribute the superior preservation of ATP

to the diminished rate of utilization. This "sparing" effect results in a true economy in the energy resources of the cell.

While a considerable amount of attention has been directed towards determining the rate and extent of glycolysis in blood during storage in the cold, comparatively little attention has been paid to the glycolytic power of preserved erythrocytes returned to body temperature. In an effort to evaluate the overall metabolic condition of preserved erythrocytes, Rapoport (1947a,b,d) tested the glycolytic power of the blood by incubating a sample for 90 minutes at 37° C. and measuring the disappearance of glucose and the production of lactic acid. Although the results were variable, a fairly definite pattern of behaviour was evident as is illustrated in Table XVII, in which the values represent milligrams of glucose utilized by 100 ml. of erythrocytes per hour at 37° C.

TABLE XVII

The glycolysis rate at 37° C. of bloods stored in three preservative solutions at 4° C.

| Duration of Storage in Days | Glucose utilized in mg./100 ml. cells/hour | | |
|---|---|---|---|
| | Citrate* | Citrate-dextrose | Acid Citrate dextrose |
| 1 | 50 | 40 | 18 |
| 5 | 25 | 30 | 18 |
| 7 | 15 | | 18 |
| 10 | 5 | 20 | 18 |
| 15 | 0 | 10 | 18 |
| 20 | | 5 | 18 |
| 25 | | 0 | 18 |
| 30 | | | 10 |
| 45 | | | 0 |

* Glucose added immediately before incubation.

It can be seen from the above data that blood stored without additional glucose rapidly loses its glycolytic power, in spite of the addition of adequate substrate immediately before incubation. Glucose in the preservative medium enables the red blood cells to retain their glycolytic power for a longer period, but not for more than three weeks. The effect of acidification on the maintenance of the glycolytic capacity is quite pronounced. Although the initial rate of glucose disappearance was lower than in the neutral blood mixtures, Rapoport's data suggest that no impairment in the glycolytic power is evident during the first month of cold-storage. However, the "scatter" of the data is so great that interpretations based on the mean values may be misleading. Thus it would appear that some specimens were comparable with fresh blood in glycolytic capacity after 30 days of storage, while others had lost this faculty completely.

There is a striking degree of correlation between the glycolytic capacity of stored blood and its ATP content. Also, there is a reasonable degree of correlation between the preservation of these two factors and the ability of the preservative medium to maintain cell viability during storage, as tested by the capacity of the cells to remain in the circulation after transfusion.

In general, the preservative mixtures that favour the maintenance of ATP in the erythrocytes during storage are superior

by in vitro testing as well. It is evident then, that the general metabolic status of the cells affords a truer index of their preservation and viability than do other in vitro criteria such as spontaneous hemolysis, cell fragility or cation diffusion.

Several authors have studied the changes in the concentration of the esterified and inorganic phosphate in the blood during storage. These studies were carried out not so much from an interest in the glycolytic mechanism, as in the changes in the electrolyte and osmatic equilibrium of the red cells during storage. Maizels and Whittaker (1940a) followed the changes in the inorganic P and the organic acid-soluble P content of the plasma and the cells of blood stored in the cold, and after incubation of the preserved specimens for 90 minutes at 37° C. The preservation medium used was that of Harington and Miles (1939), which is a hypertonic solution of sodium citrate and sodium chloride, mixed with two volumes of blood.

The changes in the content of the inorganic phosphorus and organic acid-soluble phosphorus fraction (OASP) during storage and after 90 minutes incubation at 37° C. are shown in Table XIX.

TABLE XVIII

Inorganic and organic phosphorus changes in blood during cold-storage and after 90 minutes at 37° C.

| Duration of Storage in Days | Inorganic P mg./100 ml. | | OASP mg./100 m. |
|---|---|---|---|
| | Plasma | Cells | Cells |
| 1 | 2.2 | 1.5 | 54.9 |
| 1 ≠ I* | 3.1 | 5.3 | 48.0 |
| 6 | 2.8 | 3.1 | 50.1 |
| 6 ≠ I | 4.1 | 6.9 | 43.1 |
| 7 | 2.4 | 8.6 | 39.4 |
| 7 ≠ I | 5.0 | 10.6 | 32.6 |
| 15 | 3.3 | 29.7 | 12.8 |
| 15 ≠ I | 7.2 | 20.0 | 13.0 |
| 21 | 4.5 | 33.6 | 6.9 |
| 21 ≠ I | 8.6 | 24.6 | 6.8 |
| 26 | 9.4 | 32.9 | 6.1 |
| 26 ≠ I | 13.2 | 26.9 | 4.2 |

* I= 90 minutes incubation at 37° C.

It can be seen from the above table that during two weeks of storage the inorganic P of the plasma increased but slightly, while that of the red cells increased twenty-fold due to the precipitous liberation of inorganic P from the phosphate esters between the 6th to the 15th day of storage. The ratio of the inorganic P in the cells to that in the plasma at the 15th day was about 9:1, and when one considers that the water content of the erythrocyte is only 70%, the ratio of the concentration of

the inorganic P in the cells to that in the plasma, that is, the "phosphate gradient" is of the order of more than 10:1.

Incubation of the blood had a dual effect with regard to the content of inorganic P in the erythrocytes. In the first place the rate of breakdown of the organic phosphate esters exceeds the rate of their breakdown; the OASP fraction decreases and the inorganic P fraction increases. Secondly, the red cell membrane, which in the cold is almost impermeable to phosphate ions, becomes much more permeable to this ion at $37^{o}$ C., and on incubation at this temperature the inorganic phosphate which had accumulated in the cells during storage in the cold is rapidly released into the plasma. This is in accord with the earlier findings of Maizels (1932) and Halpern (1936) who showed that at $2^{o}$ C. the diffusion of inorganic P through the erythrocyte membrane is extremely slow, at $20^{o}$ C. the rate is increased by 60%, and at $30^{o}$ C. the rate is $2\frac{1}{2}$ times as rapid as at $20^{o}$ C.

In the fresher blood specimens (less than one week old), the release of phosphate from the organic compounds predominates during incubation and the cellular inorganic P increases. After prolonged storage, incubation while favouring a rapid breakdown of the organic phosphates, causes an even more rapid release of inorganic phosphate into the plasma, the net effect being to reduce the steep concentration gradient of inorganic phosphate that had arisen during cold-storage.

Aylward and his colleagues (1940a) studied the alterations in the phosphate fractions in blood during storage in various preservative media. Their results agree in general with earlier findings of Maizels as referred to above, except that they observed that the inorganic P usually underwent a decrease in concentration during the first day of storage of citrated blood, and that in the case of blood stored with added glucose the initial diminution in inorganic P often persisted for a week. The decrease in the inorganic P content of the red cells is due to the utilization of phosphate in the synthesis of more of the organic acid-soluble P fraction in the erythrocytes. The amount of organic acid-soluble phosphate always varied inversely with that of the inorganic phosphorus fraction. Within the first day of storage the organic acid-soluble P increased from an initial value of 36 mg. P per 100 ml. to 39 mg. P per 100 ml., while the inorganic P content fell from 4 mg. P per 100 ml to 2 mg. P per 100 ml. When the organic acid-soluble P content of the cells had decreased during storage to a level of 4 mg. per 100 ml., the concentration of inorganic P had risen to 30 mg. per 100 ml. While presenting data for the behaviour of the phosphate fractions in citrated blood only, the authors state that similar results were obtained with heparinized blood and with specimens enriched with glucose.

In another series of experiments, Aylward, Mainwaring and Wilkinson (1940b) measured the plasma inorganic P in a great variety of preservative media, described in detail in an earlier section. The results of their studies showed that glucose and dextrin, added to the blood, had only a slight effect in retarding the rate of increase in the inorganic P of the plasma. This, of course, does not necessarily imply that the added glucose did not exert a beneficial effect on the preservation of the phosphate esters within the cells, since the plasma inorganic P is at best a very unreliable index of the concentration of inorganic P in the red cells.

In heparinized blood, the inorganic P was released into the plasma still more rapidly than in citrated blood during cold-storage. It is not apparent from the data whether heparin favours a more rapid hydrolysis of the organic phosphates in the cells, or facilitates the escape of inorganic phosphate by altering the membrane permeability or whether it facilitates both these processes.

Maizels (1941) endeavoured to further elucidate the role of glucose in inhibiting the breakdown of the phosphorylated esters. He showed that the amount of inorganic phosphate liberated during the first two weeks of storage was about $2\frac{1}{2}$ times greater in blood to which no glucose had been added than in blood which the glucose

concentration had been increased by 100 mg. per 100 ml. This difference in the rate of hydrolysis of the organic phosphate cannot be attributed to any difference in the $p^H$ of the two specimens, since this difference was too small ( $p^H$ 7.08 compared to $p^H$ 6.95) to produce a significant effect (Martland et al., 1924). Maizels attributed the effect of glucose in preserving the phosphate esters to the inhibition of the phosphatases of the blood. If the dephosphorylation of these esters is brought about by phosphatase action, one would expect the process to proceed from the beginning of storage, and not be delayed for periods of one or two weeks. Also, one would expect the products of diphosphoglyceric acid breakdown to be glyceric acid and inorganic phosphate, instead of the pyruvic acid and inorganic phosphate as found by Andreae (1946).

Maizels postulated further that another way in which glucose aids in the preservation of stored erythrocytes is by being absorbed onto the cell surface, and enabling the membrane to stretch and allow the cells to swell. He and Whittaker (1940a;1940b) showed that the critical bursting volume, or the volume when the membrane ruptures, can be increased by 20% in the presence of glucose. On placing the cells in a series of saline solutions of graded concentrations he found that the maximum degree of swelling that the cells could attain before bursting was 164% of their original volume. On the addition of 1% glucose to the hypotonic saline solution, the cells were capable

of swelling to 184% of their original volume before rupture occurred.

The claim that glucose helps maintain the relative impermeability of the erythrocyte membrane to cations has not been well substantiated. The results of most workers indicate that the addition of glucose has no influence on the rate of loss of potassium from the erythrocytes during storage. (See preceding section for a complete discussion).

The effect of glucose on the phosphate distribution is not due to any influence on the permeability of the cell membrane, but is due to its participation in the cell metabolism. Acidification of the preservative medium similarly exerts its effect on the phosphate partition by influencing the metabolism of glucose. However, Loutit et al.(1943) have shown that acidification in excess of that in the usual ACD solution, does accelerate the diffusion of inorganic phosphate from the cells.

D. Active Cation Transport in Stored Blood.

Maizels and Whittaker (1940) attempted to take advantage of the acquired high sodium content of preserved erythrocytes to test the capacity of the cells to remain in the circulation after transfusion. On transfusing red cells with a high sodium content, Maizels and Paterson (1940) had observed that the sodium content of the circulating erythrocytes in the recipient was increased in proportion to the sodium content of the transfused cells. They found, however, that in a few hours the sodium concentration in the circulating erythrocytes had returned to that in the recipients red cells prior to the transfusion. Since it was known that the transfused cells had not been destroyed, they must have undergone a chemical reconstitution, with the result that the excess of intracellular sodium had been removed. Maizels points out that such an ionic transfer cannot take place against the steep ionic gradient by any simple physical process of diffusion.

An example of this phenomenon is cited, in which red blood cells containing 142 mg. of sodium per 100 ml. (62 m. eq. per liter) were transfused into a recipient with a presumably normal plasma sodium content of 300-350 mg. per 100 ml. (130-150 m. eq. per liter). The infused cells were "reconditioned" to such an extent that their sodium content was decreased to 33 mg. per 100 ml. (14 m. eq. per liter), implying the expulsion of 109 mg. of sodium per 100 ml. (48 m. eq. per liter) from the cells. While recognizing the need for a complex biological system to

accomplish this ionic transfer against the concentration gradient, Maizels failed to visualize the erythrocytes ability to perform this function of their own accord. He suggested, at the time, that the erythrocytes may possibly be "reconditioned" in the spleen.

Maizels (1943) has further shown that the "reconditioning", of erythrocytes after transfusion occurred with respect to potassium as well as to sodium. That this takes place in opposition to the concentration gradients was demonstrated by the following direct method. A sample of group - O blood was stored for one week, during which time the cells lost a considerable amount of potassium and gained a similar amount of sodium from the plasma. The preserved specimen was then infused into a group - A recipient, and samples of blood were withdrawn thereafter at intervals during a period of 48 hours. The group - A cells of the recipient were selectively agglutinated by the method of Ashby, and the unagglutinated group - O cells in the sample were separated and analyzed for their sodium and potassium content. The findings are tabulated in Table XIX,

Table XIX

The cation content of stored erythrocytes before and after transfusion.

| Time of analysis | Erythrocyte cation in m. eq./liter | |
|---|---|---|
| | K⁄ | Na⁄ |
| Before transfusion | 47 | 55 |
| 1 hour after | 44 | 60 |
| 24 hours after | 76 | 34 |
| 48 hours after | 82 | 26 |

These analyses indicate that the electrolyte composition of the transfused red cells tended to be restored to normal, the cells having regained 35 m. eq. of potassium per liter, and having expelled 30 m. eq. of sodium per liter within 48 hours after transfusion. The plasma of the recipient was found to contain 3.6 m. eq. of potassium and 144 m. eq. of sodium per liter, so it is obvious that the uptake of potassium and the expulsion of sodium by the preserved cells had occurred in each case against a steep ionic gradient.

Harris (1940; 1941a; 1941b) demonstrated that the normal distribution of potassium and sodium between the red cells and the plasma is maintained by the metabolic activity of the cells. Thus, when the metabolic activity was markedly decreased, as when the cells are kept in the cold, the control over the distribution of potassium and sodium is diminished and these ions tend to diffuse along the concentration gradients. When the metabolic activity was partially restored by returning the blood cells to 25° C. or 37° C., the direction of movement of each of the ions was reversed so that the movement occurred against their respective concentration gradients and tended to reestablish the original normal distribution. Thus Harris' experiments afford in vitro confirmation of the findings of Maizels and Paterson (1940) and Maizels (1943), and proves that the "reconditioning" of preserved erythrocytes is brought about by the cells themselves through their metabolic activity.

The metabolism of glucose was found to be essential for the expulsion of sodium and the recovery of potassium by the previously cold-stored erythrocytes, but Harris believed that glycolysis per se was probably not the fundamental process by which the reconstitution is achieved. The prolongation of a normal metabolic activity by the addition of glucose allows the cells to maintain the normal distribution of cations for many hours when fresh blood is kept at body temperature. The depletion of glucose or the inhibition of glycolysis causes the erythrocytes to lose the ability to retain potassium and to exclude sodium.

Danowski (1941) also showed that the erythrocytes can maintain their potassium content when incubated at 37° C., and that they continue to do so as long as the normal glycolytic activity is maintained. With freshly drawn blood glycolysis continues for about five hours, when the natural glucose content becomes depleted, but the activity may be prolonged further by the addition of glucose.

Maizels (1948; 1949a; 1949b; 1951), Flynn and Maizels (1949) and Ponder (1950) have extended the investigations of Harris on the recovery of potassium and the expulsion of sodium by erythrocytes whose cation compositions had been altered during storage in the cold. These authors have re-emphasized that the movements of cations against their concentration gradients cannot be attributed to any simple physical process,

but must involve an active transport mechanism which requires energy for it's operation. The optimum pH for this metabolically activated transfer of cations is about 7.5, although the process does occur, but at a diminished rate, over a wider pH range of from about pH 7 to 8. Since the utilization of glucose is greatly accelerated at the higher pH levels, it is obvious that the rate of glycolysis is not in itself a direct index of active cation movements. As these publications have appeared almost simultaneously with the work being reported in this thesis, their findings will be discussed more fully in a discussion of the writer's results.

## Methods

### A. Collection of the Blood:

All the donors were healthy students, 20 to 30 years of age. The blood was withdrawn from the median basilic vein through a wide needle (no. 14-16) and a rubber tube, into a bottle containing the anticoagulant solutions. Detailed descriptions of these solutions will be given with each experiment.

In the majority of the storage studies the blood was dispensed immediately after withdrawal into Wasserman tubes, stoppered and sealed with paraffin wax. In others, the blood was dispensed into small Erlenmeyer flasks, stoppered and covered with gauze pads held in place with rubber bands. A few specimens of blood were stored in bulk lots of 200-400 ml., and aliquots were removed as required for analysis or further manipulations. In instances where only a small amount of blood was required, the specimen was drawn from the vein into a dry syringe and transferred to a vessel containing an anticoagulant.

The strictest aseptic precautions were observed throughout all manipulations.

### B. Storage of the Blood:

The blood specimens were stored in an electric refrigerator at 5° C. ( $\pm$ 1° C.).

The specimens were mixed daily in order to resuspend the cells. In the cases where the blood was in small tubes, the mixing was effected by inverting the tubes several times, while this was accomplished by gentle swirling of the blood in the flasks.

C. Incubation of the Blood:

The blood specimens for incubation were removed from the refrigerator and immediately fixed to a rocking apparatus powered by an electric motor and fitted to a constant-temperature water bath. Incubation of the specimens was carried out for varying lengths of time, in all cases at 37° C. The speed of rocking and the arc traversed was set to keep the cells completely suspended, and also to keep mechanical traumatization of the cells to a minimum. The usual rate of rocking was 15-20 excursions per minute through an arc of 180° for the sealed tubes, and through an arc of 90° for the gauze-covered flasks. The sealed tubes were completely immersed in the water of the bath, while the small flasks were adjusted to keep only the portion containing the blood immersed.

Each tube, or flask, upon removal from the bath was opened and aliquots for whole blood determinations were removed immediately. The remaining blood was centrifuged within a few minutes, the plasma removed, recentrifuged and kept for further analyses.

In the cases where the blood was stored in bulk, aliquots were placed in small tubes immediately before incubation and subsequently handled as described above. All such transfers were made as aseptically as possible.

D. Isotonic Solutions:

Numerous investigators have determined the concen-

tration of many of the anticoagulant solutions that possess the same osmotic pressure as blood serum. The writer, reinvestigated this by the freezing-point depression method, using the Beckmann thermometer. Blood serum freezes at $-0.565^{\circ}$ C. so the isotonic or isosmotic freezing point depression is $0.565^{\circ}$ C.

The concentrations of some of the solutes investigated that produce the same freezing point depression in aqueous solutions are as follows:

| | |
|---|---|
| Sodium chloride (NaCl) | 0.88 gm. per 100 ml. solution |
| Trisodium citrate ($Na_3C_6H_5O_7 3H_2O$) | 3.12 gm. per 100 ml. solution |
| Sodium oxalate ($Na_2C_2O_4$) | 1.58 gm. per 100 ml. solution |
| Potassium oxalate ($K_2C_2O_4 H_2O$) | 2.22 gm. per 100 ml. solution |
| Potassium oxalate ($K_2C_2O_4$) | 2.00 gm. per 100 ml. solution |
| Glucose | 5.15 gm. per 100 ml. solution |

The blood was usually mixed with isotonic solutions of the anticoagulants as determined above. It should be pointed out, however, that such solutions are only isosmotic to the erythrocytes if they do not permeate the red cell membrane. In the case of oxalate ions and glucose this is not so, since these distribute themselves rapidly on either side of the membrane and are therefore osmotically inactive. Dilute aqueous solutions of oxalate and glucose thus cause the erythrocytes to swell, and oxalate and glucose should therefore be added to blood either dry or in concentrated solutions.

E. Analytical Methods:

1. Potassium:

Potassium was determined by the cobaltinitrite method (Breh and Gaebler, 1930; Zwemer and Truzkowski, 1937) as modified by Dr. A. Neufeld and furnished to the writer by Dr. O.F. Denstedt. The potassium-silver-cobaltinitrite precipitate is formed during 30 minutes at 20° C. (± 1° C.). After four washings with a mixture of peroxide-free diethyl ether and ethyl alcohol, the precipitate is dissolved in dilute acid. Ammonium thiocyanate is then used to develop a deep blue color with the cobalt of the precipitate, which is read in the Evelyn colorimeter using the 620 filter.

A standard curve for potassium in plasma or blood was constructed and found to be reproducible under standardized conditions. Individual potassium determinations were done in duplicate and if these did not fall within ± 2% of each other the determination was repeated in duplicate.

All the potassium analyses were done in "sets" of from four to ten determinations, in duplicate. As an additional check on the accuracy of the methods, representatives of several of these "sets" were redetermined together. This technique of checking the results can be best illustrated by an example. A blood specimen might be stored for four days, and on each day of storage four aliquots would be incubated for varying lengths of time, making sixteen specimens in all. Using an eight-place centrifuge, the potassium can be determined in four of the specimens in duplicate

making one "set". Thus, the potassium determinations for each day are made in one "set", making four "sets" in all. A fifth "set" of potassium determinations is then made on a representative of each of the previous four "sets". This precaution has eliminated instances of otherwise undetected errors very efficiently. This concept of cross-checking has been adopted for all the analytical procedures where it was deemed necessary.

2. Sodium

Sodium was determined by modifications of Weinbach's (1935) procedure, based on the zinc-uranyl-acetate method of Barber and Kolthoff (1928; 1929). Several of the accepted procedures were considered, but found unsatisfactory because they were on the whole too time consuming, in view of the number of determinations anticipated. The practicable modification of Fowweather and Anderson (1948) was employed in some of the preliminary studies. While this method is quite satisfactory for clinical purposes, for which it was designed, it was found to be not sufficiently sensitive, and was abandoned.

The procedure finally adopted was the colorimetric modification of Stone and Goldzieher (1949). The sodium-zinc-uranyl acetate salt is formed during twenty minutes at 20° C. ($\pm$ 1° C.). After washing the precipitate a color is developed in the presence of ammonium carbonate and hydrogen peroxide. The method is based on the Rosenheim-Daehr reaction in which

the uranyl ion, in strongly alkaline solution, is treated with hydrogen peroxide to produce a complex of intensely reddish yellow color. This method was found to be very satisfactory and reproducible within ± 2% when the proper precautions were observed.

3. Magnesium

A method for the determination of magnesium that was both practical and had promise of reliability was the Titan yellow method of Hirschfelder and Serles (1934), employing a visual colorimeter. The writer undertook to modify this procedure in order to obtain a color readable in the Evelyn photoelectric colorimeter.

The principle of the determination lies in the ability of magnesium hydroxide to form a bright red complex with an acridine-sulfo dye, Titan yellow. A systematic study of this reaction was undertaken to determine the optimum conditions for color development, the spectroscopic properties of the red color formed, the influence of interfering ions, notably calcium and oxalate, and the concentration range of magnesium that would give a proportional color. The Titan yellow method thus developed for estimating magnesium in plasma and blood is rapid, easy and accurate. The proteins are precipitated with trichloroacetic acid, the dye is added to the filtrate, and upon addition of alkali a red magnesium hydroxide-Titan yellow lake is formed which can be measured colorimetrically. Soluble starch serves to stabilize the insoluble lake. Calcium in concentration up to at least 25 mg. per 100 ml.

and oxalate up to at least 15 mg. per 100 ml. do not affect the determination of magnesium by this technique.

The procedure as devised and used by the author is as follows:

1.0 ml. of blood or plasma is added to 9 ml. of 10% trichloroacetic acid in a 15 ml. conical centrifuge tube. After thorough mixing the protein precipitate is removed by filtration or centrifugation. 5 ml. of the clear filtrate is transferred to an Evelyn colorimeter tube and 3 ml. of 0.5% soluble starch in 90% saturated sodium chloride solution, 1 ml. of 0.02% Titan yellow solution (made daily from a stock 0.1% solution) and 1 ml. of 6 N sodium hydroxide are added in succession. After mixing and allowing ten minutes for color development, the color is read in the Evelyn colorimeter using the 10 ml. aperture and the 520 filter. The blank is prepared by using 1 ml. of water instead of the 1 ml. of plasma, and is set at 100% transmission in the colorimeter. A standard curve is contructed by using 1 ml. of the appropriate magnesium solutions in place of the 1 ml. of plasma. The results are reproducible within $\pm$ 2% and 95-105% magensium added to plasma can be recovered.

About 20% of serum magnesium is protein-bound (Soffer, 1939; Dine 1942), but is completely liberated by the trichloroacetic acid. This has been demonstrated by acid digestion and ashing of plasma or blood and determining the magnesium content

of the digest or ash. These results checked within ± 5% of the values determined by trichloroacetic acid extraction.

After the development of this method for the determination of magnesium in blood and plasma, the writer found that Garner (1946) had made practically identical observations two years previously, using gum ghatti as the dispersing agent and reading the resulting color in the Spekker absorptiometer. Heagy (1948) further modified the technique, using polyvinyl alcohol as the dispersing agent and reading the color in the Evelyn colorimeter. No comparative studies of the three techniques have been attempted by the present writer.

4. Iron

Iron was determined on ashed blood and plasma by the O - phenanthroline method of Barkan and Walker (1940). The plasma iron determined by this technique is not to be confused with the "serum iron" of the above authors, which is a dilute acid-extract of serum or plasma and does not include hemoglobin iron. The determination of iron in the plasma, as modified by the writer includes organically bound iron and was used as an index of hemolysis in the blood plasma. This method for the determination of hemoglobin in blood or plasma is accurate provided the appropriate corrections for non-hemoglobin iron are made.

The procedure adopted was as follows:

0.5 to 1.0 ml. of plasma or 0.02 ml. of blood are introduced into a pyrex boiling tube and ashed on an electric hot-plate by repeated additions of concentrated nitric acid, until a pure white residue remains and all the nitrogen dioxide fumes have been driven off. After cooling, 1 ml. of 10% hydrochloric acid is added to dissolve the salt and the volume is made up to 5 ml. with water. Then, in succession, are added 1 ml. of saturated sodium acetate, 1 ml. of 1% hydrazine sulphate in 2 m sodium acetate buffer (pH 4.5) and 1 ml. of 0.1% of O - phenanthroline. The tubes are stoppered, mixed and allowed to stand for two hours for color development. Finally an additional 2 ml. of water are added, the contents are transferred to Evelyn cuvettes and the color is read in the Evelyn colorimeter using the 10 ml. aperature and the 490 filter and setting the blank at 100% transmission. The blank must be carried throughout the entire procedure, including the exact amount of nitric acid added for digestion, since iron-free nitric acid was unobtainable.

A standard curve was constructed over the range of 2 to 20 micrograms of iron, which is equivalent to a range in plasma of 200 to 2000 micrograms of iron per 100 ml. when 1 ml. of plasma is used. This curve was reproducible, and duplicate determinations checked within $\pm$ 5%. Micrograms of iron were converted to milligrams of hemoglobin, by dividing by the factor 3.35.

5. Chloride

Chloride was determined by the method of Schales and Schales (1941), which involves a titration of the chloride ions in a Folin-Wu filtrate of plasma or blood, using diphenylcarbazone as indicator. Deproteinization of the plasma is not necessary, but it enhances the ease of titration and the accuracy of the determination. The method is accurate to $\pm$ 2%.

6. Phosphorus

The estimation of phosphorus as inorganic phosphate was carried out by the method of Fiske and SubbaRow (1925). The blue phosphomolybdate color was read in the Evelyn colorimeter, filter 660.

The analytical procedure for the partition of the organic acid-soluble phosphorus compounds in blood is based on their different rates of hydrolysis by mineral acids. This method was devised by Lohmann (1928) and first applied to blood by Bomskov (1932). It was shown that during seven minutes at 100° C. in 1 N hydrochloric acid, two of the three phosphate groups of adenosine triphosphate are liberated as inorganic phosphates. This phosphorus fraction will be referred to as the "Labile Phosphate" (L.P.), and represents the easily hydrolyzable phosphate groups of adenosine di- and triphosphate. On prolongation of this hydrolysis for one hundred minutes, one of the phosphate groups of hexose diphosphate is liberated as inorganic phosphate.

This phosphorus fraction liberated by the additional ninety-three minutes of acid hydrolysis will be referred to as the "Hydrolyzable Phosphate" (H.P.), and serves as an index of the content of hexose diphosphate in the blood. Only complete digestion will liberate the phosphate from the stable diphosphoglyceric acid of blood. This will be referred to as the "Stable Phosphates" (S.P.) and gives an estimate of the 2,3 diphosphoglyceric acid in blood.

It is obvious that such a procedure provides only an inaccurate estimation of the various phosphate fractions of blood, since no one compound is hydrolyzed specifically during the somewhat arbitrarily chosen periods. Great as this defect of the procedure on fresh blood may be, it is probably open to even greater criticism when applied to stored blood, where the amounts of organically-bound phosphorus are very different from that in fresh blood, which is what the method was designed for. Nevertheless, one is able to obtain estimates of the magnitude of the alterations in the various phosphate esters of the blood during storage and incubation by this method that are not only reproducible, but are in good agreement with other biochemical findings (Andrea, 1946).

The procedure adopted for the complete partition of the acid-soluble phosphate of blood was as follows:

0.5 ml. of blood or plasma are added to 5 ml. of ice-cold trichloroacetic acid in a 15 ml. conical centrifuge

tube. The tube is stoppered, shaken vigorously and immediately centrifuged at a high speed for three minutes. Steps a.) and b.) as described below are carried out immediately upon removal of the tube from the centrifuge. Itwas found that steps c.)and d.) could be postponed for several hours at least.

a.) Inorganic Phosphate (I.P.)

A 1 ml. aliquot of the filtrate is placed in an Evelyn colorimeter tube, and 9 ml. of water, 1 ml. of 2.5% ammonium molybdate and 0.4 ml. of the sulphonic acid reagent of Fiske and SubbaRow are then added in that order. After mixing and allowing 15 minutes for color development, the tubes are read in the colorimeter.

b.) Labile Phosphate (L.P.)

A 1 ml. aliquot of the filtrate is placed in a colorimeter tube and 1 ml. of 2 N hydrochloric acid is added. The tube is then immersed in boiling water for exactly seven minutes, cooled immediately and made to 10 ml. with water. The phosphate liberated is then determined as inorganic phosphate. The "Labile Phosphate" equals the inorganic phosphate after seven minutes hydrolysis, minus the preformed "Inorganic Phosphate" (a), and serves as an index of the adenosine triphosphate (ATP) and adenosine diphosphate (ADP) content of the blood.

c.) Hydrolyzable Phosphate (H.P.)

This determination is done in the same way as the "Labile Phosphate", except that the tubes are heated in the water bath for one hundred minutes. The "Hydrolyzable Phosphate" equals the inorganic phosphate after hydrolysis for one hundred

minutes minus the inorganic phosphate after hydrolysis for seven minutes, and serves as an index of the amount of hexose diphosphate in the blood.

d.) Stable Phosphate (S.P.)

A 1 ml. aliquot of the filtrate is placed in a pyrex boiling tube and digested with 0.2 ml. of 70% perchloric acid on a hot-plate until colorless. After cooling, the contents are made to 10 ml. with water and the inorganic phosphate is determined as described above, giving the total acid-soluble phosphate (TASP). This fraction does not change in blood during storage or incubation, and therefore serves as a check on the sampling of the blood. The organic acid-soluble phosphate (OASP) is the difference between the TASP and the IP. The "Stable Phosphate" is calculated as the difference between the TASP and the phosphate found after hydrolysis for one hundred minutes. The "Stable Phosphate" is due mainly to the two phosphate groups of the 2,3 diphosphoglyceric acid of the blood, but also includes the phosphate of hexose monophosphate and of adenylic acid.

7. Glucose

Glucose was determined by Nelson's (1944) modification of Somogyi's copper reduction method. The proteins and the non-glucose reducing substances are precipitated with zinc sulphate and barium hydroxide, and the copper-reducing activity is determined in the filtrate. The final color of the reduced arsenomolybdate is read in the Evelyn colorimeter, filter 520.

This method was found to be accurate to ± 2% over a wide range of glucose concentrations, and does not include any of the nonfermentable reducing substances of blood.

8. Erythrocyte Fragility

The fragility of the erythrocytes to hypotonic salt solutions was determined by the method of Waugh and Asherman (1938). The blood is added to various dilutions of Simmel's solution (a complex salt solution adjusted to p H 7.35) and the percentage of hemolysis in each dilution is determined in the Evelyn photoelectric colorimeter. This procedure is more reproducible than the more common fragility tests using simple sodium chloride solutions.

9. Hemoglobin

Hemoglobin was estimated by the oxyhemoglobin method of Evelyn (1936). Twenty cubic millimeters of blood are introduced into 10 ml. of water, a drop of ammonia water is added, and the color read against a water blank in the Evelyn colorimeter, filter 540. The method was calibrated with a hemoglobin solution whose concentration was determined by Van Syke's oxygen capacity method.

The hemoglobin content of the plasma was estimated by diluting 0.1 - 0.5 ml. of plasma to 10 ml. with water and reading it against a blank containing the same amount of non-hemolyzed plasma, obtained from the same blood sample before it was either stored or incubated. The percentage of hemolysis is then calculated from the amount of hemoglobin in the plasma.

10. Hematocrit

The hematocrit was determined in Wintrobe tubes, by centrifugation of the blood until no change in the red cell volume occurred over a 10 minute period. The gravitational field was the same in all determinations, but the time of centrifugation differed. Fresh blood cells usually reach a minimum volume more rapidly (30 minutes) than stored or incubated specimens (up to one hour), and it was decided to standardize the technique to attain maximum packing rather than by centrifuging for a predetermined time. No corrections for trapped plasma were made, as it is believed that these prescribed factors apply only to fresh cells and not to the more spherical cells of stored or incubated blood.

11. Oxygen

The oxygen content of the blood was determined in the Van Slyke volumetric apparatus (Hawk and Bergeim, 1937). The blood specimens for oxygen analysis were stored and incubated in sealed immunological tubes. In order to avoid oxygenation or deoxygenation of the blood during sampling, a procedure was devised that is described briefly as follows:

After thorough suspension of the blood cells by repeated inversion of the sealed tubes, the stopper is removed and immediately replaced with a tightly fitting cap or diaphragm of thin rubber. Through this is inserted the sampling pipette

which reaches to the bottom of the tube. The blood is then subjected to a positive pressure by means of a syringe and needle also inserted through the rubber cap, forcing the blood to rise in the pipette to the required height. The object of this procedure is to avoid subjecting the blood to a negative pressure as occurs when drawn into a pipette in the usual manner. A heavy walled Ostwald pipette graduated to deliver between marks and provided with a stopcock was used.

The determinations of the oxygen content of the blood were not entirely satisfactory, since the Van Slyke volumetric apparatus is not as accurate as the Van Slyke manometric apparatus generally recommended for oxygen determinations. Duplicate determinations were reproducible to only $\pm$ 8%.

12. Glycogen

Glycogen was determined by a modification of the method of Good, Kramer and Somogyi (1933).

The procedure followed was to centrifuge the blood and remove the plasma and white cells by suction. The cells are then made up to the volume of the original whole blood specimen with water and thoroughly mixed. A 2 ml. aliquot of

this hemolysate is transferred to a 15 ml. graduated conical centrifuge tube and 1 ml. of 60% potassium hydroxide is added. The tube is then immersed in boiling water for 20 minutes to dissolve the tissue and to destroy any glucose present. 3.5 ml. of 95% ethanol are then added, the contents mixed and again brought to boiling. After cooling the tube is centrifuged at high speed for 15 minutes. The supernatant fluid is then decanted off and allowed to drain. The precipitate is washed with 60% ethanol and again centrifuged, decanted and drained. The alcohol still present is expelled by heating momentarily in the boiling water bath. The precipitate is then hydrolyzed by heating with 2 ml. of 2/3 N sulphuric acid for 2 hours in a boiling water bath. Covering the tube with a glass agate prevents evaporation during the hydrolyzing period. After cooling, the resulting solution is neutralized to phenol red with 10% sodium hydroxide, and the volume brought to 3 ml. with water. The reducing power of this solution was determined on 1 ml. aliquots by the method of Nelson (1944) for the determination of glucose.

13. Carbonic Anhydrase

The carbonic anhydrase activity of the blood was determined by a method that is essentially that of Meldrum and Roughton (1933). This technique measures the catalytic effect of the blood on the rate of evolution of carbon dioxide from a sodium bicarbonate solution when mixed with a phosphate buffer

of p H 6.8. A two compartment glass vessel or "boat", fitted with a ground-glass stopper and connected by a length of pressure tubing to a water manometer is the main part of the apparatus. The "boat" is held in a constant temperature bath and is connected to an electric shaker.

One compartment of the "boat" holds the bicarbonate solution, the other the phosphate buffer and the enzyme solution. With the solutions in their proper compartments the "boat" is connected to the manometer, immersed in the water bath and connected to the shaking mechanism. Determinations were made at $0^{o}$ C. and $15^{0}$ C., the temperature being controlled fairly well with ice and an electric stirrer. After temperature equilibration had been reached in the "boat", the reaction was started by rapid mixing of the two solutions and shaking with the electric motor, standardized at four back and forth swings per second through an angle of $90^{o}$. The manometer is read at five second intervals. The activity of the enzyme solution is expressed in units, E, which is defined by the equation:

$$E = \frac{(R - R_o)}{R_o}$$

Where $R_o$ is the reciprocal of the time taken for the second quarter of the carbon dioxide to be evolved when no catalyst is present (water substituted for blood), and R is the reciprocal of the time taken for the second quarter of the evolution when the catalyst is present. For E to be proportional to the amount of carbonic anhydrase present the blood

must be diluted extensively, and in most of the determinations was diluted 500 fold in distilled water.

The writer wishes to acknowledge the helpful suggestions of Dr. O.F. Denstedt in the design of the apparatus used and the assistance of Dr. E.A. Hosien in it's construction and maintenance.

14. Cholinesterase

The cholinesterase activity of the blood and plasma was measured manometrically in the Warburg apparatus by a method that is essentially that of Ammon (1933), as modified by Du Bois and Mangun (1947). For practical reasons, this method has been further modified by the writer, and as finally adopted was as follows:

2.1 ml. of Ringer-bicarbonate buffer of pH 7.4 (0.02 M sodium bicarbonate, 0.15 M sodium chloride and 0.04 M magnesium chloride) are placed in the main compartment of the Warburg vessel and 0.1 ml. of the enzyme solution (blood, plasma, cell suspension, hemolysate, etc.) is added. Into the sidearm is introduced 0.3 ml. of 0.1 M acetylcholine chloride solution. Appropriate control vessels are prepared: enzyme without substrate and substrate without enzyme.

After gassing with 95% nitrogen and 5% carbon dioxide for 10 minutes and equilibration in the bath at 38° C. for 15 minutes, the manometers are set at 50 mm. and read at "zero" time. The substrate is then tipped in from the side arm and readings are taken at five minute intervals for thirty minutes. Cholinesterase activity is expressed as microliters of carbon dioxide evolved per unit of time, or in the storage experiments, as the percentage of the original activity.

F. Calculation of Cellular Contents

The concentration of potassium and the various phosphate fractions in the red cells was calculated from the formula:

$$E = \frac{B - (1 - H) P}{H}$$

where E, B and P are the erythrocyte, blood and plasma concentrations respectively, and H is the hematocrit value expressed as a fraction of one.

Only in the experiments where the blood cells were suspended in buffers were the potassium determinations done directly on the packed cells. A portion of the cell mass was diluted with water, and one aliquot taken for the potassium analysis. Another aliquot of the diluted cells was dried to constant volume over-night at 100° C., and the potassium content was corrected for the dry weight of the sample.

## RESULTS

### A. Electrolyte metabolism of blood stored at 0° C. and incubated at 37° C.

The effect of storage of whole citrated blood with added glucose at 5° C., and of subsequent incubation at 37° C. on some of the plasma electrolytes is shown in Table 1A. The blood was drawn into a mixture of 0.2 parts of 3.2% sodium citrate solution and 0.2 parts of 5.4% glucose solution and dispensed in 10 ml. aliquots into a series of 50 ml. Erlenmeyer flasks. The flasks were tightly stoppered, covered with gauze pads and the samples stored at 5° C., as described under Methods. On the first day, and at subsequent weekly intervals, five flasks were taken, the first for immediate analysis and the other four were immediately transferred to a water bath at 37° C. and incubated, with rocking, for 30, 60, 90 and 120 minutes respectively. The contents of each tube were analyzed and the results are shown in Tables 1A and 1B.

It may be seen from Table 1A that when blood is stored at refrigerator temperatures, potassium diffuses# from the red cells into the plasma. The rate of diffusion is most rapid during

#Throughout our discussion we have assumed that the movement of cations along the concentration gradient, potassium out of the erythrocytes and sodium into them, obeys the laws of simple diffusion. Solomon (1952b) has suggested that such movements involve active transport mechanisms. If this is the case, all our references to "diffusion along the concentration gradient" should be regarded as "transport along the concentration gradient".

the first week and thereafter becomes slower and more constant. During the first seven days of cold-storage the potassium in the plasma increases by more than 25 m.eq. per liter, while during each subsequent week of storage, an increase in the plasma of only about 3 m.eq.per liter occurs. The form of the diffusion curve indicates that the entry of potassium into the plasma does not represent cellular degeneration, but rather a process of diffusion (Harris,1941). Similarly, the increase in the potassium content of the plasma cannot be explained by the release of this ion from hemolyzed cells, since a low degree of spontaneous hemolysis occurs, as evinced by the low concentration of iron in the plasma.

The total concentration of iron in the plasma was originally determined to serve as an index of spontaneous hemolysis. Barkan and Walker (1939) have shown that iron is released from hemoglobin during storage and incubation which results in an increase in the concentration of iron in the plasma of the same order of magnitude as is shown in Table 1A. The concentration of iron in the plasma, not having been corrected for non-hemoglobin iron, is therefore not a true index of the degree of hemolysis, and has not been considered as such. However, the relatively low amounts of total iron in the plasma demonstrate the virtual absence of spontaneous hemolysis during the storage period. The constancy of the concentration of hemoglobin in the whole blood specimen during storage and incubation demonstrates that the disintegration of hemoglobin,

as described by Barkan and Walker, is of an extremely low order of magnitude.

The changes in the concentration of chloride in the plasma are more ambiguous (Table 1A) (see Denstedt et al.,1943). This ion apparently leaves the cells against the concentration gradient during the first two weeks in the cold, then diffuses back during the third week of storage. The concentration of chloride in the plasma shows an increase of six milliequivalents per liter during the first fortnight of cold-storage, while during the same period the concentration of potassium in the plasma increases by twenty-nine milliequivalents per liter. Sodium and potassium ions tend to change places in the cells during cold-storage, but the membrane may be more permeable to the latter species of ions, since they are the smaller of the two due to their lower degree of hydration. Thus, more potassium ions might diffuse out of the cells than is made up by the entry of sodium ions, and to maintain electrical neutrality, the deficit is met by the leakage of chloride ions into the plasma. Subsequent experiments, however, indicated that the shift of chloride from the cells to the plasma, under these conditions, is due to the gradual increase in the oxygenation of the blood in the flasks.

During the third week of storage of the blood in the cold the concentration of chloride in the plasma returns to the

level of that in the freshly drawn blood. This phenomenon might be explained by the decrease in the oxygen content of the blood and the air in the flask due to the metabolic activity of the cells. The oxyhemoglobin becomes progressively reduced and the "chloride-shift" is elicited. Also, the concentration of chloride in the plasma is almost three times that of the erythrocytes, and as the permeability of the membrane is altered and other anions leave the cells, chloride ions diffuse in along the concentration gradient.

When fresh citrated blood is incubated at 37° C. for a two hour period, there is no obvious change in the concentration of potassium in the plasma (Table 1A). This probably represents a steady state, in which the cells have a full complement of potassium and are unable to take up significant amounts. By maintaining the metabolic activity of the erythrocytes, the cation distribution may be kept at the existing level (Danowski,1941; Harris,1941). Red cells stored for seven days are capable of recovering potassium ions from the plasma when incubated at 37° C. When stored for longer periods the ability of the cells to recover lost potassium decreases until no recovery was apparent in three week old blood specimens. Blood cells stored for four weeks tend progressively to lose potassium during a two hour period of incubation.

The concentration of chloride in the plasma tends to increase during the incubation of fresh blood. This is probably a demonstration of the well-known "chloride-shift" as the venous blood is being oxygenated by the air in the flasks during the rocking.

No such shift in the chloride ions occurs during the incubation of the preserved specimens.

The instability of the erythrocytes to hypotonic saline does not parallel the rate of potassium loss during cold-storage (Table 1B), and it seems unlikely that the potassium was leaking out of the erythrocytes in a prehemolytic stage (Ponder and Robinson,1934; Davson,1940; Ponder,1937,1949,1951). The fragility of the red cells increases to a somewhat greater extent during the first seven days of storage than during the subsequent three weeks. This is roughly analogous to the loss of potassium from the cells, but during the last three weeks of storage the fragility increases at a proportionately greater rate than the potassium is leaking out of the cells. Furthermore, during the incubation of the blood at 37° C., the fragility of the cells increases markedly, while there is not only no accompanying loss of potassium, but in most cases an uptake.

B. Magnesium in stored blood.

Table 2A shows the effect of storing blood in various anticoagulant and preservative mixtures on the whole blood and plasma magnesium content. The blood was collected into the following preservative mixtures:

1) 0.2 parts of 3.2% sodium citrate solution.
2) 0.2 parts of 3.2% sodium citrate solution and 0.1 parts of of 5.4% glucose solution.
3) 0.2 parts of 1.85% potassium oxalate solution.

It may be observed from the data presented (Table 2A) that the anticoagulants used do not cause an alteration in the magnesium content of the blood during cold-storage, that is to say, the magnesium is not sequestered in an insoluble form by either citrate or oxalate. A slight and possibly insignificant decrease in the concentration of magnesium in the plasma of the citrated blood occurs during the storage period. Whether this is due to the entry of magnesium into the cells against the concentration gradient, or to the removal of magnesium from the plasma as a citrate or phosphate complex, could not be ascertained. In the case of the oxalated blood, the increase in the magnesium content of the plasma may be attributed to the marked hemolysis of the magnesium-rich red cells.

Incubation of the fresh and stored specimens of blood brought about no changes in the concentration of magnesium in the plasma (Table 3A), other than that attributable to the augmented hemolysis in the oxalated samples.

C. Electrolyte metabolism of blood stored in citrate and in oxalate, without additional glucose.

The effect of storing and incubating blood, using citrate and oxalate as the anticoagulant, without additional glucose, on the concentration of some of the electrolytes in the plasma is illustrated in Table 4A. The blood was collected into the following anticoagulant mixtures:

1) 0.2 parts of 3.2% sodium citrate solution.

2) 0.2 parts of 1.58% sodium oxalate solution.

These blood mixtures were dispensed into sterile tubes which were then stoppered and sealed with wax and stored and incubated as described under Methods.

The rapid loss of potassium from the erythrocytes into the plasma occurs at about the same rate in citrated and oxalated blood (Table 4A). Since oxalate, at the concentration used, inhibits glycolysis in blood (Kwiecinska,1948), it appears that the low temperature of storage is alone responsible for the loss of potassium from the red blood cells and that the further inhibition of the metabolism of the cells by oxalate exerts no further effect. Hemolysis is very pronounced in the oxalated blood specimens, becoming most evident during the second week of storage, when the concentration of potassium in the plasma exceeds the concentration of this

ion in the plasma of the citrated specimens. After three weeks in the cold, the concentration of potassium in the plasma of both specimens equals the concentration of potassium in the cells, and thus further hemolysis has no effect on the concentration of potassium in the plasma.

During the first day of cold-storage there is a slight increase in the concentration of chloride in the plasma as the blood becomes oxygenated. During further storage there is a progressive loss of chloride ions from the plasma, which results in an increase in the cellular chloride content. (Table 4A). Deoxygenation of the blood by oxidative processes could account for a considerable movement of chloride from the plasma into the cells. When the oxygen saturation of the hemoglobin is reduced from the characteristic 95% of arterial blood to the characteristic 65% of venous blood, about 6 mg. of chloride enter 100 ml. of the cells (Henderson et al,1924). Further deoxygenation will presumably result in a further ingress of chloride ions. The erythrocytes have a considerably lower chloride content than the plasma(about 1/3), and during storage, ionic and membrane changes take place that allow chloride to flow along its concentration gradient into the cells.

As phosphate ions leave the cells and enter the plasma during storage, chloride ions may enter to replace them. The rate of the entry of phosphate ions into the plasma depends on the rate

of the breakdown of organic phosphate compounds in the cells, and the permeability of the erythrocyte membrane to inorganic phosphate ions. Relatively little phosphate enters the plasma of citrated blood until the third week of storage, while in oxalated blood the concentration of inorganic phosphate in the plasma begins to increase immediately. (Table 4A). The erythrocyte membrane is normally quite impermeable to phosphate ions at 5° C., so it is probable that oxalate affects the membrane, allowing these ions to escape the cell interior readily, while augmenting the rate of the breakdown of the phosphate esters as well.

The progressive increase in the iron concentration of the plasma during the cold-storage period is mainly attributable to spontaneous hemolysis, especially where hemolysis is marked (Table 4A).

During two hours of incubation at 37° C. of the fresh citrated blood specimens, the concentration of potassium in the plasma changes very little (Table 4B). Incubation of the cold-stored specimens, however, results in a decrease in the potassium concentration of the plasma of blood stored for as long as eight days. Incubation of older blood specimens results in net losses of potassium from the cells. The ability of the red cells to recapture lost potassium from the plasma is lost by the eleventh day of storage with citrate alone.

The erythrocytes of the oxalated blood specimens at no time demonstrate the capacity to take up potassium from the plasma during two hours incubation at 37° C. (Table 4A). The concentration of potassium in the plasma invariably increases during this period, at a rate of from 3.6 to 8.4 mg. per 100 ml. The inhibition of glycolysis by the oxalate may be responsible for the inability of the cells to regain potassium; also the membrane may have been rendered more permeable to this ion for passive diffusion, possibly by the removal of calcium.

It should be emphasized that the changes in the potassium concentration of the plasma during incubation are the resultant of two factors, working in opposition to each other. In the first place there is a tendency for potassium ions to diffuse out of the cells along the concentration gradient. Opposed to this is the ability of the cells, when depleted of potassium, to recapture it against the concentration gradient. Such a process requires "work", and must be driven by energy derived from metabolism. In fresh blood the two factors of passive diffusion and active uptake of potassium apparently balance each other. During cold-storage the process of passive diffusion predominates, and the concentration of potassium in the plasma increases. Incubation of citrated blood at body temperature, if stored for not more than eight days, results in an active uptake of potassium by the cells, overcoming the passive diffusion out of

the cells that invariably is taking place. The magnitude of the passive diffusion during two hours at 37° C. may be equal to that which occurs in non-glycolyzing blood cells, such as in citrated blood after eleven days of storage, when no glucose is present, (Table 4A and 4B), and possibly in the oxalated cells when glycolysis has been inhibited.

The increase in the inorganic phosphate concentration of the plasma during incubation of whole blood is the result of two simultaneous processes (Table 4A). The breakdown of the organic phosphate compounds results in an increase in the intracellular inorganic phosphate content, while the elevated temperature increases the permeability of the membrane to these ions (Halpern, 1936; Maizels, 1935). Which factor predominates during the incubation of stored blood is impossible to ascertain without a knowledge of the extent of the breakdown of the organic phosphate compounds.

D. Electrolyte metabolism of blood stored in citrate and in oxalate, with additional glucose.

The effect of storing and of incubating blood with citrate and with oxalate as anticoagulant, with added glucose in the medium, on the concentration of some of the electrolytes in the plasma is illustrated in Table 5A. The two blood preservative mixtures investigated were as follows:

1) 200 ml. of blood were drawn into a mixture of 28 ml. of 3.2% sodium citrate and 12 ml. of 5.4% glucose solution.
2) 200 ml. of blood were drawn into a mixture of 28 ml. of 1.58% sodium oxalate and 12 ml. of 5.4% glucose solution.

The anticoagulant solutions and the glucose solutions were autoclaved separately and mixed before adding the blood. The blood mixtures were dispensed into small sterile tubes, which were then stoppered and sealed with wax. The specimens were stored and incubated as was previously described under Methods.

The rate of loss of potassium from the citrated cells at 5° C. is more rapid than from the oxalated cells, as is seen in Table 5A. (see also Table 4A). The reason for this is unknown at the present time and awaits further investigation of the osmotic, electrostatic, metabolic and permeability effects of both citrate and oxalate. The degree of hemolysis is appreciably higher in the oxalated than in the citrated specimens, which makes the difference in the rate of potassium diffusion all the more puzzling. Added glucose plays a protective role against the hemolytic effect of oxalate, however, as hemolysis is not as extensive as is depicted in Table 4A.

Chloride does not shift out of the cells at any time in this experiment (Table 5A) because the precaution was taken to fill the tubes of blood completely so that no oxygenation of the hemoglobin occurs. During the storage period the characteristic decrease in the concentration of plasma chloride takes place, again demonstrating the diffusion of chloride ions into the cells along the concentration gradient.

The increase in the concentration of the acid-soluble phosphates in the plasma during storage appears to be due entirely to the diffusion of inorganic phosphate ions from the cells (Table 5A). The cell membrane does not, then, become permeable to the organic phosphate compounds, located within the interior of the erythrocytes, at any time during the storage period. The presence of added glucose in the preservative medium retards the spontaneous increase in the concentration of inorganic phosphate in the plasma during the storage of both oxalated and citrated blood (compare Tables 4A and 5A).

The effect of incubation for two hours at 37° C. on the concentration of potassium in the plasma may be seen in Table 5A and still more clearly in Table 5B. It is noteworthy that the uptake of potassium by the cells which had been stored in citrate with added glucose is greater than in the specimens that were stored without added glucose (compare Tables 4B and 5B). Also, the ability of the cells to recapture lost potassium is maintained

for a longer time when blood is stored with additional glucose in the medium, and is only lost after three weeks of cold-storage (Table 5B). The oxalated cells were incapable of taking up potassium from the medium, but, on the other hand, lost less by passive diffusion than oxalated cells stored without added glucose in the medium (compare Tables 4A and 5A).

During the incubation of the blood specimens for two hours at 37° C. the concentration of inorganic phosphate in the plasma rises quite sharply, especially in the older specimens (Table 5A). As mentioned in the preceding section, this is due not only to the accelerated breakdown of the organic phosphate compounds in the erythrocytes, but also to the fact that the inorganic phosphate that has accumulated in the cells during the cold-storage period is being rapidly released into the plasma. The incubation of citrated blood stored for fourteen and twenty days results in a greater amount of inorganic phosphate entering the plasma than in the case of the oxalated blood specimens. This is a reflection of the inhibitory effect of oxalate on the metabolism of all the organic phosphate compounds.

The concentration of chloride in the plasma of both the citrated and oxalated specimens remains essentially unchanged during incubation at 37° C. (Table 5A). There is little if any oxygenation of the specimens, as the air space in the tubes was kept to a minimum.

E. Glycolysis, phosphorolysis and electrolyte exchanges in citrated blood stored with and without added glucose.

A series of experiments was undertaken to investigate the relationship between the rate of potassium diffusion from the cells during cold-storage, the rate of the disappearance of glucose and the breakdown of the phosphate esters. Furthermore, it was of interest to determine the degree of correlation between the rates of glycolysis and phosphorolysis, and the uptake of potassium by stored cells during their incubation at 37° C. Typical results of these studies are presented in Tables 6A and B, 7A and B and 8A and B.

Tables 6A and 6B show the results of the analyses on two aliquots of blood from the same donor. Both were mixed with isotonic sodium citrate and glucose solutions in the ratio of 5:1:0.5. The anticoagulant and the glucose solutions had been autoclaved separately and cooled. In one case (Table 6A) the glucose solution was mixed with the anticoagulant solution before the blood was added, while in the other (Table 6B) glucose was added after the blood was mixed with the anticoagulant. Tables 7A and 7B depict the results of analyses on separate aliquots of blood from another donor. For one aliquot (Table 7A), 200 ml. of blood were drawn into 40 ml. of isotonic sodium citrate solution, while for the other (Table 7B), 200 ml. of blood were drawn into a mixture of 38 ml. of isotonic sodium citrate solution and 2 ml. of 54% glucose solution. The data in Tables 8A and 8B represent the analyses on separate aliquots of blood

from yet another donor. In one case (Table 8A), 200 ml. of blood were mixed with 40 ml. of 3.2% sodium citrate solution, while in the other (Table 8B), 200 ml. of blood were taken into a mixture of 38 ml. of 3.4% sodium citrate solution and 2 ml. of 54% glucose solution.

In all cases, the blood specimens were thoroughly mixed with the preservative solution and dispensed into small serological tubes, which were then stoppered and sealed. The samples were then stored and incubated for various periods of time, as described under Methods. The contents of each tube were analyzed and the results are depicted in Tables 6A and B, 7A and B and 8A and B.

The movement of potassium from the red cells into the plasma during storage at 5° C. has already been described, and is again well illustrated. The presence of added glucose in the preservative medium retards the rate of escape of potassium from the cells only slightly (compare Tables 7A and B; 8A and B). It is difficult to attribute the influence of the added glucose on potassium diffusion to an altered glycolytic rate, since the rate of the disappearance of glucose is not consistently increased in the glucose-enriched specimens. Similarly,when the glucose is depleted in the citrated specimens without additional glucose, the rate of increase in the concentration of potassium in the plasma (Tables 7A and 8A) does not exceed that of the blood with an excess of glucose still present

(Tables 7B and 8B). The presence of glucose in concentrations up to about 400 mg. per 100 ml. in the preserved blood samples does not alter the rate of the egress of potassium from the erythrocytes by affecting their glycolitic rate, but more probably has a less specific action on the permeability properties of the cellular membrane.

During the storage of citrated blood in the cold, the sodium of the plasma tends to enter the erythrocytes in exchange for the potassium that escapes. The transposition of these two cations does not appear to be a reciprocal one, as the number of potassium ions entering the plasma is slightly greater than the number of sodium ions leaving (Tables 8A and B). The exchange# of sodium and potassium is more equivalent in the blood specimen with glucose added (Table 8B) than in the simple citrated specimen, due to the slower rate of loss of potassium from the red cells in the former.

#We have used the term "exchange of cations" to mean the exchange of one species of cation, e.g. potassium, for another species of cation, e.g. sodium, and vice versa. To those working with isotopes, "exchange of cations" means the exchange of members of one species of cation inside of the cell for members of the same species outside of the cell, and vice versa.

The progressive decrease in the concentration of the indigenous glucose in citrated blood during cold-storage may be seen from the data in Tables 7A and 8A. The normal complement of blood sugar is completely consumed before the first seven to ten days of storage have elapsed. The glucose disappears at a fairly regular rate of about 10-15 mg. per 100 ml. per day, except for what appears to be an analytical error on the fourth day of storage (see Andreae, 1946).

The rate of the disappearance of glucose from citrated blood brought to a glucose concentration of 300-400 mg. per 100 ml. is shown in Tables 6A and B, 7B and 8B. During the first two days of cold-storage glucose is consumed at a rate of 20-45 mg. per 100 ml. per day, and except for minor fluctuations, this rate falls progressively. The rate of the glucose disappearance is about 10 mg. per 100 ml. per day during the remainder of the first week, about 5 mg. per 100 ml. per day during the second week and about 2 mg. per 100 ml per day during the third and fourth weeks of the storage period.

The phosphorylated compounds deserve special attention in an appraisal of the metabolic state of the erythrocytes. The concentration of inorganic phosphate in the stored blood reflects the state of preservation of the phosphorylated intermediates of glycolysis and of the high-energy phosphate groups of adenosine triphosphate, but does not afford an index of the behaviour of

the individual phosphate fractions, which may vary quite independently of one another. Thus, there is a general tendency for the amount of stable phosphate, mainly representing 2,3-diphosphoglyceric acid, to increase during the first few days of storage, while the labile and hydrolyzable phosphates, representing adenosine triphosphate and hexose phosphate, decrease slightly. The inorganic phosphate concentration usually decreases during the first few days of cold-storage. Maizels (1943) has observed a similar increase in the stable phosphate fraction of unacidified blood during storage. The rate of the phosphorylation of glucose exceeds the rate of the metabolic degradation of the diphosphoglycerate during the early part of the storage period, and accounts for the accumulation of this glycolytic intermediate.

As the phosphorylation of glucose fails progressively during the storage period, there is a concomitant decrease in the hydrolyzable and stable phosphate fractions in the blood. As the rate of dephosphorylation of adenosine triphosphate exceeds the rate of rephosphorylation of adenylic acid and adenosine diphosphate, the labile phosphate fraction decreases as well. In citrated blood, without additional glucose in the medium, the hydrolyzable and labile phosphate compounds disappear at about the same time as the depletion of the glucose

occurs. The store of diphosphoglyceric acid continues to be metabolized, and the stable phosphate fraction falls to a low level after two weeks of storage.

When glucose is added to blood the glycolytic rate still tends to fall off with time, and eventually fails to replenish the glycolytic intermediates (Tables 6A and B, 7B and 8B). The hydrolyzable phosphate fraction begins to decrease during the second week of storage and usually reaches an immeasurable value by the twentieth day. The stable phosphate fraction is maintained for a few more days and usually doesn't decrease until the third week of cold-storage. During the first few days of the storage period glycolysis proceeds at a relatively rapid rate while the level of adenosine triphosphate, as indicated by the concentration of the labile phosphate fraction, tends to decrease. This indicates that under such circumstances the utilization of the high-energy phosphate groups of adenosine triphosphate proceeds more rapidly than does the rephosphorylation of adenylic acid. As the supply of adenosine triphosphate becomes depleted, the phosphorylation of glucose is diminished and the phosphorylated esters fail to be synthesized at the same rate as they are being metabolized. The glycolytic intermediates phosphorylate adenylic acid as they are metabolized and are able to partially maintain the level of the labile phosphate fraction for two or three weeks.

In tables 7A and 8A it may be seen that during the

incubation of fresh citrated blood at 37° C., glycolysis proceeds at a rate of about 10 mg. per 100 ml. per hour. No potassium is taken up by the red cells and no sodium is expelled by them. This statement refers to quantitative changes of these ions, not to the normal inward and outward flux demonstrable by isotope studies (Sheppard and Martin, 1950; 1951; Hastings et al. 1950; Solomon, 1951; 1952). The concentration of inorganic phosphate in the cells increases as some of the hexose phosphate and diphosphoglyceric acid is hydrolyzed, but some of this newly formed inorganic phosphate diffuses into the plasma as the membrane is readily permeated by phosphate ions at 37° C. (Table 7A). The concentration of adenosine triphosphate is generally high and well maintained during the incubation of fresh blood for periods up to four hours.

Glucose disappears at the same rate (10 mg. per 100 ml. per hour) during the incubation of two-day old citrated blood as occurs during the incubation of fresh blood. There is a concomitant decrease in the concentration of potassium in the plasma (0.75 to 1.5 m. eq. per liter per hour) and an increase in the concentration of sodium (about 1 m.eq. per liter per hour). The concentration of inorganic phosphate in the cells and the plasma rises steeply as the organic phosphate compounds are being metabolized more rapidly than they are being replenished (Tables 7A and 8A).

Incubation at 37° C. of citrated blood stored for four, five and six days at 5° C. results in a rapid depletion of the glucose present, while potassium is being removed from the plasma

at a rate of 1 to 1.5 m.eq. per liter per hour and sodium is entering the plasma at a similar rate. The amount of organically bound phosphate tends to decrease during the incubation period and the amount of inorganic phosphate to increase in the cells and the plasma (Tables 7A and 8A).

Citrated blood stored for periods longer than one week do not contain any glucose, and on being returned to body temperature, the erythrocytes are no longer able to accumulate potassium and expel sodium. The phosphorylated glycolytic intermediates are slowly metabolized during the incubation period, liberating additional inorganic phosphate. This ion is rapidly released from the cells at 37° C., so that the concentration of inorganic phosphate in the plasma increases and the concentration in the cells actually decreases (Table 7A). Eventually all of the organic acid-soluble phosphate compounds are hydrolyzed and the inorganic phosphate that they give rise to is distributed equally between the intra- and the extracellular phases.

During the incubation at 37° C. of fresh blood, with glucose added to a concentration of 300-400 mg. per 100 ml., glycolysis proceeds at a rate of 5 to 20 mg. per 100 ml. per hour (Tables 6A and B, 7B and 8B). The cells show little tendency to accumulate potassium, not because of the relatively low concentration of this ion in the plasma, but because their capacity to contain it is limited and probably already is at a maximum. In other words, the normal diffusion of potassium out of the cells is quantitatively

balanced by the active uptake of potassium against the concentration gradient (Hastings et al. 1950; Solomon, 1951). Similarly, the sodium content of the cells is at a minimum in fresh erythrocytes and they are unable to expel this ion to a degree other than the capacity to maintain the normal sodium flux (Table 8B). There is a slight increase in the concentration of inorganic phosphate in the cells and the plasma as the organic phosphate fractions tend to decrease during the incubation period.

Blood specimens with added glucose stored at 5° C. for two days glycolyze at approximately the same rate as fresh blood when returned to body temperature. The preserved erythrocytes now demonstrate a capacity to recapture potassium from the plasma, and to expel sodium, at a rate of about 1 m. eq. per liter per hour. The organic phosphate compounds tend to undergo hydrolysis during the incubation period and the inorganic phosphate content of both the cells and the plasma increases. The stable phosphate fraction is generally well maintained, and the increase in the inorganic phosphate concentration is mainly at the expense of the labile and hydrolyzable phosphates.

During the incubation of glucose-enriched blood stored for four to six days, glucose usually disappears at a rate of about 10 mg. per 100 ml. per hour, while the sodium concentration of the plasma increases at a rate of 1-2 m.eq. per liter per hour, and the potassium concentration of the plasma decreases at a similar rate (Tables 6A and B, 7B and 8B). It has been noticed

that occasionally the glycolytic rate of a blood specimen is greater after a few days of storage than it was in the fresh specimen. This phenomenon will be discussed in a later section dealing with the Pasteur effect in blood. The organic phosphate compounds continue to be metabolized more rapidly than they are being replenished and thus the concentration of inorganic phosphate increases in the cells and the plasma.

When blood that has been stored with added glucose for one to two weeks is incubated at 37° C., the utilization of glucose proceeds at a rate of 5 to 10 mg. per 100 ml. per hour, while the cells demonstrate the capacity to recapture potassium and expel sodium at a rate of 1-2 m.eq. per liter per hour. During the incubation period, the organic phosphate compounds in the red cells generally tend to decrease, increasing further the concentration of inorganic phosphate in both the cells and the plasma.

Incubation of the blood specimens during the third week of storage results in a rate of glucose disappearance of about 2-7 mg. per 100 ml. per hour, accompanied by a decrease in the concentration of potassium in the plasma of about 1 m.eq. per liter per hour, and an increase in the sodium concentration in the plasma of the same order of magnitude. During the incubation period a further breakdown of the organic phosphate compounds occurs, giving rise to more inorganic phosphate in the blood. However, the cells contain a large amount of inorganic phosphate

that has accumulated during the cold-storage period, and during incubation at 37° C., more inorganic phosphate ions are released into the plasma than are formed from the further metabolism of the organic phosphates. The concentration of inorganic phosphate in the cells therefore decreases during the incubation period.

After three weeks of storage at 5° C. the cells have practically lost their ability to glycolyze when incubated at 37° C., and have also lost their ability to regain potassium and expel sodium. By this time the adenosine triphosphate content has usually fallen to an immeasurable level, as has the hydrolyzable phosphate fraction. Small amounts of stable phosphate present in the blood after three weeks of storage are slowly hydrolyzed during the incubation period. The inorganic phosphate that has accumulated in the cells during the cold-storage period is released into the plasma as the membrane becomes permeable to these ions at 37° C.

After four weeks in the cold the blood cells are no longer capable of regaining potassium when incubated for four hours. Glucose utilization is negligible and only traces of the organic phosphate compounds remain. The only significant change produced on warming the blood cells to body temperature is the release of inorganic phosphate into the plasma.

Despite the apparent loss of the metabolic activity of the cells, it is of incidental interest to point out that about 60% of them would be expected to be viable if transfused.

It is obvious from the data in Tables 6A and B, 7A

and B and 8A and B that potassium is not held in the erythrocytes by being bound to organic phosphate anions as has been so often stated (Maizels and Farmer, 1939). These are generally well maintained, and may even increase in amount, at a time when potassium is rapidly leaving the cells. Similarly, the uptake of potassium by preserved erythrocytes, when brought back to 37° C., is usually accompanied by a breakdown of the organic phosphate esters and a release of inorganic phosphate from the cells.

Conway (1947) has proposed, with reference to frog sartorius muscle, that the high potassium and low sodium levels in the cells are maintained entirely by physical forces of electrostatic attraction and selective permeability of the cellular membrane. Potassium is accumulated by being brought in with inorganic phosphate anions. The inorganic phosphate is then built into non-diffusable organic phosphate anions within the cells which retain potassium ions electrostatically (Boyle and Conway, 1941). Roberts and his colleagues (1949; 1950) have shown that Escherichia coli take up potassium by binding it with various hexose phosphates. Steinbach (1951) stated that he ".... knows of no justification in the chemistry of these compounds to indicate such a binding". In any case, such a mechanism obviously does not exist in human erythrocytes, as organic phosphate compounds are being decomposed, and inorganic phosphate leaving the cells, at a time when potassium is being accumulated.

However, the maintenance of the organic phosphate

compounds appears to be essential to some degree for cation movements against the concentration gradient. Maizels (1949) is of the opinion that active cation movements "...require that phosphorylated compounds break down actively, yet not so rapidly as to overtake resynthesis". Of course, this is simply another way of saying that glycolysis is essential for active cation exchanges to occur. The relationship between glycolysis and potassium uptake or sodium output by the erythrocytes, however, is by no means direct. In some instances potassium accumulation may occur at the maximum rate while glycolysis proceeds relatively slowly; at other times glycolysis may be at a maximum and active cation movements virtually at a standstill. Of the organic phosphate fractions measured, the behaviour of the labile phosphate or adenosine triphosphate fraction appears to be best correlated with the active cation movements. The relationship between high-energy phosphate compounds and reactions involving ion transport is obvious. Whether the adenosine triphosphate is necessary to provide energy for another metabolic system involved in cation transport or whether it is used directly in the synthesis of an ion-carrier complex, is at present unknown.

Harris (1941) was of the opinion that the metabolism of glucose is an important factor, either directly or indirectly, in energizing the passage of cations across the erythrocyte membrane. Maizels (1949, 1950) supported this view, with the reservation that the active process in cold-stored erythrocytes is the expulsion

of sodium from the interior, and that the uptake of potassium is merely a passive process (to maintain osmotic and electrical neutrality). He incubated six-day old blood only, for periods of six to twenty-four hours, and found a poor correlation between glycolysis and cation movements. However, he measured the glucose concentration in the cells after incubation and in many cases the blood specimens were not rocked. The amount of glucose found in the cells could therefore not be a true index of the concentration of glucose in the blood, or the rate of glucose utilization. Our data show more clearly that the rate of glycolysis, per se, does not determine the rate of either potassium accumulation or sodium expulsion.

Hastings and his co-workers (1950) have attempted to correlate glucose consumption with the maintenance of the potassium gradient in fresh incubated erythrocytes, while Solomon (1952) has calculated the amount of energy required by the erythrocytes for the expulsion of sodium and the uptake of potassium in order to maintain the status quo. Glycolysis in fresh cells at 37° C. provides energy to maintain the normal flux of sodium and potassium between the cells and plasma, the energy being used presumably in the outward transport of sodium and the inward transport of potassium. Less than ten percent of the energy provided by glycolysis in fresh blood cells is used in this process (Solomon, 1952). Glycolysis in stored cells brought to 37° C. provides energy not only for the maintenance of the normal cation flux, but more potassium is transported inward than diffuses out along the

concentration gradient and more sodium is transported outward than passively diffuses in. Stored erythrocytes, therefore, not only maintain the status quo regarding their cation content, but are able quantitatively to expel sodium and accumulate potassium while glycolyzing at a diminished rate. How can one reconcile the lower metabolic activity of the stored cells with what appears to be a greater amount of osmotic work? Is the cell able to divert a greater proportion of its metabolic energy toward re-establishing its normal cation composition, or are the energy requirements for active cation movements in stored erythrocytes less than in fresh ones? This latter suggestion might imply that the membrane of stored cells is less permeable to cations than that of fresh cells, and the passive diffusion of cations along the concentration gradient is correspondingly slower. Bond (1952) has pointed out that the energy required for potassium accumulation may be less in stored erythrocytes than in fresh ones if the membrane becomes altered in such a way as to offer less resistance to the entry of potassium. It should be emphasized however, that the movement of potassium into stored cells is against a much less steep concentration gradient than exists for the maintenance of the potassium flux in fresh blood. In the differences in the gradients involved, as well as the permeability properties of the membrane, may lie the explanation of the ability of stored cells to accumulate potassium and expel sodium at a lower metabolic level. The resolution of this problem awaits an investigation of

the energy requirements to maintain the flux of sodium and potassium in stored blood cells, as has been studied by Solomon (1952) with fresh red cells.

Davidsen and Kjerulf-Jensen (1950) have studied the uptake of radioactive potassium by red cells with a low content of potassium brought on by chronic hypokalemia. By raising the concentration of potassium in the plasma, the depleted cells were found to take up more radioactive potassium than did the normal ones. A net gain of potassium by the cells resulted, while the passive exchange rate was unaffected.

In the opinion of Maizels (1949) the correlation between cation movements and the concentration of the organic acid-soluble phosphate compounds in six-day-old blood incubated at 37° C. for eighteen hours is not good, but he concluded that when the concentration of the organic acid-soluble phosphates falls below 1.5 mg. per 100 ml., the active transport of cations fails. In our experiments we have separated the components of the organic acid-soluble phosphate fraction into the labile, hydrolyzable and stable phosphate fractions and are of the opinion that the level of labile phosphate is probably the factor which determines whether or not active cation movements occur. In many cases the stable phosphates were not entirely depleted, but the labile phosphates were exhausted at the time when the movement of the cations against their concentration gradients no longer occurred during incubation.

Flynn and Maizels (1949) were able to provide further evidence that the outward transport of sodium by cold-stored

erythrocytes incubated for periods up to twenty hours is an active process, and the uptake of potassium by these cells only secondary to it, and passive. Their conclusions are based to a large extent on the repeated observations that the output of sodium by the cells may exceed the uptake of potassium during incubation.

Ponder (1950) also has investigated the ability of stored erythrocytes to regain lost potassium, but reserves judgment on whether the uptake of potassium is secondary to sodium expulsion or also is an active process requiring energy. In our experiments, the movements of the cations were nearly always found to be almost reciprocal to each other, and no conclusions regarding the nature of the primary process can be drawn. Maizels' (1949) argument for the need of a sodium-expelling mechanism in all cells is very convincing, and he believes that this would necessarily entail the passive uptake of potassium against the concentration gradient. Parpart and Green (1952) point out that for potassium uptake to be passive, a normally high impermeability of the membrane to potassium as compared to sodium must exist. Studies on the permeability of the erythrocyte membrane to sodium and potassium do not bear this out (Solomon, 1951; Maizels and Harris, 1951).

Maizels has not reported on the stability of the cation transport system in blood stored in the cold, but used six- to eight-day old specimens. Such specimens, we have observed, demonstrate the maximum capacity of the cells to take up potassium from the medium, and in the opinion of the writer, Maizels must have established this in preliminary studies

involving the incubation of specimens stored for various periods of time. However, Ponder (1950) claims to have shown that the accumulating mechanism for potassium deteriorates during ten days of cold-storage, at a time when the cells are still able to glycolyze. This confirms our findings for citrated blood, where the cells are unable to recapture lost potassium after ten days of storage. However, this failure occurs only because their glucose content has been depleted during the storage period. When blood cells are stored in a glucose-enriched medium, the cation transport mechanisms are preserved for over twenty days, and fail only when the glycolytic activity falls to a low level. The failure of both these systems, glycolysis and ion transport, may be caused by a lowering of the pH due to the accumulation of acid products of glycolysis. We have not attempted to measure the pH of our systems, plasma pH not being a true index of red cell pH, but it is apparent that the products of glycolysis themselves do not alter the pH of the cells sufficiently to abolish either glycolysis or active cation movements. Our results indicate that glucose is being broken down during the incubation period without decreasing the rate or extent of the uptake of potassium and the output of sodium by the cells.

F. Potassium exchanges and glycolysis in stored erythrocytes incubated in a buffered system.

Maizels (1949) and Ponder (1950) have emphasized the dependence of cation movements between the red cells and the plasma on the $p^H$ of the system. They agree that the optimum $p^H$ range for the active uptake of potassium by cold-stored erythrocytes is from $p^H$ 7.3 to 7.8, and that above or below this $p^H$, active cation movements are less or abolished. In other words, at high or low $p^H$, either the cation transport fails or the permeability allowing passive diffusion increases. The possibility that the $p^H$ of the blood mixtures under study was not optimum for active cation movements should be seriously considered, since in some instances the amount of potassium taken up by the cells per hour was not as great as was observed by Ponder, who incubated cells in a phosphate buffer of $p^H$ 7.5, or by Maizels who added alkali to the cell suspensions to maintain a high $p^H$. It is possible that during the storage of the blood in the cold the accumulation of lactic acid and pyruvic acid overcame the buffering capacity of the blood and lowered the $p^H$ of the cells sufficiently to inhibit both their glycolytic mechanism and their cation transport mechanism.

Experiments were designed to assess the ability of cold-stored red blood cells to recapture potassium at 37° C. when the $p^H$ of the medium was kept constant during the incubation period. Krebs-Ringer phosphate buffer at $p^H$ 7.5 (Umbreit et al., 1945) was used

in some of the preliminary experiments, but on the whole proved unsatisfactory because of the considerable degree of hemolysis that occurred during the incubation period. While these studies were in progress, Ponder (1950) pointed out that the most satisfactory concentration of phosphate buffer to maintain the volume of the red cells is 0.11 M, and not 0.067 M, the concentration often employed in buffer mixtures. The sodium phosphate buffer at $p^H$ 7.5 recommended by Ponder was used and no hemolysis or cell volume changes were encountered.

The procedure finally adopted was to draw the blood into the anticoagulant solution, 3.2% sodium citrate, with or without added glucose, and to store these two mixtures in the refrigerator at 5° C. At various time intervals about 30 ml. of each mixture was taken from the refrigerator and the cells washed three times with cold 0.11 M sodium phosphate buffer at $p^H$ 7.5 containing about 20 m. eq. of potassium per liter, added as isotonic potassium chloride. The cells were diluted to the original volume of the blood with the potassium containing phosphate buffer. To 15 ml. of this cell suspension was added 0.6 ml. of 5.4% glucose solution, while 0.6 ml. of water were added to the remaining 15 ml. to serve as a glucose-free control. Each of these mixtures was transferred to three small tubes in 5 ml. aliquots. One tube of each set was taken for the determination of glucose on the suspension and the cells separated for the determination of their potassium content. The other tubes were stoppered and incubated for two and four

hours at 37° C. and analyzed for the concentration of glucose in the suspension and potassium in the packed cells.

The results of incubating blood stored without additional glucose in the medium are presented in Table 9A. They are essentially confirmatory of the conclusions arrived at from the incubation of stored blood cells in the native plasma. In the absence of glucose, the cells lose potassium slowly at a rate of about 1 m. eq. per liter per hour, except the fresh cells which are able to retain their potassium content even in the absence of glucose. The phosphate esters and the adenosine triphosphate present in the fresh cells provide energy for the back-transport of the potassium ions diffusing out, enabling cells to maintain their potassium content.

When glucose is added to the fresh cells, they are unable to take up more potassium, despite the presence of about 20 m.eq. of potassium per liter in the surrounding medium. However, after being partially depleted of their potassium content during storage in the cold for periods up to one week, the cells, on incubation at 37° C. are able to recover potassium at a rate of from 1 to 2 m.eq. per liter per hour. Erythrocytes stored for ten days without additional glucose in the plasma, however, lose their ability to take up potassium from the medium on incubation. The ability to maintain the potassium flux likewise appears to be failing. The glycolytic power of the erythrocytes falls progressively though somewhat irregularly with the duration of cold-storage and appears to be related to the potassium accumulating capacity of the cells only to the extent that both

mechanisms fail almost simultaneously.

Table 9B depicts the results obtained when glucose-enriched blood was used for the incubation studies. The fresh erythrocytes again were unable to accumulate appreciable amounts of potassium despite an active glycolytic rate. When stored for periods of up to two weeks with added glucose, the cells are able to accumulate potassium at a rate of from 1 to 2 m.eq. per liter per hour when incubated at 37° C. When stored in the cold for twenty days the erythrocytes, on incubation, still demonstrate the capacity to glycolyze and to accumulate potassium from the medium, though at a diminished rate. It is doubtful whether the loss of these capacities in blood cells stored for twenty days and incubated in their native plasma can be attributed to $p^H$ changes alone. The writer holds that the maintenance of both these systems depends on the presence of adenosine triphosphate in the cells, and when this becomes depleted, glycolysis and cation transport fail.

Previous attempts to demonstrate an uptake of potassium by erythrocytes stored in an acid-citrate-dextrose medium when incubated at 37° C., in their native plasma, had failed, due to the low $p^H$ of the medium. These specimens glycolyzed, albeit slowly, but at no time was evidence obtained of a decrease in the concentration of potassium in the plasma. The technique of incubating the stored cells in a buffer at $p^H$ 7.5 was therefore ideally suited to test the preservation of the glycolytic activity and the potassium accumulating mechanism in blood stored in the acid-citrate-dextrose (ACD) medium.

The disodium citrate anticoagulant used was a mixture of 5.9 gm of trisodium citrate dihydrate and 2.1 gm of citric acid, ground together intimately, and made into a 3.4% solution. Two hundred ml. of blood were drawn into a mixture of 40 ml. of this 3.4% disodium citrate solution and 2 ml. of 54% glucose solution and stored in a refrigerator maintained at $5^{\circ} \pm 1^{\circ}$ C. At appropriate intervals during storage about 30 ml. of the blood mixture were removed from the bottle and divided into two lots. One lot was divided into three 5 ml. aliquots, dispensed into small tubes, two of which were stoppered and incubated for two and four hours at $37^{\circ}$ C. These represent the red blood cells incubated in their native plasma. The cells in the second 15 ml. lot were washed three times with the phosphate buffer described above, and made up to the initial volume of the blood, 15 ml. with the phosphate buffer at $p^{H}$ 7.5, containing about 20 m.eq. of potassium per liter and about 200 mg. of glucose per 100 ml. This cell suspension was then dispensed in three 5 ml. aliquots into small test tubes, two of which were stoppered and incubated at $37^{\circ}$ C. for two and four hours. The concentration of glucose was determined on the whole blood or on the cell suspension in each case, and the packed cells were analyzed for their potassium content. The results of this experiment may be found in Table 10A.

When blood is stored in the acidified citrate medium enriched with glucose, potassium diffuses out of the cells as previously described for the cells stored in neutral citrate media.

However, the rate of diffusion of this ion from the cells is slower in the acidified medium than in the neutral media. Similarly, glycolysis proceeds at a slower rate at the lower $p^H$ of the blood stored in ACD. Although no data for the same blood stored in neutral media are presented as a control, the effect of the $p^H$ on the diffusion of potassium from the erythrocytes and on the glycolytic rate of the blood has been observed in some preliminary studies, and confirms the findings of several other investigators (see Historical Introduction).

In none of the experiments did the incubation of whole blood stored in the acidified medium result in an appreciable uptake of potassium by the erythrocytes. The pH of the blood specimens was not measured, but was probably in the vicinity of $p^H$ 7 or less (Rapoport,1947a), which is unfavourable to active cation movements (Maizels,1949; Ponder,1950). Glycolysis proceeds during the incubation of the stored blood, despite the lack of active accumulation of potassium by the cells. Although the rate of glycolysis during the incubation period is slower than with the neutral blood mixtures, the ability to glycolyze is preserved for a longer period of storage. (Rapoport,1947a).

When the erythrocytes, preserved in the acidified medium, are suspended in a buffer at $p^H$ 7.5 containing about 20 m.eq. of potassium per liter and about 200 mg. of glucose per 100 ml. and then incubated at $37^o$ C., they show an augmented capacity to break

down glucose and demonstrate the ability to take up potassium from the surrounding medium. These metabolic functions are performed to a similar degree by cells stored in neutral media (Tables 9A and B), but it would appear that the acidified medium favours the preservation of the capacity of the cells to glycolyze and to accumulate potassium. The duration of the storage experiment was four weeks, and after this period glycolysis and potassium accumulation still occur during the incubation period, though at a diminished rate.

G. Glycolysis, phosphorolysis and electrolyte exchanges in citrated blood stored with metabolic inhibitors.

It was of interest to investigate the effect of certain well known metabolic inhibitors on the cation exchange in blood during prolonged storage at 5° C. and during incubation at 37° C. To this end, 300 ml. of blood were mixed with 60 ml. of 3.2 sodium citrate solution and divided into six equal portions. One portion of the blood mixture was retained to serve as a control, while to each of the five remaining 60 ml. aliquots of the citrated blood was added one of the following metabolic inhibitors.

1) 1.2 ml. of 0.5 M sodium fluoride solution, to give a final concentration of 0.01 M sodium fluoride in the blood mixture.
2) 0.6 ml. of 0.5 M sodium fluoride solution, to give a final concentration of 0.005 M sodium fluoride in the blood mixture.
3) 0.6 ml. of 0.5 M iodoacetic acid (IAc) solution, to give a final concentration of 0.005 M iodoacetate in the blood mixture.
4) 0.12 ml. of 0.5 M iodoacetic acid (IAc) solution, to give a final concentration of 0.001 M iodoacetate in the blood mixture.
5) 0.24 ml. of M sodium cyanide solution (neutralized with hydrochloric acid), to give a final concentration of 0.004 M sodium cyanide in the blood mixture.

The mixtures were dispensed in 5 ml. aliquots into small tubes which were then stoppered and sealed. Within one hour of the drawing of the blood, one tube from each lot was taken for immediate analysis, and two tubes from each lot were incubated at 37° C. for two and four hours. The remaining tubes were stored at 5° C., and after two, five and nine days of storage respectively one tube from each lot was taken for immediate analysis and two tubes from each lot were incubated at 37° C. for two and four hours respectively.

The rate of loss of potassium from the erythrocytes during the cold-storage of the citrated specimen without any inhibitors added is quite typical. The potassium content of the cells falls from an initial level of 96 m.eq. per liter to about 60 m.eq. per liter by the ninth day. (Table 11A and C). This rate of potassium diffusion may be compared with that which occurs in blood specimens stored under similar conditions (Table 6B, 9A,etc), but any direct comparison of the changes which occur in blood specimens from different donors is impossible because of individual variations. The decrease in the potassium content of the cells during the storage period has generally been attributed to the retardation of glycolysis in the cold, resulting in the failure of the metabolically driven "pump" to maintain the intracellular potassium against the concentration gradient ( Harris,1941). However, it may be seen in Table 11A that the loss of potassium from the cells in the presence of metabolic inhibitors was retarded. With 0.01 M sodium fluoride, 0.005 M sodium fluoride or 0.001 M iodoacetate, the

loss of potassium from the cells is approximately 50% less after two days, and 30% less after nine days at 5° C., than from the unpoisoned cells. In the presence of 0.005 M iodoacetate, the diffusion of potassium from the red cells also appears to be delayed for a few days, but hemolysis becomes quite pronounced and the results are difficult to interpret. These findings are similar to those obtained when oxalate, which also is a glycolytic inhibitor, was used as the anticoagulant (Table 4A). Similarly, the glycolytic activity of blood cells stored at a low $p^H$, as in the ACD mixture, is diminished, and the rate of loss of potassium retarded (Table 10A).

Obviously, the diffusion of potassium from the erythrocytes during cold-storage cannot be due simply to a lowering of the glycolytic rate, and the inhibition of a system for the back-transport of potassium. The effect of the glycolytic inhibitors on potassium diffusion in the cold is not due to the absence of the acid products of glycolysis. On the contrary, an increased acidity tends to decrease the rate of potassium loss from the erythrocytes during storage. It appears then that these metabolic inhibitors decrease the rate of diffusion of potassium from the cells during cold-storage either by rendering the membrane less permeable to this ion at 5° C., or by preventing the breakdown of a component of some system that helps the cell retain potassium. The effect is similar to that of storing blood at a low $p^H$. Both glycolysis and potassium diffusion are diminished, and it is felt that potassium is retained by the erythrocytes stored in ACD because the

adenosine triphosphate is well preserved in the cells by the acidified medium (Rapoport,1947a). A consideration of the effect of the glycolytic poisons on the preservation of the labile phosphate fraction of the blood led to equivocal findings (Tables 11C-G). On the whole,however, it would appear that adenosine triphosphate is not well preserved in the poisoned specimens, and although the possibility cannot at present be ruled out, it seems that the preservation of high-energy phosphate is not the explanation for the decreased loss of potassium from poisoned cells at 5° C.

Sodium enters the cells from the plasma at practically the same rate in the poisoned and unpoisoned cells (Table 11B). The rate of sodium diffusion into the cells in the cold is relatively unaffected by glycolytic poisons, in contrast to the effect of the poisons on potassium diffusion.

Erythrocytes, with added cyanide, recapture lost potassium at the same rate as normal cells when incubated at 37° C., while accompanied by an increased glycolytic rate. In the presence of 0.01 M sodium fluoride the loss of potassium from the erythrocytes when incubated at 37° C. is most profound. While a downhill gradient from the cells to the plasma exists, potassium leaves the cells at the rate of approximately 3 m.eq. per liter per hour. (Table 11a) This rate appears to be independent of the storage-age of the cells and is determined by the prevailing ionic gradient. Incubation of erythrocytes in the presence of 0.005 M sodium fluoride or 0.001 M iodoacetate results in a loss of from 1-2 m.eq. of potassium per liter per hour from

the cells (Table 11A). The rate of diffusion of potassium from the erythrocytes in the presence of 0.005 M iodoacetate is about the same as in the presence of 0.001 M iodoacetate but is again complicated by hemolysis.

At the concentrations of inhibitors used, glycolysis at 37° C. was inhibited to varying degrees. With 0.01 M sodium fluoride, the inhibition was about 80%, while with 0.005 M sodium fluoride, about 50%. The utilization of glucose appeared to be completely blocked at all the concentrations of iodoacetic acid used. Obviously the rate of diffusion of potassium from the cells is not directly dependent on the degree of inhibition of glycolysis. As described earlier, blood cells incubated without glucose in the medium (Tables 9A and B) lose potassium at a rate of about 1 to 2 m. eq. per liter per hour and at a similar rate when glycolysis has been inhibited by oxalate (Tables 4A and 5A). The loss of potassium from the cells incubated with 0.005 M sodium fluoride, 0.005 M iodoacetate or 0.001 M iodoacetate may therefore be largely attributable to the inhibition of glycolysis, while the massive loss of potassium from the cells incubated with 0.01 M sodium fluoride may be due to a direct effect of the poison on the cellular membrane, causing it to become more permeable to potassium at 37° C.

Sodium continues to enter the poisoned cells during the incubation period ( Table 11B), in contrast to the tendency of unpoisoned cells to expel it. The exchange of sodium and potassium, moving along their respective concentration gradients, is almost reciprocal in the blood specimens in which only the glycolytic capacity

has been impaired by the poisons. However, in the case of the blood with 0.01 M sodium fluoride, sodium does not enter the cells as fast as potassium escapes. If a metabolic "pump" expelling only sodium was the sole factor operating, potassium would leak out only as fast as sodium enters. We must accept then, that a concentration of fluoride in the region of 0.01 M alters the permeability of the erythrocyte membrane to potassium but not to sodium, and the loss of potassium cannot be attributed to the blocking of a metabolically-driven transport mechanism alone. This view confirms the observations of Wilbrandt (1937;1940), who found that relatively high concentrations of sodium fluoride (0.015-0.007 M) increase the osmotic resistance of erythrocytes by releasing potassium. This observation in itself implies that sodium had not entered the cells in excessive amounts, and that the loss of potassium is not necessarily preceded or followed by the entry of sodium. A comparable effect on the potassium content of erythrocytes, in vivo, was produced by Henriques and Orskov(1936) by injecting lead salts. After thirty-five minutes the potassium content of the red cells had decreased by eighty percent, with a corresponding shrinkage in the volume of the cells, indicating that the outflow of potassium exceeded the inflow of sodium. During the following twenty-three hours there was a marked increase in the potassium content of the erythrocytes which could not be accounted for by the formation of new red blood cells, but must have been brought about by the uptake of potassium from the plasma. Under such circumstances

the accumulation of potassium by the erythrocytes must be a primary process and not secondary to an active sodium "pump".

Wilbrandt (1937;1940) attributed the effect of glycolytic inhibitors to the interference with the nutrition of the membrane, which he considered necessary for the maintenance of its normal impermeability to cations. However, he also found that concentrations of fluoride from 0.007 - 0.005 M inhibit glycolysis, but had little effect on the permeability of the membrane to potassium. Our observations are essentially in agreement with those of Wilbrandt, but we cannot regard the effect of 0.01 M sodium fluoride on the loss of potassium from the red cells to be due only to the inhibition of their metabolism. Parpart and Green (1950) have demonstrated similar effects with metabolic poisons on the permeability of the erythrocyte membrane to potassium. The reader is referred to Davson and Danielli (1943) for a discussion of their observations on the effect of metabdic inhibitors on the cation gradient in erythrocytes with low potassium and high sodium content.

Greig (1949 et seq.) has suggested that the effect of these poisons on the cholinesterase of the red cells is responsible for the increased permeability of the membrane to potassium ions. She and her co-workers have amply developed this idea in recent years (Greig and Holland,1949; 1950;1951; Holland and Greig,1950;1951) in connection with the osmotic fragility of the erythrocytes. Our findings do not contradict her theory, but it must be borne in mind that a clear distinction must be made between passive permeability phenomena and

active transport mechanisms. Recently a large volume of new information has been added to our understanding of the relationship between the cholinesterase system and the permeability of cellular membranes to sodium and potassium ions. Unfortunately, a good deal of confusion has been introduced through a failure to distinguish between the metabolic processes underlying active cation movements against the concentration gradient and the rate of sodium and potassium exchanges in the direction of the diffusion gradient.

Teorell (1949) and Steinbach (1951) have emphasized the need for a clearer definition and usage of the term "permeability", but their otherwise excellent reviews do not always make the distinction between active transport and permeability phenomena clear. Ussing (1949) and Sheppard (1951) have, on the whole, avoided this ambiguity in their discussions of the permeation, accumulation and expulsion of ions. Parpart and Green (1952) have made a stimulating and provocative contribution to the controversial question, whether the relative impermeability of the red cell membrane, or active transport mechanisms, play the dominant role in maintaining the high potassium concentration and the low sodium concentration in human erythrocytes. Of course, neither process is dominant in so far as one simply acts to offset the other. Recent studies with radioactive isotopes of sodium and potassium (Sheppard and Martin,1950; 1951; Hastings et al.1950; Solomon,1951;1952; Maizels and Harris, 1951)have demonstrated the degree of the permeability of the

erythrocyte membrane to these cations, while the active transport mechanisms normally balance the passive diffusion.

Attempts to poison the active transport mechanism have led to contradictory results because of the failure to realize that the metabolic poisons used also are capable of altering the permeability of the membrane to passive cation diffusion. Our observations should help resolve this problem in that certain concentrations of metabolic poisons affect active transport by inhibiting the source of energy, glycolysis, while higher concentrations of these poisons, in addition to inhibiting glycolysis and active transport, render the membrane more permeable to potassium for passive diffusion along the concentration gradient.

The effect of fluoride and iodoacetate on glycolysis and phosphorolysis at 5° C. and at 37° C. can be seen by comparing the data in Table 11C with those in Tables 11D-G. The findings are essentially in keeping with current concepts of the action of these agents on the glycolytic cycle in erythrocytes (Gourley,1951; Mueller and Hastings,1951), and do not contribute to an understanding of cation transport mechanisms.

The action of cyanide on glycolysis in blood deserves further comment, since this poison interferes with the oxidative metabolism of actively respiring cells. Cyanide, in a concentration that would inhibit oxidative processes in most cells, does not affect the active transport of potassium by stored, incubated erythrocytes. This was to be expected, since human red cells have a low oxygen consumption and are considered to derive most of their energy from

aerobic glycolysis. The small amounts of energy arising from oxidative processes are replaced by a more active glycolysis in the presence of cyanide. Maizels (1951) has shown a similar effect with carbon monoxide and azide, as well as with cyanide.

Fluoride and iodoacetate have a profoundly deleterious effect on the stability of the erythrocyte membrane. The spontaneous hemolysis of the poisoned cells during the cold-storage and the incubation periods was very great (Table 11I). The removal of calcium by fluoride has been suggested as the reason for the increased fragility of the cells (also seen in oxalated cells (Tables 4A and 5A)), but the even more profound hemolytic effect of iodoacetate indicates that the damage to the membrane is probably metabolic in origin, possibly related to the nutrition of the membrane.

The fragility of the poisoned cells in various dilutions of buffered saline after ten days of cold-storage is shown in Table 11J. The cells stored with the glycolytic inhibitors demonstrate a greatly increased fragility, not the decreased fragility found by Wilbrandt (1937;1940) in poisoned fresh cells. His experiments were done at room temperature, where potassium leaves the poisoned cells rapidly. This decreases the amount of osmotically active material in the cells, which attract water and cause the cells to swell beyond their critical volume, when exposed to hypotonic saline. In our storage experiments in the cold, the poisoned cells do not lose potassium at an abnormal rate and could not be expected to be more

stable to hypotonic saline than unpoisoned cells. On the contrary, the poisoned cells are more prone to osmotic hemolysis than the normal ones, not because of any increase in the amount of osmotically active material inside, but because the membrane itself is more fragile and unable to withstand the inevitable swelling that occurs in hypotonic saline.

Sodium cyanide has little, if any, effect either on the degree of spontaneous hemolysis of the erythrocytes during storage and incubation, or on the fragility of the cells when exposed to hypotonic saline.

H. The influence of hormones on the electrolyte exchanges in erythrocytes.

It has long been recognized that the concentration of potassium in the plasma generally follows the carbohydrate cycle, rising and falling with the blood sugar level (Fenn,1940). As early as 1923, Harrop and Benedict (1923;1924) and Briggs, et al. (1923) had shown that the concentration of potassium in the plasma decreased after the injection of insulin. During the last decade many clinical reports have indicated that during diabetic acidosis the potassium concentration in the plasma may be increased (Danowski et al.,1927; Nadler et al.,1948), while during insulin therapy this concentration may be reduced (see Overman,1951, for numerous references; Groen et al.,1952).

The loss of potassium from diabetic cells is, for the most part, due to changes in cellular metabolism (Elkinton and Winkler,1944) and during the recovery of the cells, under the influence of insulin, potassium is recovered. It is generally thought that under such circumstances potassium migrates from the extracellular to the intracellular space where it becomes fixed with newly formed glycogen and perhaps protein. Kamminga and his co-workers (1950) have shown that the increased glucose utilization by isolated rat diaphragm in the presence of added insulin is accompanied by an uptake of potassium from the medium.

As early as 1926, Kerr (1926a;1926b) demonstrated that the injection of insulin could increase the potassium content of

circulating erythrocytes, while low red blood cell potassium levels have been reported in diabetic acidosis (Schmitt,1936; Danowski et al.,1941; Guest and Rapoport, 1948). The rate of glycolysis in diabetic erythrocytes has been increased, in vitro, by the addition of insulin (Bevilotti and Citarda,1948). The accumulation of potassium by previously depleted erythrocytes has been demonstrated, in vivo, by Maizels (1943) and Davidsen and Kjerulf-Jensen (1950).

It was considered profitable to investigate the influence of insulin on the uptake of potassium by erythrocytes that had previously been depleted of some of their potassium during a period of storage in the cold. The effect of insulin on the glycolytic rate and the glycogen content of the erythrocytes was also investigated. Insulin had no detectable effects when added to blood in a concentration of 10 units per 100 ml. The insulin did not affect the rate of potassium diffusion, glucose breakdown and glycogen disappearance during cold-storage. Nor did this amount of added insulin affect either the rate of potassium uptake, glycolysis or the breakdown of glycogen in incubated specimens. Diabetic blood cells have not been investigated in this respect.

There is some question as to the exact nature of the glycogen found in the erythrocytes. Attempts to demonstrate its presence by histo-chemical methods have invariably failed (Wachstein, 1949; Gibb and Stowell,1949). The material we measured was, like glycogen, resistant to alkali hydrolysis, insoluble in ethanol and gave rise to copper-reducing substances on acid hydrolysis. We were

able to demonstrate the presence of an amount of this material that yields the equivalent of 30 mg. of glucose per 100 ml. of erythrocytes. This level falls to zero during ten days of cold-storage, but is rapidly depleted during incubation at 37° C. Andreae (1946) found similar amounts in blood, which also disappeared during ten days of storage.

Yamagata (1949), using another technique, found 30 to 80 mg. of glycogen in 100 ml. of blood. We have not subjected the red cell glycogen we isolated to salivary digestion and are therefore not certain of its true chemical nature.

The effect of the adrenal cortical hormones on the ionic composition of the body is well known. Loeb (1932) showed that hyponatremia and hyperkalemia were part of the symptom complex of Addison's disease in humans. He advanced the hypothesis that the hormones of the adrenal cortex act directly on the kidney, causing sodium retention and potassium excretion. Swingle and his co-workers (1937) were of the opinion that the cortical hormones affected the distribution of cations between the intracellular and extracellular compartments of the body directly.

It is well known that adrenalectomized animals show changes in the concentration of electrolytes in the intracellular as well as extracellular fluids. Muscles and erythrocytes show an increased potassium and decreased sodium content, while desoxycortico sterone acetate increases the intracellular sodium and decreases the intracellular potassium of muscles (Miller and Darrow, 1941; Muntwyler et al., 1944). The cortical hormones may act only on the reabsorptive

mechanism in the kidney tubules, but there is evidence that they have a direct effect on the electrolyte composition of other cells as well. Hegnauer (1943) decreased the potassium content of the muscles and erythrocytes of rats by a potassium deficient diet. Injections of desoxycorticosterone acetate did not allow a fall in the concentration of potassium in the red cells in the potassium deficient animals, although the muscle potassium was low. Krogh (1946) suggested that the hormone may stimulate the potassium transport mechanism in the erythrocytes of the potassium depleted animals.

The cation distribution in blood stored at 5° C. is unaffected by the presence of 2 mg. of either desoxycorticosterone or its acetate in 100 ml. of the blood mixture. Similarly, the addition of either of these two steroids had no effect on the cation exchanges in erythrocytes incubated at 37° C.

I. Other sources of energy for active cation transport in the erythrocyte.

Maizels(1951) has shown that mannose and fructose can replace glucose in energizing cation transport in human erythrocytes. Galactose, pentoses, disaccharides, pyruvate and lactate will not energize active cation transport.

Krebs and his co-workers (1949;1950a;1950b) showed that glutamic acid plays an important role in the transport of potassium in brain and retinal cells. Cold-stored retinal cells, which had lost 50% of their potassium, regained it after incubation with glucose and glutamate, but not well with either alone. Glucose can be replaced by lactate or pyruvate, glutamate by aspartate.

We have been unable to demonstrate any effect of glutamic acid on the uptake of potassium by previously depleted erythrocytes. There is no evidence that erythrocytes can utilize glutamate as an energy source.

J. Oxygen consumption and the Pasteur effect in erythrocytes.

The mature human red cell demonstrates a low consumption of oxygen, and some investigators doubt that this respiratory activity is of any significance in the energy metabolism of these cells (see Denstedt,1952,for numerous references). On the other hand, evidence both direct and indirect, indicates that although the metabolism of the erythrocyte is mainly conducted through aerobic glycolysis, this is accompanied by a small amount of respiration, a dual metabolic route normally followed by many cells such as spermatozoa, retina and pus, and resorted to by other tissues in times of insufficient oxygen.

It has been known for many years that the respiration of mammalian erythrocytes is accompanied by the liberation of carbon dioxide and heat (Harrop and Barron,1928; Ramsay and Warren,1930). Warren and Ponder (Ponder,1948) have estimated that 60% of the energy generated by erythrocyte metabolism is derived from oxidative processes, feeble as they may seem. Warburg (1949) has referred to the oxygen consumption of mammalian erythrocytes in connection with the effect of respiratory poisons. Within forty hours at 37° C. all the oxygen in arterialized blood is used up by oxidative processes in the red cells. These processes can be inhibited by carbon monoxide and cyanide (Onaka,1911). When the respiration of the erythrocyte is thus inhibited, glycolysis increases from the aerobic to the anaerobic value. Drabkin (1951) has shown

that the concentration of cytochrome C, a member of the biocatalytic system responsible for the utilization of oxygen, is proportional to the rate of oxygen consumption or oxidative metabolism in numerous tissues, including mammalian red blood cells.

The effect of ageing on the oxygen consumption of rat erythrocytes has been studied by Angelone and Angerer (1949). The oxygen consumption of the red cells was measured intermittently in the Warburg manometric apparatus . Fresh erythrocytes showed a $QO_2$ (dry weight) of -0.16 $mm.^3$ After two days of storage at 2° -8° C. in Kreb's solution ($p^H$ 7.4), the $QO_2$ fell to -0.138 $mm.^3$ and after four days storage, to -0.095 $mm.^3$ The $QO_2$, after storage for a week, was reduced to - 0.053 $mm.^3$, while after two weeks, the $QO_2$ had fallen to -0.03 $mm.^3$

The writer studied the rate of oxygen consumption in whole blood during cold-storage and subsequent incubation at 37° C. Citrated blood, with and without added glucose, was dispensed into small tubes, which were almost completely filled with the mixture, stoppered and sealed with paraffin wax. These tubes were stored at 5° C. and incubated at 37° C. as described under Methods. The decrease in the oxygen content of the specimens during the storage period is seen in Table 12. The changes are quite irregular due to the limitations of the technique used, and a change in the oxygen content of the blood during four hours at 37° C. could not be demonstrated unequivocally. It may be readily observed, by plotting a graph of the oxygen content of the

blood against the duration of the storage, that the rate of oxygen consumption falls off quite rapidly during the storage period, and, in citrated blood, stops within a week or two. The data indicate that the oxygen consumption proceeds for a longer period in blood enriched with glucose. We have not attempted to measure the oxygen content of blood stored in the presence of respiratory inhibitors, but have noticed that the bright red color of oxyhemoglobin persists in cyanide-containing blood, whereas the color of citrated blood progressively darkens during storage. Rochlin (1942) has shown that methemoglobin does not form in blood during prolonged storage in the cold.

The significance of the oxygen consumption of human erythrocytes has been questioned by some, especially since Alivisatos# (1951) was unable to demonstrate succinic dehydrogenase activity in these cells. However, the demonstration that anaerobic glycolysis exceeds aerobic glycolysis (Bird, 1947; Warburg, 1949), and that inhibitors of oxidative enzymes accelerate glycolysis in blood, elevating it from the aerobic to the anaerobic level (compare Table 11C with 11H) (Onaka, 1911; Maizels, 1951), are evidences of a Pasteur effect in human erythrocytes (see Burk, 1939). Moreover the writer has observed, occasionally, that the incubation of two to four day old blood specimens may result in a greater decrease in the glucose content than in fresh specimens. We have suggested that this may be again related to the Pasteur mechanism, whereby the rapid failure

# Personal communication.

of the respiratory systems is compensated for, in part at least, by an accelerated glycolytic activity. The reader is referred to a further discussion by the writer, on the possible significance of the low respiratory rate in the energy metabolism of human erythrocytes (see Denstedt,1952).

It is possible, then, that respiratory processes normally contribute to active transport mechanisms in the erythrocyte, although agents such as cyanide do not seem to affect the accumulation of potassium nor the expulsion of sodium by these cells. If the transport mechanisms are regulated by the amount of adenosine triphosphate available, the accelerated glycolysis that occurs anaerobically, will meet these requirements.

K. Carbonic anhydrase in stored blood.

The erythrocytes contain a number of enzymes associated with respiration, one of which, carbonic anhydrase, is important in the transport of carbon dioxide. Meldrum and Roughton (1933) first demonstrated its existence, and it was crystallized from the red cells by Keilin and Mann (1940). This enzyme catalyzes the reversible formation of carbonic acid from carbon dioxide and water, driving it forward in the tissues, and driving it backward, liberating carbon dioxide, in the lungs. There is sufficient carbonic anhydrase in the erythrocytes to accelerate this reaction about 1,500 times at 38° C. An extensive review of all aspects of carbonic anhydrase distribution and activity is available (van Goor, 1948).

The role of carbonic anhydrase in certain ionic exchanges in the erythrocyte has been studied by Jacobs and Stewart (1941), but this did not include an investigation of the role of this enzyme in cation exchanges. The role of carbonic anhydrase in ion transport mechanisms in the gastric mucosa, (Anderson and Wilbur,1948; Martinson, 1950; Davies 1948), and in the kidney (Pitts and Lotspeich,1946; Pitts,1948; Davies,1950) has been investigated extensively. The systems described by these investigators involve ion-exchange mechanisms, hydrogen ions from carbonic acid, formed under the agency of carbonic anhydrase, exchanging for sodium ions.

The possibility that carbonic anhydrase is involved in cation transport mechanisms in the erythrocyte prompted the writer to determine the stability of the enzyme during the preservation of blood

in the cold. It was found that the activity of the enzyme remains unaltered in stored blood long after cation transport has failed. Furthermore, the writer found that normal activity is retained in specimens of human blood stored aseptically in the refrigerator for over a year. Keilin and Wang (1947) found that a forty-two year old specimen of horse blood had retained 83% of the normal activity when stored in glass ampules without continuous refrigeration.

Cyanide and sulfanilamide inhibit the activity of carbonic anhydrase (see Lewis and Altshule, 1949, for numerous references), but we have shown that neither substance affects the uptake of potassium by stored erythrocytes. The conclusion, therefore, is warranted that carbonic anhydrase plays no role in cation movements in the human erythrocyte.

L. Cholinesterase in Stored Blood.

Greig and Holland (1949-1951; see also Holland and Greig) have introduced a concept which relates the activity of the cholinesterase-choline acetylase system to the permeability properties of mammalian erythrocytes, much as Nachmansohn (1945; 1946; 1950) has described the participation of this enzyme system in axonal conduction. Working with dog erythrocytes, which have a low potassium and a high sodium content, Greig has related the osmotic stability of the cells to a functioning cholinesterase system. The inhibition of the cholinesterase activity of the red cells by various well known inhibitors, presumably resulting in the accumulation of acetyl choline, or the addition of acetyl choline, results in an increase in the fragility and the rate of spontaneous hemolysis of the erythrocytes when suspended in an isotonic sodium chloride-sodium bicarbonate solution. On the other hand, these drugs, plus certain other choline and non-choline esters, increase the resistance of the erythrocytes in an isotonic potassium chloride-potassium bicarbonate solution.

Greig and Holland have further demonstrated that the effect of these drugs on the fragility of the red cells of cats, dogs and rabbits may be related to their effect on the permeability of the membrane to cations, and have correlated the rate of the hydrolysis of acetyl choline and certain other choline and non-choline esters with a slower rate of loss of potassium

from the cells. In contrast, Parpart and Hoffman (1952) have shown that the rate of diffusion of potassium from human erythrocytes may be retarded to the same extent with appropriate amounts of acetic acid. They attribute the effect of the acetyl choline to the $p^H$ change brought about by its hydrolysis. However, neither acetic acid nor choline alone was effective in delaying cation diffusion in the dog, cat and rabbit erythrocytes (Holland and Greig, 1950).

A study demonstrating a correlation between the cholinesterase activity of the red cells of various mammalion species and the stability of these cells in hemolytic solutions has been published in Russian (Koshtoyants and Bulatova, 1950).

Taylor and Weller (1950) found that physostigmine, a cholinesterase inhibitor, promoted the escape of potassium from human erythrocytes, but the findings of Christensen and Riggs (1951) indicate that this effect is not mediated through the inhibition of the cholinesterase system, but by the competition of the cationic alkaloid with potassium for a place in the potassium accumulating mechanism. Thus, at low concentrations, physostigmine interferes with the transport of potassium into the cell, but not with the diffusion of potassium out of the cell (Taylor and Weller, quoted by Christensen and Riggs, 1951).

The results of further investigations into the role of the cholinesterase-choline acetylase system in the maintenance of the potassium content of human red blood cells

have recently been reported (Taylor, Weller and Hastings, 1952). These investigators were able to measure qualitative as well as quantitative changes in the potassium content of the erythrocytes by the use of a radioactive isotope of potassium. They found that the addition of agents that will inhibit the choline acetylase system, results in a rapid decrease in the potassium content of the cells, by increasing the rate at which this ion diffuses from them; the addition of agents that will inhibit the cholinesterase system, results in a net decrease in the potassium content of the cells, by interfering with the accumulating mechanism for potassium. The Harvard group was able to demonstrate that, at 37° C., high concentrations of fluoride decrease the rate of entry of potassium and increase the rate of its exit, both effects leading to a fall in the potassium content of the cells. This observation is in accord with the writer's findings, as described above (section G). Fluoride affects the transport of potassium into the cells by inhibiting glycolysis (the energy source) and cholinesterase (role unknown). The implication is that the diffusion of potassium from the cells is accelerated by the action of fluoride on the choline acetylase system.. Flucride may inhibit this particular system indirectly, by blocking glycolysis and the production of adenosine triphosphate and pyruvate, both necessary in the synthesis of acetyl choline by choline acetylase (Harpur and Quastel, 1949). How the choline acetylase system operates to keep the cell membrane

relatively impermeable to potassium ions is unknown at the present time. Its function may be related to the maintenance of a positively charged area in the cell membrane (see Discussion,

Recently Korey (see Nachmansohn and Wilson, 1951) and Greig and Holland (1952) have demonstrated the existence, in human erythrocytes, of a system capable of the enzymatic acetylation of choline. What the natural substrates and products of this system are is as yet unknown. The work of Christensen and Hastings (1940) on the complexing of cations by certain phospholipids may be of significance in this connection. Also, the existence of choline-containing phospholipids in the erythrocyte membrane should not be overlooked.

Although the work of some (Taylor, Weller and Hastings, 1952) has been more definitive in this subject than that of others, it is still safe to say that the role of the cholinesterase-choline acetylase system in the maintenance of the cation gradients in cells, by active transport mechanisms and by the maintenance of a relatively impermeable membrane, is unknown.

The writer's main object in studying the cholinesterases of blood was to determine the stability of these enzymes during cold-storage. Pritchard (1949) had shown that the younger erythrocytes in the blood of the rat have a higher cholinesterase activity than the older ones. Keilin and Wang (1947) found that specimens of horse blood that had been stored in ampules for forty-two years, still retained 85% of the activity.

The writer measured the cholinesterase activity of the blood, the cells and the plasma during a period of cold-storage of whole citrated blood. The non-specific substrate, acetyl-choline chloride, was used since the main object was to determine the changes in terms of relative values only. The effects of thirty-three days of cold-storage on the cholinesterase activity of blood, erythrocytes and plasma are illustrated in Table 13. The cholinesterase activity of the whole blood does not change significantly during the storage period. The writer also determined the cholinesterase activity of a specimen of human blood which had been stored aseptically for two years, and found it to be normal.

Of greater interest is the finding that the cholinesterase gradually leaves the erythrocytes and enters the plasma during the first three weeks of storage. During the following twelve-day period the rate of loss of the cholinesterase from the cells into the plasma is greatly accelerated. By the thirty-third day of storage the activity of the enzyme in the plasma is almost 200% that of fresh plasma, and the activity of the cells is about 60% that of fresh cells. A simple calculation (Table 13) shows that all the cholinesterase activity that leaves the cells can be accounted for in the plasma and that none was lost in the washing of the cells. Davies and his co-workers (1951) noticed a decrease in the cholinesterase activity of the cells and an increase in the plasma of oxalated blood specimens during short periods of storage. They attributed this to the progressive

hemolysis, which was appreciable under their conditions. The writer's findings do not correlate with the degree of spontaneous hemolysis occuring in the specimens, which was negligible, but the possibility that the cholinesterase leaves the cells in a pre-hemolytic stage cannot be overlooked.

The cholinesterase activity of plasma, stored separately from the erythrocytes, does not change, even after long periods at 4° C. Scudamore and his colleagues (1950) stored plasma in a "deep-freeze" and found no change in the cholinesterase activity of the plasma.

The writer hemolyzed erythrocytes by repeatedly freezing and thawing them in a dry-ice-alcohol mixture and found that this procedure does not affect the total cholinesterase activity of the specimen. Removal of the cell debris from such a hemolysate, by high-speed centrifugation, does not alter the activity of the hemolysate, indicating that the enzyme, which is considered to be situated in or on the red cell membrane (Krauer and Root, 1945; Paleus, 1947) becomes detached from the stroma during the process of freezing hemolysis. According to Mentha and his colleagues (1947), the cholinesterase can be extracted from chilled red cells at $p^H$ 8.3 with little hemolysis. This indicates that the enzyme is quite readily detachable from the stroma of the cells.

The significance of the departure of the cholinesterase from the erythrocyte surface during cold-storage, and the relationship between this process and the permeability properties of such stored cells to cations, remains to be elucidated.

TABLE 1A

The effect of cold-storage and of incubation at 37°C on the concentration of plasma electrolytes*

| Storage Period (days) | Incub. Period (mins) | Whole Blood | | Plasma | | |
|---|---|---|---|---|---|---|
| | | H. Crit | Hb gm.% | K meq /l | Cl meq/l | Fe mg % |
| 0 | 0 | 37 | 11.5 | 7.1 | 102 | 0.22 |
| | 30 | 37 | 11.7 | 7.5 | 101 | 0.26 |
| | 60 | 38 | 11.5 | 6.8 | 105 | 0.33 |
| | 90 | 37 | 11.3 | 6.5 | 110 | 0.22 |
| | 120 | 38 | 11.8 | 6.8 | 115 | 0.33 |
| 7 | 0 | 37 | 11.4 | 33.4 | 105 | 0.21 |
| | 30 | 37 | 11.6 | 33.2 | 104 | 0.28 |
| | 60 | 37 | 11.5 | 32.2 | 104 | 0.37 |
| | 90 | 37 | 11.5 | 30.2 | 104 | 0.41 |
| | 120 | 38 | 11.5 | 29.4 | 103 | 0.37 |
| 14 | 0 | 38 | 11.4 | 36.3 | 108 | 0.35 |
| | 30 | 37 | 11.5 | 35.9 | 108 | 0.43 |
| | 60 | 37 | 11.5 | 34.9 | 108 | 0.45 |
| | 90 | 38 | 11.6 | 34.8 | 108 | 0.47 |
| | 120 | 38 | 11.5 | 33.5 | 108 | 0.39 |
| 21 | 0 | 37 | 11.5 | 39.6 | 102 | 0.49 |
| | 30 | 38 | 11.4 | 40.2 | 103 | 0.58 |
| | 60 | 38 | 11.5 | 38.9 | 103 | 0.51 |
| | 90 | 37 | 11.5 | 38.9 | 102 | 0.47 |
| | 120 | 39 | 11.4 | 39.6 | 103 | 0.58 |
| 28 | 0 | 39 | 11.5 | 43.3 | 103 | 0.49 |
| | 30 | 37 | 11.6 | 45.6 | 103 | 0.53 |
| | 60 | 38 | 11.7 | 46.9 | 102 | 0.52 |
| | 90 | 38 | 11.4 | 45.4 | 102 | 0.75 |
| | 120 | 39 | 11.5 | 45.0 | 102 | 0.72 |

* Blood: Citrate: Glucose = 5:1:1

TABLE 1B

The effect of cold-storage and of incubation at 37° C. on the fragility of the red cells

| Storage Period (days) | Incub. Period (Mins.) | Percentage of Isotonic Saline (Simmel's) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 100 | 70 | 65 | 60 | 55 | 50 | 45 | 40 |
| | | % hemolysis | | | | | | | |
| 0 | 0 | | | | 0 | 6 | 39 | 90 | 98 |
| | 30 | | | | 2 | 4 | 38 | 96 | 100 |
| | 60 | | | | 0 | 7 | 60 | 95 | 100 |
| | 90 | | | | 0 | 17 | 70 | 95 | 100 |
| | 120 | | | | 0 | 12 | 67 | 90 | 100 |
| 7 | 0 | | 2 | 4 | 6 | 17 | 51 | 91 | 98 |
| | 30 | | 0 | 4 | 6 | 15 | 55 | 96 | 100 |
| | 60 | | 0 | 8 | 9 | 19 | 55 | 98 | 100 |
| | 90 | | 0 | 6 | 9 | 22 | 70 | 98 | 100 |
| | 120 | | 2 | 7 | 9 | 20 | 71 | 100 | |
| 14 | 0 | 0 | 3 | 5 | 9 | 22 | 55 | 97 | 98 |
| | 30 | 0 | 3 | 5 | 9 | 20 | 65 | 97 | 100 |
| | 60 | 0 | 3 | 5 | 9 | 20 | 65 | 100 | 100 |
| | 90 | 0 | 3 | 4 | 8 | 20 | 70 | 98 | 100 |
| | 120 | 2 | 9 | 15 | 20 | 32 | 72 | 98 | 100 |
| 21 | 0 | 2 | 4 | 9 | 16 | 45 | 88 | 98 | 100 |
| | 30 | 2 | 3 | 5 | 13 | 32 | 76 | 97 | 100 |
| | 60 | 2 | 3 | 7 | 13 | 37 | 80 | 98 | 100 |
| | 90 | 2 | 3 | 5 | 18 | 50 | 85 | 98 | 100 |
| | 120 | 2 | 3 | 8 | 14 | 40 | 78 | 96 | 100 |
| 28 | 0 | 3 | 5 | 8 | 20 | 48 | 82 | 98 | 100 |
| | 30 | 3 | 7 | 10 | 20 | 52 | 86 | 96 | 100 |
| | 60 | 2 | 6 | 8 | 20 | 52 | 85 | 96 | 100 |
| | 90 | 3 | 6 | 12 | 22 | 54 | 87 | 96 | 100 |
| | 120 | 3 | 12 | 15 | 32 | 68 | 91 | 98 | 100 |

Blood-citrate-glucose - 5:1:1

TABLE 2A

The effect of various preservatives on the concentration of Magnesium in the blood and plasma during cold-storage.

| Storage Period | Citrate | | Citrate-Glucose | | Oxalate | |
|---|---|---|---|---|---|---|
| | Blood | Plasma | Blood | Plasma | Blood | Plasma |
| (days) | mg% | mg% | mg% | mg% | mg% | mg% |
| 0 | 3.0 | 1.3 | 2.7 | 1.0 | 3.3 | 2.0 |
| 1 | 3.2 | 1.2 | 2.8 | 1.1 | 3.3 | 2.0 |
| 4 | 3.0 | 1.0 | 2.8 | 0.9 | 3.5 | 1.9 |
| 7 | 3.1 | 1.1 | 2.8 | 0.8 | 3.3 | 2.0 |
| 10 | 3.1 | 1.2 | - | - | 3.3 | 2.2 |
| 13 | 3.0 | 1.1 | - | - | 3.3 | 2.5 |
| 14 | - | - | - | - | 3.3 | 2.6 |

1. Blood-Citrate = 5:1 2. Blood-citrate-glucose = 5:1:0.5

3. Blood-oxalate = 5:1

TABIE 3A

The effect of storing and of incubating blood in various preservative mixtures on the concentration of magnesium in the plasma.

| Storage Period (days) | Incub. Period (hours) | Citrate mg.% mg% | Citrate-glucose mg.% mg% | Oxalate mg.% mg% |
|---|---|---|---|---|
| 0 | 0 | 1.5 | 1.4 | 1.5 |
| | 2 | 1.5 | 1.4 | 1.5 |
| | 4 | 1.4 | 1.4 | 1.5 |
| 2 | 0 | 1.4 | 1.4 | 1.5 |
| | 2. | 1.3 | 1.5 | 1.5 |
| | 4 | 1.5 | 1.4 | 1.3 |
| 5 | 0 | 1.2 | 1.3 | 1.5 |
| | 2 | 1.2 | 1.3 | 1.6 |
| | 4 | 1.2 | 1.3 | 1.5 |
| 9 | 0 | 1.3 | 1.4 | 1.6 |
| | 2 | 1.3 | 1.4 | 1.8 |
| | 4 | 1.3 | 1.4 | 1.9 |
| 14 | 0 | 1.3 | 1.4 | 1.7 |
| | 2 | 1.3 | 1.4 | 1.8 |
| | 4 | 1.3 | 1.4 | 1.8 |
| 21 | 0 | 1.2 | 1.5 | 1.9 |
| | 2 | 1.2 | 1.4 | 1.9 |
| | 4 | 1.2 | 1.4 | 2.0 |
| 28 | 0 | 1.3 | 1.4 | 2.0 |
| | 2 | 1.2 | 1.4 | 2.1 |
| | 4 | 1.2 | 1.3 | 2.2 |

1.Blood-citrate = 5:1 2. Blood-citrate-glucose = 5:1:0.5
3. Blood-oxalate = 5:1

TABLE 4A

The effect of cold-storage and incubation at 37° C. on the concentration of plasma electrolytes.

| Storage Period (days) | Incub. Period (Mins.) | $K^+$ | | $Cl^-$ | | I P | | $Fe^{++}$ (?) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Cit. | Oxal. | Cit. | Oxal. | Cit. | Oxal. | Cit. | Oxal |
| | | mg% | mg% | mg% | mg% | mg% | mg% | mg% | mg% |
| 0 | 0 | 16.2 | 15.0 | 333 | 365 | 2.3 | 4.9 | 0.25 | 0.25 |
| | 30 | 15.9 | 19.8 | 328 | 370 | 2.4 | 4.6 | 0.32 | 0.29 |
| | 60 | 16.2 | 20.1 | 333 | 365 | 1.9 | 5.5 | 0.39 | 0.35 |
| | 90 | 16.5 | 21.6 | 329 | 363 | 2.4 | 7.7 | 0.35 | - |
| | 120 | 17.1 | 23.6 | 327 | 366 | 2.9 | 6.2 | 0.90 | 0.55 |
| 1 | 0 | 36.9 | 36.0 | 340 | 368 | 3.6 | 5.6 | 0.35 | 0.42 |
| | 30 | 32.4 | 37.8 | 340 | 372 | 3.6 | 6.9 | 0.23 | 0.64 |
| | 60 | 30.9 | 39.8 | 339 | 370 | 4.5 | 8.0 | 0.32 | 0.69 |
| | 90 | 28.8 | 37.8 | 333 | - | 5.4 | 8.0 | 0.39 | 0.67 |
| | 120 | 29.4 | 43.2 | 335 | 372 | 4.8 | 8.4 | 0.38 | 0.84 |
| 2 | 0 | 50.4 | 44.1 | 328 | 368 | 3.4 | 4.8 | 0.32 | 0.56 |
| | 30 | 46.8 | 45.9 | 331 | 370 | 3.5 | 5.7 | 0.41 | 0.62 |
| | 60 | 44.1 | 46.8 | 331 | 366 | 4.7 | 8.2 | 0.35 | 0.69 |
| | 90 | 42.0 | 46.2 | 328 | 364 | 5.3 | 8.3 | 0.35 | 0.77 |
| | 120 | 42.0 | 47.7 | 326 | 368 | 6.8 | 9.2 | 0.41 | 1.0 |
| 5 | 0 | 73.5 | 69.0 | 323 | 363 | 3.6 | 5.5 | 0.35 | 1.4 |
| | 30 | 72.0 | 72.0 | 326 | 365 | 4.0 | 7.1 | 0.32 | 1.6 |
| | 60 | 69.9 | 72.0 | 324 | 366 | 5.3 | 7.9 | 0.32 | 1.8 |
| | 90 | 61.2 | 72.9 | 326 | 363 | 6.5 | 7.9 | 0.45 | 1.9 |
| | 120 | 66.6 | 72.9 | 326 | 359 | 8.5 | 8.8 | 0.50 | 2.0 |
| 8 | 0 | 93.0 | 117.0 | 322 | 359 | 4.8 | 8.6 | 0.36 | 2.8 |
| | 30 | 92.1 | 121.2 | 322 | 361 | 7.8 | 11.0 | | |
| | 60 | 91.8 | 118.8 | 318 | 361 | 9.9 | 11.6 | | |
| | 90 | 88.5 | 120.0 | 324 | 363 | 11.7 | 12.0 | | |
| | 120 | 91.5 | 123.3 | 322 | 359 | 12.9 | 11.6 | 0.39 | 3.1 |

cont'd

TABLE 4A - Continued.

| Storage Period (days) | Incub. Period (Mins.) | K+ | | Cl- | | I P† | | Fe++ (†) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Cit. mg% | Oxal. mg% | Cit. mg% | Oxal. mg% | Cit. mg% | Oxal. mg% | Cit. mg% | Oxal. mg% |
| 11 | 0 | 109.8 | 113.1 | 322 | 357 | 3.6 | 7.4 | 0.35 | 3.6 |
| | 30 | 110.7 | 117.0 | 320 | 350 | 13.2 | 9.0 | - | - |
| | 60 | 109.5 | 116.1 | 318 | 355 | 16.7 | 9.6 | - | - |
| | 90 | 118.8 | 120.9 | 316 | 352 | 18.2 | 11.0 | - | - |
| | 120 | 115.2 | 120.0 | 316 | 350 | 18.0 | 10.4 | 0.45 | 6.5 |
| 14 | 0 | 121.5 | 127.8 | 315 | 353 | 6.0 | 8.4 | 0.38 | 7.6 |
| | 30 | 123.3 | 127.8 | 315 | 350 | 14.4 | 10.8 | - | - |
| | 60 | 125.1 | 128.7 | 313 | 352 | 19.2 | 11.7 | - | - |
| | 90 | 127.8 | 132.0 | 307 | 352 | 19.2 | 11.4 | - | - |
| | 120 | 127.8 | 134.1 | 311 | 348 | 22.0 | 12.3 | 0.57 | 9.4 |
| 21 | 0 | 149.4 | 144.0 | 307 | 338 | 11.4 | 13.5 | 0.77 | 9.9 |
| | 30 | 153.9 | 146.7 | 305 | 338 | 20.4 | 13.5 | - | - |
| | 60 | - | 147.6 | - | 336 | - | 15.6 | - | - |
| | 90 | - | 148.5 | - | 337 | - | 20.1 | - | - |
| | 120 | 156.6 | 151.2 | 302 | 338 | 26.4 | 19.6 | 1.0 | 14.2 |

† I P - inorganic phosphate P

1. Blood - citrate = 5:1 2. Blood-oxalate = 5:1

TABLE 4B

The effect of incubation at 37° C. for 2 hours on the concentration of potassium in the plasma of citrated blood # stored for various periods.

| Storage Period (days) | Before Incubation mg% | After Incubation mg% | Net Change mg% |
|---|---|---|---|
| 0 | 16.2 | 17.1 | +0.9 |
| 1 | 36.9 | 29.4 | -7.5 |
| 2 | 50.4 | 42.0 | -8.4 |
| 5 | 73.5 | 66.6 | -6.9 |
| 8 | 90.0 | 91.5 | -1.5 |
| 11 | 109.8 | 115.2 | +5.4 |
| 14 | 121.5 | 127.8 | +6.3 |
| 21 | 149.4 | 156.6 | +7.2 |

# Blood-citrate = 5:1

TABLE 5A

The effect of cold-storage and of incubation at 37° C. on the concentration of plasma electrolytes of citrated and oxalated blood stored with added glucose.

| Storage Period (Days) | Incub. Period (Mins.) | $K^+$ | | $Cl^-$ | | IP | | TASP# | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 mg% | 2 mg% | 1 mg% | 2 mg% | 1 mg% | 2 mg% | 1 mg% | 2 mg% |
| 0 | 0 | 18.6 | 24.6 | 407 | 410 | 3.6 | 3.8 | 4.6 | 5.9 |
| | 30 | 19.8 | 27.0 | 408 | 410 | 3.1 | 4.1 | 3.8 | 6.5 |
| | 60 | 21.3 | 28.2 | 407 | 411 | 2.9 | 5.3 | 4.1 | 7.3 |
| | 90 | 19.8 | 30.0 | 406 | 410 | 3.1 | 5.0 | 4.8 | 8.3 |
| | 120 | 21.0 | 27.9 | 408 | 411 | 4.8 | 5.6 | 4.9 | 8.0 |
| 1 | 0 | 54.0 | 39.3 | 400 | 410 | 3.2 | 3.8 | 4.0 | 4.9 |
| | 30 | 51.3 | 40.5 | 402 | 408 | 2.9 | 4.9 | 5.3 | 6.4 |
| | 60 | 45.9 | 42.6 | 402 | 409 | 3.7 | 5.2 | 4.0 | 7.2 |
| | 90 | 42.6 | 40.5 | 400 | 410 | 4.6 | 6.1 | 7.5 | 7.7 |
| | 120 | 40.5 | 42.3 | 402 | 408 | 5.9 | 6.3 | 9.1 | 7.7 |
| 2 | 0 | 77.4 | 54.3 | 400 | 408 | 2.5 | 3.6 | 4.3 | 6.9 |
| | 30 | 72.0 | 56.7 | 400 | 408 | 2.9 | 4.3 | 5.0 | 7.1 |
| | 60 | 66.0 | 54.0 | 398 | 406 | 4.1 | 5.5 | 6.5 | 7.8 |
| | 90 | 63.9 | 58.5 | 398 | 407 | 5.2 | 5.8 | 7.5 | 8.5 |
| | 120 | 61.2 | 57.9 | 400 | 407 | 6.3 | 6.4 | 9.2 | 9.7 |
| 5 | 0 | 128.7 | 86.4 | 392 | 402 | 3.5 | 4.8 | 4.5 | 5.7 |
| | 30 | 115.2 | 90.0 | 393 | 404 | 3.4 | 5.5 | 4.6 | 7.4 |
| | 60 | 113.4 | 90.0 | 393 | 402 | 4.9 | 5.7 | 6.9 | 7.4 |
| | 90 | 111.9 | 91.2 | 392 | 402 | 6.6 | 6.7 | 8.6 | 9.0 |
| | 120 | 106.8 | 90.8 | 392 | 404 | 7.9 | 7.3 | 9.5 | 9.6 |
| 8 | 0 | 150.9 | 112.8 | 387 | 402 | 3.5 | 4.6 | 6.7 | 8.8 |
| | 30 | 147.3 | 116.1 | 387 | 401 | 7.1 | 5.8 | 7.0 | 10.6 |
| | 60 | 146.1 | 109.8 | 386 | 401 | 5.6 | 6.4 | 9.1 | 9.4 |
| | 90 | - | 112.8 | - | 400 | - | 7.3 | - | 11.3 |
| | 120 | 133.5 | 111.9 | 388 | 402 | 7.3 | 7.8 | 11.9 | 10.6 |

cont'd

TABLE 5A - Continued

| Storage Period (Days) | Incub. Period (Mins.) | K≠ | | Cl- | | IP | | TASP# | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 mg% | 2 mg% | 1 mg% | 2 mg% | 1 mg% | 2 mg% | 1 mg% | 2 mg% |
| 11 | 0 | 165.6 | 130.5 | 383 | 394 | 3.9 | 6.4 | 6.3 | 9.4 |
| | 30 | 160.8 | 133.5 | 385 | 394 | 6.0 | 7.0 | 7.6 | 10.4 |
| | 60 | 156.9 | 131.4 | 383 | 394 | 7.6 | 7.4 | 8.8 | 10.8 |
| | 90 | - | 128.7 | - | 396 | - | 8.1 | - | 11.6 |
| | 120 | 154.5 | 131.4 | 385 | 393 | 9.4 | 9.5 | 9.8 | 11.5 |
| 14 | 0 | 170.1 | 140.4 | 372 | 386 | 5.3 | 7.5 | 7.3 | 10.2 |
| | 30 | 166.8 | 141.0 | 373 | 384 | 8.4 | 8.0 | 10.6 | 12.0 |
| | 60 | 165.9 | 141.0 | 374 | 386 | 11.3 | 7.5 | 13.3 | 12.2 |
| | 90 | - | 139.8 | - | 386 | - | 9.4 | - | 12.8 |
| | 120 | 162.9 | 139.2 | 372 | 387 | 13.8 | 9.0 | 16.4 | 12.8 |
| 20 | 0 | 173.1 | 164.4 | 361 | 378 | 9.1 | 8.8 | 9.3 | 9.7 |
| | 30 | 176.7 | 165.0 | 360 | 376 | 14.7 | 8.8 | 14.9 | 10.3 |
| | 60 | 176.7 | 166.8 | 363 | 377 | 18.1 | 9.4 | 18.0 | 10.8 |
| | 90 | - | 166.8 | - | 378 | - | 9.7 | - | 11.9 |
| | 120 | 173.1 | 167.7 | 361 | 378 | 19.3 | 10.3 | 19.0 | 12.3 |

# TASP - total acid-soluble phosphate P

≠ Blood-citrate=glucose - 200:28:12
Blood-oxalate-glucose - 200:28:12

TABLE 5B

The effect of cold-storage and incubation at 37° C. on the concentration of plasma electrolytes with glucose.#

| Storage Period (Days) | Before Incubation mg% | After Incubation mg% | Net Change mg% |
|---|---|---|---|
| 0 | 19.8 | 19.2 | -0.6 |
| 1 | 54.0 | 40.5 | -13.5 |
| 2 | 77.4 | 61.2 | -16.2 |
| 5 | 128.7 | 106.8 | -21.9 |
| 8 | 150.9 | 133.5 | -17.4 |
| 11 | 165.6 | 154.5 | -11.1 |
| 14 | 170.1 | 162.9 | -7 .2 |
| 20 | 173.1 | 173.1 | 0.0 |

# Blood-citrate-glucose = 200:28:12

TABLE 6A

The effect of cold-storage and of incubation at 37° C. on glycolysis, phosphorolysis, and potassium and inorganic phosphate movements in citrated blood stored with glucose #

| Storage Period | Incub. Period | Plasma | | Cells | Whole Blood | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | K | IP[1] | IP[1] | IP[1] | LP[2] | HP[3] | SP[4] | Glucose |
| (Days) | (Hours) | meq/l | mg% | mg% | mg% | mg% | Mg% | mg% | mg% |
| 0 | 0 | 3.3 | 2.0 | 0.8 | 1.5 | 3.0 | 1.7 | 11.0 | 360 |
| | 1 | 3.4 | 2.0 | 1.3 | 1.8 | 2.2 | 1.9 | 10.9 | 330 |
| | 2 | 3.5 | 2.2 | 3.0 | 2.5 | 2.1 | 2.4 | 10.2 | 316 |
| 2 | 0 | 10.6 | 2.5 | 0.0 | 1.5 | 1.3 | 2.8 | 13.7 | 320 |
| | 1 | 8.9 | 3.0 | 2.3 | 2.7 | 1.5 | 1.5 | 13.0 | 306 |
| | 2 | 8.7 | 4.0 | 4.5 | 4.2 | 0.9 | 1.4 | 11.0 | 290 |
| 5 | 0 | 18.3 | 2.1 | 0.6 | 1.5 | 1.4 | 3.2 | 13.2 | 314 |
| | 1 | 16.2 | 3.1 | 4.4 | 3.6 | 1.0 | 1.6 | 12.9 | 294 |
| | 2 | 15.7 | 4.6 | 5.9 | 5.1 | 1.0 | 0.9 | 12.3 | 276 |
| 8 | 0 | 21.8 | 2.1 | 1.6 | 1.9 | 1.7 | 3.0 | 10.9 | 278 |
| | 1 | 19.8 | 4.4 | 4.2 | 4.3 | 1.1 | 2.0 | 9.9 | 274 |
| | 2 | 19.0 | 5.6 | 5.9 | 5.7 | 1.2 | 2.4 | 8.9 | 266 |
| 12 | 0 | 24.5 | 2.8 | 4.3 | 3.4 | 1.7 | 1.1 | 11.8 | 243 |
| | 1 | 22.8 | 5.2 | 7.0 | 5.9 | 0.3 | 0.9 | 10.1 | 236 |
| | 2 | 21.5 | 7.2 | 7.7 | 7.4 | 0.0 | 1.5 | 8.8 | 230 |
| 16 | 0 | 28.7 | 3.7 | 15.5 | 8.4 | 0.8 | 1.0 | 11.3 | 222 |
| | 1 | 28.0 | 6.7 | 15.0 | 10.0 | 0.5 | 0.8 | 9.7 | 214 |
| | 2 | 27.0 | 10.2 | 11.0 | 10.5 | 0.0 | 1.7 | 5.8 | 210 |
| 20 | 0 | 30.2 | 7.0 | 19.8 | 12.1 | 0.6 | 0.0 | 5.5 | 210 |
| | 1 | 30.2 | 12.3 | 12.3 | 12.3 | 0.8 | 0.0 | 5.4 | 196 |
| | 2 | 29.8 | 13.0 | 11.8 | 12.5 | 0.4 | 0.0 | 5.4 | 194 |
| 27 | 0 | 35.5 | 7.5 | 25.0 | 14.5 | 0.3 | 0.0 | 2.3 | 194 |
| | 1 | 36.0 | 13.8 | 16.8 | 15.0 | 0.3 | 0.0 | 1.9 | 192 |
| | 2 | 35.2 | 14.8 | 16.6 | 15.5 | 0.0 | 0.0 | 1.4 | 192 |

#1. IP - inorganic phosphate P 2.LP - labile phosphate P 3 HP hydrolyzable phosphate P
4. SP - stable phosphate P
#Blood-citrate-glucose - 5:1:0.5

TABLE 6B

The effect of cold-storage and of incubation at 37° C. on glycolysis, phosphorolysis, and potassium and inorganic phosphate movements in citrated blood stored with glucose #

| Storage Period (Days) | Incub. Period (Hours) | Plasma | | Cells | | Whole Blood | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | K meq/l | IP mg% | K meq/l | IP mg% | LP mg% | Hp mg% | SP mg% | Glucose mg% |
| 0 | 0 | 3.2 | 2.2 | 102.2 | 0.8 | 2.4 | 2.6 | 10.1 | 406 |
| | 1 | 3.4 | 2.5 | 102.1 | 1.8 | 1.9 | 2.3 | 11.8 | 380 |
| | 2 | 3.3 | 2.4 | 102.0 | 2.6 | 1.6 | 2.6 | 10.4 | 360 |
| 2 | 0 | 10.4 | 2.6 | 89.0 | 0.6 | 1.8 | 2.0 | 13.1 | 360 |
| | 1 | 8.9 | 2.9 | 92.1 | 3.7 | 1.2 | 1.7 | 12.3 | 346 |
| | 2 | 8.4 | 4.2 | 92.6 | 4.7 | 1.3 | 1.0 | 10.8 | 330 |
| 5 | 0 | 17.8 | 2.2 | 74.5 | 1.2 | 1.3 | 2.4 | 14.0 | 300 |
| | 1 | 16.2 | 3.1 | 78.0 | 4.3 | 1.2 | 1.5 | 12.6 | 212 |
| | 2 | 15.6 | 4.6 | 79.4 | 5.9 | 0.9 | 0.7 | 12.4 | 190 |
| 8 | 0 | 21.0 | 2.4 | 68.0 | 2.2 | 1.4 | 3.1 | 10.2 | 272 |
| | 1 | 19.5 | 4.9 | 71.6 | 4.9 | 0.7 | 2.0 | 10.9 | 268 |
| | 2 | 18.0 | 5.8 | 73.5 | 5.8 | 1.3 | 3.1 | 7.7 | 256 |
| 12 | 0 | 24.5 | 2.9 | 63.2 | 3.2 | 1.6 | 1.5 | 11.9 | 256 |
| | 1 | 23.2 | 5.6 | 66.0 | 6.3 | 0.0 | 0.7 | 11.1 | 256 |
| | 2 | 22.2 | 7.9 | 68.5 | 6.7 | 0.0 | 0.7 | 9.9 | 246 |
| 16 | 0 | 28.5 | 3.9 | 55.2 | 16.2 | 0.0 | 0.7 | 8.9 | 250 |
| | 1 | 27.0 | 6.5 | 56.8 | 15.5 | 0.0 | 1.1 | 10.3 | 246 |
| | 2 | 26.0 | 10.7 | 58.3 | 11.7 | 0.2 | 0.8 | 8.3 | 236 |
| 20 | 0 | 30.2 | 6.2 | 52.0 | 20.5 | 0.6 | 0.0 | 6.5 | 236 |
| | 1 | 30.2 | 12.9 | 52.0 | 9.9 | 0.7 | 0.0 | 5.7 | 220 |
| | 2 | 29.8 | 14.0 | 54.4 | 12.3 | 0.4 | 0.0 | 5.6 | 212 |
| 27 | 0 | 35.5 | 8.3 | 45.5 | 23.8 | 0.2 | 0.0 | 2.7 | 210 |
| | 1 | 34.8 | 13.6 | 44.8 | 16.9 | 0.3 | 0.0 | 2.4 | 212 |
| | 2 | 35.0 | 14.7 | 45.7 | 16.7 | 0.0 | 0.0 | 2.1 | 209 |

#Blood-citrate -glucose = 5:1:0.5

TABLE 7A

The effect of cold-storage and of incubation at 37° C. on glycolysis, phosphorolysis and potassium and inorganic phosphate movements in citrated blood stored without glucose.*

| Storage Period (Days | Incub. Period (hours) | Plasma | | Cells | Whole Blood | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | K | IP | IP | IP | LP | HP | SP | Glucose |
| | | meq/l | mg% | mg% | mg% | mg% | mg% | mg% | mg% |
| 0 | 0 | 4.7 | 3.9 | 1.2 | 2.8 | 3.4 | 5.0 | 13.1 | 50 |
| | 2 | 5.0 | 3.5 | 5.5 | 4.3 | 3.3 | 3.7 | 13.3 | 29 |
| | 4 | 5.1 | 5.4 | 6.9 | 6.0 | 3.4 | 2.4 | 12.7 | 9 |
| 2 | 0 | 12.4 | 4.6 | 0.0 | 2.6 | 2.4 | 5.4 | 16.6 | 20 |
| | 2 | 9.9 | 6.9 | 7.7 | 7.2 | 1.7 | 3.4 | 15.5 | 0 |
| | 4 | 8.9 | 12.6 | 15.6 | 13.8 | 0.1 | 2.8 | 9.1 | 0 |
| 5 | 0 | 19.5 | 4.0 | 2.8 | 3.5 | 2.5 | 5.3 | 10.5 | 5 |
| | 2 | 17.7 | 9.9 | 11.4 | 10.5 | 0.0 | 4.2 | 9.7 | 0 |
| | 4 | 18.1 | 15.0 | 18.2 | 16.3 | 0.8 | 0.0 | 9.2 | 0 |
| 9 | 0 | 22.0 | 4.8 | 23.8 | 12.4 | 0.0 | 1.2 | 11.6 | 0 |
| | 2 | 22.0 | 16.6 | 21.9 | 18.7 | 0.1 | 0.0 | 8.4 | 0 |
| | 4 | 22.0 | 25.0 | 19.2 | 21.7 | 0.0 | 0.0 | 3.8 | 0 |
| 14 | 0 | 24.0 | 2.4 | 37.9 | 16.6 | | | 8.2 | |
| | 2 | 23.8 | 13.3 | 38.3 | 21.7 | | | 2.4 | |
| | 4 | 24.2 | 14.2 | 33.7 | 22.0 | | | 3.5 | |
| 21 | 0 | 28.5 | 10.7 | 28.0 | 17.6 | | | 6.0 | |
| | 2 | 28.5 | 22.1 | 10.6 | 17.8 | | | 2.3 | |
| | 4 | 28.9 | 22.9 | 14.9 | 19.7 | | | 5.5 | |
| 28 | 0 | 33.0 | 12.6 | 36.6 | 22.2 | | | 0.0 | |
| | 2 | 32.8 | 21.3 | 29.3 | 24.2 | | | 1.0 | |
| | 4 | 33.8 | 23.9 | 23.4 | 23.7 | | | 1.4 | |
| 35 | 0 | 35.0 | 20.3 | 29.0 | 23.8 | | | 0.3 | |

*Blood-citrate - 5:1

TABLE 7B

The effect of cold-storage and of incubation at 37° C. on glycolysis, phosphorolysis and potassium and inorganic phosphate movements in citrated blood stored with glucose.

| Storage Period (days) | Incub. Period (hours) | Plasma K+ meq/l | Plasma IP mg% | Cells IP mg% | Whole Blood IP mg% | Whole Blood LP mg% | Whole Blood HP mg% | Whole Blood SP mg% | Whole Blood Glucose mg% |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 4.3 | 4.2 | 0.7 | 2.7 | 3.2 | 7.2 | 12.0 | 332 |
| | 2 | 4.3 | 3.8 | 5.3 | 4.3 | 3.3 | 8.2 | 7.8 | 312 |
| | 4 | 4.4 | 5.5 | 6.3 | 5.8 | 3.5 | 5.0 | 10.0 | 296 |
| 2 | 0 | 10.8 | 4.0 | 0.5 | 2.8 | 2.2 | 8.6 | 15.1 | 274 |
| | 2 | 8.9 | 6.9 | 2.2 | 5.0 | 0.9 | 4.1 | 16.2 | 263 |
| | 4 | 8.1 | 10.0 | 11.0 | 10.4 | 1.0 | 6.4 | 11.8 | 231 |
| 5 | 0 | 18.3 | 3.8 | 0.0 | 2.0 | 3.5 | 3.6 | 15.3 | 256 |
| | 2 | 16.2 | 7.6 | 7.4 | 7.5 | 0.4 | 2.9 | 14.9 | 207 |
| | 4 | 15.2 | 11.6 | 12.1 | 11.8 | 5.0 | 0.0 | 12.4 | 202 |
| 9 | 0 | 22.0 | 3.9 | 3.4 | 3.7 | 1.6 | 3.6 | 13.3 | 245 |
| | 2 | 20.1 | 7.9 | 5.2 | 6.8 | 1.1 | 1.3 | 13.8 | 228 |
| | 4 | 19.1 | 12.0 | 13.0 | 12.4 | 1.6 | 0.6 | 9.5 | 206 |
| 14 | 0 | 22.5 | 3.4 | 16.9 | 8.8 | 2.0 | 2.5 | 15.3 | 190 |
| | 2 | 21.8 | 8.8 | 14.8 | 11.0 | 2.3 | 1.4 | 11.6 | 178 |
| | 4 | 21.2 | 14.6 | 3.1 | 10.0 | 2.6 | 0.2 | 14.6 | 170 |
| 21 | 0 | 25.6 | 8.8 | 20.1 | 13.3 | 0.5 | 0.5 | 8.4 | 155 |
| | 2 | 25.2 | 19.3 | 7.8 | 14.7 | 0.6 | 1.8 | 8.6 | 145 |
| | 4 | 25.2 | 19.8 | 11.1 | 16.3 | 0.7 | 2.3 | 6.4 | 145 |
| 28 | 0 | 27.9 | 10.7 | 36.0 | 20.8 | 1.3 | 0.3 | 0.2 | 147 |
| | 2 | 28.8 | 19.8 | 19.3 | 19.6 | 1.3 | 0.9 | 2.5 | 141 |
| | 4 | 29.0 | 20.4 | 23.7 | 21.7 | 0.0 | 0.8 | 1.3 | 135 |
| 35 | 0 | 30.0 | 19.0 | 28.0 | 22.6 | 1.0 | 0.5 | 1.6 | 131 |

Blood-citrate-glucose = 5: 0.95:0.05

TABLE 8A

The effect of cold-storage and of incubation at 37° C. on glycolysis, phosphorolysis and potassium, sodium and inorganic phosphate movements in citrated blood stored without glucose.

| Storage Period (days) | Incub. Period (Hours) | Plasma | | | Whole Blood | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | K | Na | IP | IP | LP | HP | SP | Glucose |
| | | meq/l | meq/l | mg% | mg% | mg% | mg% | mg% | mg% |
| 0 | 0 | 3.9 | 198 | 3.0 | 1.9 | 2.3 | 10.0 | 8.0 | 81 |
| | 1 | 3.4 | 200 | 3.6 | 2.4 | 2.7 | 8.1 | 9.0 | 69 |
| | 2 | 3.4 | 202 | 3.3 | 2.8 | 2.0 | 7.2 | 10.2 | 63 |
| | 4 | 4.0 | 200 | 4.2 | 3.9 | 2.6 | 8.9 | 5.6 | 45 |
| 2 | 0 | 18.5 | 189 | 1.5 | 1.3 | 1.5 | 2.5 | 17.7 | 44 |
| | 1 | 14.3 | 190 | 1.8 | 3.0 | 1.5 | 2.0 | 16.5 | 35 |
| | 2 | 14.6 | 192 | 4.4 | 4.8 | 1.0 | 2.0 | 15.1 | 27 |
| | 4 | 12.4 | 194 | 7.5 | 6.7 | 1.1 | 2.9 | 12.2 | 13 |
| 4 | 0 | 29.4 | 178 | 3.4 | 1.3 | 1.7 | 10.1 | 11.6 | 53 |
| | 1 | 26.3 | 180 | 4.4 | 3.6 | 1.2 | 2.0 | 15.3 | 36 |
| | 2 | 25.0 | 180 | 7.1 | 5.5 | 0.9 | 1.3 | 15.9 | 23 |
| | 4 | 23.9 | 182 | 11.8 | 9.7 | 0.0 | 2.8 | 10.4 | 12 |
| 6 | 0 | 33.4 | 170 | 2.9 | 1.5 | 1.3 | 5.5 | 16.7 | 12 |
| | 1 | 30.0 | 173 | 4.3 | 4.0 | 0.7 | 3.9 | 14.3 | 7 |
| | 2 | 30.5 | 173 | 6.8 | 6.7 | 0.0 | 2.9 | 14.2 | 6 |
| | 4 | 28.6 | 175 | 12.4 | 11.1 | 0.0 | 8.0 | 7.0 | 8 |
| 9 | 0 | 40.8 | 172 | 3.7 | 5.0 | 1.2 | 6.9 | 11.9 | 0 |
| | 1 | 44.1 | 173 | 7.4 | 7.2 | 0.5 | 5.7 | 11.6 | 0 |
| | 2 | 40.0 | 170 | 9.8 | 8.9 | 0.9 | 1.7 | 13.5 | 0 |
| | 4 | 40.0 | 172 | 14.4 | 13.6 | 0.6 | 0.7 | 11.1 | 0 |

cont'd

TABLE 8A - Continued.

| Storage Period (days) | Incub. Period (Hours) | Plasma | | | Whole Blood | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | K | Na | IP | IP | LP | HP | SP | Glucose |
| | | meq/l | meq/l | mg% | mg% | mg% | mg% | mg% | mg% |
| 12 | 0 | 40.6 | 171 | 7.1 | 12.0 | 1.4 | 0.0 | 6.6 | |
| | 1 | 42.6 | 170 | 14.9 | 14.6 | 0.0 | 0.8 | 8.4 | |
| | 2 | 37.5 | 169 | 17.0 | 15.8 | 0.4 | 0.0 | 6.9 | |
| | 4 | 39.8 | 170 | 18.7 | 18.4 | 0.7 | 0.0 | 7.0 | |
| 16 | 0 | 41.3 | 169 | 8.5 | 18.0 | 0.7 | 0.0 | 4.3 | |
| | 1 | 40.3 | 168 | 18.9 | 18.7 | 0.6 | 0.1 | 2.9 | |
| | 2 | 42.5 | 166 | 20.5 | 18.9 | 0.0 | 0.6 | 2.3 | |
| | 4 | 42.5 | 168 | 20.5 | 18.9 | 0.5 | 0.0 | 2.6 | |

Blood-citrate = 5:1

TABLE 8B

The effect of cold-storage and of incubation at 37° on glycolysis, phosphorolysis and potassium, sodium and inorganic phosphate movements in citrated blood stored with glucose.

| Storage Period (days) | Incub. Period (hours) | Plasma | | | Whole Blood | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | K | Na | IP | Ip | LP | HP | SP | Glucose |
| | | meq/l | meq/l | mg% | mg% | mg% | mg% | mg% | mg% |
| 0 | 0 | 3.8 | 190 | 3.1 | 1.8 | 2.4 | 3.1 | 13.6 | 373 |
| | 1 | 3.4 | 192 | 4.2 | 2.0 | 2.6 | 7.9 | 8.0 | 373 |
| | 2 | 3.4 | 193 | 3.4 | 2.7 | 2.7 | 5.0 | 11.6 | 366 |
| | 4 | 3.8 | 191 | 4.6 | 3.9 | 2.0 | 8.2 | 6.4 | 357 |
| 2 | 0 | 15.8 | 181 | 1.7 | 1.3 | 1.5 | 5.6 | 14.6 | 280 |
| | 1 | 13.8 | 181 | 2.0 | 3.1 | 1.6 | 4.2 | 13.9 | 278 |
| | 2 | 13.4 | 184 | 4.4 | 4.9 | 1.0 | 2.7 | 15.3 | 268 |
| | 4 | 11.4 | 186 | 7.0 | 7.3 | 0.7 | 0.4 | 14.8 | 256 |
| 4 | 0 | 26.3 | 175 | 3.5 | 1.3 | 1.8 | 3.3 | 17.2 | 252 |
| | 1 | 24.5 | 175 | 4.7 | 4.0 | 0.7 | 1.7 | 11.3 | 242 |
| | 2 | 23.3 | 176 | 7.1 | 5.4 | 0.8 | 1.8 | 16.3 | 240 |
| | 4 | 22.8 | 178 | 10.3 | 8.4 | 1.4 | 0.0 | 14.0 | 233 |
| 6 | 0 | 30.0 | 167 | 3.2 | 1.7 | 1.0 | 5.7 | 16.6 | 240 |
| | 1 | 28.9 | 170 | 4.5 | 3.9 | 0.6 | 4.4 | 16.4 | 236 |
| | 2 | 27.5 | 170 | 6.3 | 5.7 | 0.1 | 2.8 | 15.6 | 236 |
| | 4 | 26.0 | 172 | 10.0 | 9.2 | 0.1 | 1.4 | 12.6 | 214 |
| 9 | 0 | 35.0 | 164 | 3.2 | 2.6 | 2.5 | 2.4 | 17.5 | 230 |
| | 1 | 34.9 | 166 | 4.3 | 4.3 | 1.4 | 4.6 | 13.8 | 224 |
| | 2 | 33.5 | 168 | 6.4 | 5.9 | 1.2 | 2.9 | 15.0 | 222 |
| | 4 | 33.0 | 166 | 9.8 | 9.2 | 0.8 | 3.9 | 9.7 | 206 |

cont'd

TABLE 8B - Continued

| Storage Period (days) | Incub. Period (hours) | Plasma | | | Whole Blood | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | K | Na | IP | IP | LP | HP | SP | Glucose |
| | | meq/l | meq/l | mg% | mg% | mg% | mg% | mg% | mg% |
| 12 | 0 | 38.5 | 163 | 4.8 | 5.7 | 1.3 | 0.0 | 13.4 | 198 |
| | 1 | 36.6 | 164 | 7.5 | 7.1 | 1.1 | 0.0 | 13.6 | 198 |
| | 2 | 31.6 | 166 | 7.9 | 8.0 | 0.0 | 1.1 | 10.1 | 202 |
| | 4 | 33.9 | 167 | 11.5 | 14.4 | 0.0 | 0.0 | 10.9 | 185 |
| 16 | 0 | 38.2 | 161 | 5.6 | 10.1 | 1.7 | 0.8 | 10.6 | 182 |
| | 1 | 37.4 | 162 | 11.9 | 11.1 | 2.0 | 0.7 | 7. 6 | 172 |
| | 2 | 37.0 | 162 | 14.0 | 12.9 | 1.7 | 1.4 | 6. 2 | 180 |
| | 4 | 35.8 | 165 | 15.3 | 14.9 | 0.9 | 0.4 | 5. 2 | 168 |
| 20 | 0 | 40.1 | 160 | 10.4 | 14.4 | 0.9 | 1.9 | 7. 8 | 160 |
| | 1 | 39.8 | 162 | 15.5 | 15.2 | 1.0 | 1.4 | 7. 4 | 161 |
| | 2 | 40.9 | 162 | 15.9 | 15.8 | 1.0 | 1.1 | 7. 2 | 160 |
| | 4 | 40.1 | 160 | 17.9 | 17.3 | 0.7 | 1.3 | 5. 7 | 160 |
| 25 | 0 | 42.6 | 159 | 11.5 | 17.8 | 0.0 | 1.8 | 6. 0 | 152 |
| | 1 | 44.4 | 159 | 15.6 | 17.4 | 0.3 | 1.8 | 5. 5 | 141 |
| | 2 | 42.1 | 158 | 17.7 | 17.7 | 0.4 | 0.1 | 6. 8 | 132 |
| | 4 | 44.3 | 158 | 18.6 | 19.1 | 0.0 | 0.6 | 5. 7 | 134 |

Blood-citrate=glucose - 5:0.95:0.05

TABLE 9A

The effect of storing blood in citrate* on the capacity of the erythrocytes to recapture potassium when incubated in phosphate buffer at $p^H$ 7.5

| Storage Period (days | Incub. Period (hours) | Glucose absent | Glucose added | |
|---|---|---|---|---|
| | | K+ | K+ | Glucose |
| | | meq/l cells | meq/l cells | mg/100 ml. |
| 0 | 0 | 98.3 | 98.0 | 216 |
| | 2 | 100.0 | 99.0 | 190 |
| | 4 | 98.7 | 98.8 | 178 |
| 2 | 0 | 87.2 | 88.2 | 238 |
| | 2 | 84.8 | 90.6 | 228 |
| | 4 | 83.1 | 93.1 | 216 |
| 6 | 0 | 72.0 | 74.1 | 222 |
| | 2 | 70.3 | 75.8 | 208 |
| | 4 | 68.8 | 77.9 | 200 |
| 10 | 0 | 69.8 | 70.2 | 128 |
| | 2 | 65.8 | 70.8 | 210 |
| | 4 | 65.0 | 69.0 | 204 |

*Blood-citrate = 5:1

TABLE 9B

The effect of storing blood in citrate-glucose* on the capacity of the erythrocytes to recapture potassium when incubated in phosphate buffer at $p^H$ 7.5.

| Storage Period (days) | Incub. Period (hours) | Glucose absent | Glucose added | |
|---|---|---|---|---|
| | | K+ | K+ | Glucose |
| | | meq/l cells | meq/l cells | mg/100 ml |
| 1 | 0 | 95.2 | 95.8 | 242 |
| | 2 | 94.8 | 96.1 | 228 |
| | 4 | 96.0 | 97.9 | 198 |
| 3 | 0 | 84.6 | 83.0 | 230 |
| | 2 | 86.2 | 88.2 | 212 |
| | 4 | 82.8 | 89.0 | 202 |
| 5 | 0 | 76.2 | 77.2 | 236 |
| | 2 | 74.0 | 80.8 | 220 |
| | 4 | 74.0 | 81.3 | 200 |
| 8 | 0 | 71.0 | 72.0 | 218 |
| | 2 | 68.2 | 76.1 | 208 |
| | 4 | 65.0 | 78.0 | 193 |
| 12 | 0 | 64.2 | 63.1 | 236 |
| | 2 | 60.0 | 66.3 | 230 |
| | 4 | 61.4 | 68.8 | 226 |
| 20 | 0 | 54.0 | 55.0 | 212 |
| | 2 | 52.6 | 58.1 | 218 |
| | 4 | 51.8 | 54.4 | 208 |

*Blood-citrate-glucose = 5:1:0.5

TABLE 10A

The effect of storing blood in ACD* on the capacity of the erythrocytes to recapture potassium when incubated in native plasma and in phosphate buffer at $p^H$ 7.5

| Duration of Storage (Days) | Incub. Period (hours) | Inc. in Native Plasma | | Inc. in Buffer-$p^H$ 7.5 | |
|---|---|---|---|---|---|
| | | K meq/l cells | Glucose mg/100 ml. | K meq/l cells | Glucose mg/11 ml. |
| 0 | 0 | 92.8 | 412 | 91.0 | 206 |
| | 2 | 93.0 | 404 | 93.2 | 185 |
| | 4 | 91.2 | 396 | 91.8 | 168 |
| 3 | 0 | 84.8 | 388 | 83.0 | 212 |
| | 2 | 80.9 | 380 | 85.0 | 200 |
| | 4 | 82.4 | 369 | 89.6 | 179 |
| 7 | 0 | 77.1 | 332 | 75.2 | 238 |
| | 2 | 78.2 | 330 | 77.0 | 222 |
| | 4 | 76.2 | 320 | 80.6 | 179 |
| 14 | 0 | 70.4 | 308 | 71.2 | 230 |
| | 2 | 68.3 | 300 | 73.8 | 220 |
| | 4 | 71.4 | 282 | 75.2 | 202 |
| 21 | 0 | 63.1 | 273 | 65.0 | 216 |
| | 2 | 62.0 | 268 | 66.8 | 200 |
| | 4 | 62.9 | 252 | 69.1 | 174 |
| 28 | 0 | 59.0 | 264 | 58.2 | 236 |
| | 2 | 56.0 | 261 | 58.8 | 229 |
| | 4 | 55.8 | 248 | 59.0 | 218 |

* Blood-disodium citrate-glucose = 200:40:2

TABLE 11A

The effect of metabolic inhibitors on the content of potassium in the erythrocytes during cold-storage and incubation at 37° C.

| Storage Period (days) | Incub. Period (hours) | Cellular K+ - m.eq./liter cells | | | | | |
|---|---|---|---|---|---|---|---|
| | | Citrate Control | 0.01M NaF | 0.005M NaF | 0.005M IAc* | 0.001M IAc | 0.004M NaCN |
| 0 | 0 | 95.7 | 98.0 | 97.1 | 94.8 | 96.0 | 96.6 |
| | 2 | 96.0 | 84.3 | 94.3 | 91.5 | 92.6 | 96.4 |
| | 4 | 97.0 | 72.6 | 90.6 | 87.4 | 89.8 | 96.0 |
| 2 | 0 | 78.6 | 87.3 | 87.5 | 84.4 | 86.5 | 82.5 |
| | 2 | 84.0 | 66.0 | 83.5 | 80.5 | 83.6 | 86.5 |
| | 4 | 85.7 | 60.3 | 80.0 | 71.9 | 80.5 | 88.5 |
| 5 | 0 | 66.1 | 78.1 | 78.0 | 68.1 | 78.4 | 72.2 |
| | 2 | 71.6 | 52.1 | 74.1 | 63.5 | 75.2 | 78.2 |
| | 4 | 73.5 | 55.5 | 70.6 | 58.8 | 70.3 | 79.9 |
| 9 | 0 | 59.4 | 68.0 | 68.0 | 56.2 | 67.8 | 67.0 |
| | 2 | 62.0 | 62.1 | 66.5 | 54.3 | 66.2 | 67.7 |
| | 4 | 62.6 | 56.6 | 62.9 | 51.7 | 62.0 | 68.5 |

*IAc - Iodoacetic acid

TABLE 11B

The effect of metabolic inhibitors on the content of sodium in the plasma of blood during cold-storage and incubation at 37° C.

| Storage Period (days) | Incub. Period (hours) | Plasma Na+ - m.eq./liter | | | | | |
|---|---|---|---|---|---|---|---|
| | | Citrate Control | 0.01M NaF | 0.005M NaF | 0.005M IAc | 0.001M IAc | 0.004M NaCN |
| 0 | 0 | 193 | 194 | 196 | 193 | 190 | 189 |
| | 2 | 194 | 192 | 193 | 192 | 192 | 193 |
| | 4 | 191 | 189 | 194 | 188 | 191 | 194 |
| 2 | 0 | 183 | 183 | 181 | 179 | 177 | 179 |
| | 2 | 189 | 177 | 181 | 168 | 174 | 181 |
| | 4 | 187 | 174 | 183 | 169 | 179 | 185 |
| 5 | 0 | 168 | 168 | 170 | 163 | 162 | 171 |
| | 2 | 165 | 160 | 156 | 157 | 156 | 165 |
| | 4 | 163 | 161 | 165 | 164 | 154 | 165 |
| 9 | 0 | 160 | 164 | 171 | 157 | 160 | 170 |
| | 2 | 162 | 163 | 168 | 150 | 162 | 168 |
| | 4 | 174 | 161 | 169 | 158 | 160 | 172 |

TABLE 11C

The effect of cold-storage and incubation at 37° C. on glycolysis, phosphorolysis and electrolyte exchanges in citrated blood without inhibitors.

| Storage Period (days) | Incub. Period (hours) | Plasma | | Cells* | | | | | Blood |
|---|---|---|---|---|---|---|---|---|---|
| | | K+ | Na+ | K+ | IP | LP | HP | SP | Glucose |
| | | meq/l | meq/l | meq/l | mg% | mg% | mg% | mg% | mg% |
| 0 | 0 | 2.9 | 193 | 95.7 | 0.3 | 7.2 | 4.8 | 28.2 | 78 |
| | 2 | 2.8 | 194 | 96.0 | 1.5 | 8.2 | 6.5 | 23.4 | 55 |
| | 4 | 2.4 | 191 | 97.0 | 4.9 | 8.1 | 4.0 | 29.2 | 40 |
| 2 | 0 | 12.1 | 183 | 78.6 | 0.3 | 5.0 | 4.2 | 28.0 | 51 |
| | 2 | 9.3 | 189 | 84.0 | 3.9 | 7.0 | 2.6 | 28.3 | 31 |
| | 4 | 8.3 | 187 | 85.0 | 4.1 | 6.7 | 1.4 | 26.8 | 15 |
| 5. | 0 | 21.0 | 168 | 66.1 | 0.0 | 6.5 | 2.4 | 30.5 | 22 |
| | 2 | 15.9 | 165 | 71.6 | 3.4 | 2.3 | 2.9 | 28.7 | 3 |
| | 4 | 14.9 | 163 | 73.5 | 5.4 | 8.5 | 0.0 | 30.2 | 0 |
| 9 | 0 | 22.5 | 160 | 59.4 | 1.4 | 7.9 | 1.7 | 28.1 | 1 |
| | 2 | 21.0 | 162 | 62.0 | 13.6 | 3.2 | 0.0 | 25.1 | 0 |
| | 4 | 20.8 | 174 | 62.6 | 9.4 | 0.5 | 0.9 | 16.2 | 0 |

*Cellular composition calculated as described under Methods (C15)

TABLE 11D

The effect of cold-storage and incubation at 37° C. on glycolysis, phosphorolysis and electrolyte exchanges in citrated blood with 0.01M NaF.

| Storage Period (days) | Incub. Period (hours) | Plasma | | Cells | | | | | Blood |
|---|---|---|---|---|---|---|---|---|---|
| | | K⁄ | Na⁄ | K⁄ | IP | LP | HP | SP | Glucose |
| | | meq/l | meq/l | meq/l | mg% | mg% | mg% | mg% | mg% |
| 0 | 0 | 2.8 | 194 | 98.0 | 1.4 | 3.9 | 5.8 | 28.4 | 78 |
| | 2 | 9.1 | 192 | 84.3 | 2.5 | 0.0 | 6.0 | 26.4 | 70 |
| | 4 | 15.4 | 189 | 72.6 | 1.7 | 0.0 | 5.4 | 24.6 | 67 |
| 2 | 0 | 7.5 | 183 | 87.3 | 2.5 | 2.3 | 2.2 | 34.0 | 79 |
| | 2 | 18.9 | 177 | 66.0 | 2.7 | 4.2 | 1.7 | 28.5 | 80 |
| | 4 | 22.0 | 174 | 60.3 | 3.3 | 1.1 | 1.9 | 25.9 | 80 |
| 5 | 0 | 12.4 | 168 | 78.1 | 0.1 | 2.3 | 3.4 | 25.2 | 73 |
| | 2 | 21.0 | 160 | 62.1 | 0.0 | 2.1 | 1.1 | 27.6 | 71 |
| | 4 | 24.6 | 161 | 55.5 | 1.3 | 0.0 | 1.3 | 25.9 | 71 |
| 9 | 0 | 17.8 | 164 | 68.0 | 2.1 | 1.1 | 2.5 | 25.8 | 70 |
| | 2 | 21.0 | 163 | 62.1 | 3.2 | 3.5 | 0.2 | 28.5 | 70 |
| | 4 | 24.0 | 161 | 56.6 | 4.4 | 0.7 | 0.0 | 25.7 | 70 |

TABLE 11E

The effect of cold-storage and incubation at 37° C. on glycolysis, phosphorolysis and electrolyte exchanges in citrated blood with 0.005 M NaF

| Storage Period (days) | Incub. Period (hours) | Plasma | | Cells | | | | | Blood |
|---|---|---|---|---|---|---|---|---|---|
| | | K⁄ | Na⁄ | K⁄ | IP | LP | HP | SP | Glucose |
| | | meq/l | meq/l | meq/l | mg% | mg% | mg% | mg% | mg% |
| 0 | 0 | 3.2 | 196 | 97.1 | 3.4 | 2.3 | 6.3 | 28.9 | 77 |
| | 2 | 3.7 | 193 | 94.3 | 4.4 | 0.2 | 5.5 | 29.1 | 67 |
| | 4 | 5.7 | 194 | 90.6 | 5.1 | 0.0 | 5.7 | 28.4 | 57 |
| 2 | 0 | 7.4 | 181 | 87.5 | 2.9 | 3.2 | 2.5 | 30.6 | 76 |
| | 2 | 9.5 | 181 | 83.5 | 2.4 | 3.9 | 2.9 | 29.6 | 76 |
| | 4 | 11.5 | 183 | 80.1 | 3.4 | 2.1 | 2.4 | 29.6 | 74 |
| 5 | 0 | 12.5 | 170 | 78.0 | 2.0 | 2.3 | 3.9 | 24.5 | 71 |
| | 2 | 14.6 | 156 | 74.1 | 2.3 | 4.0 | 1.8 | 26.4 | 64 |
| | 4 | 16.5 | 165 | 70.6 | 0.0 | 5.6 | 1.0 | 29.0 | 62 |
| 9 | 0 | 17.8 | 171 | 68.0 | 5.4 | 0.4 | 2.5 | 22.7 | 73 |
| | 2 | 18.6 | 168 | 66.5 | 5.2 | 2.3 | 0.0 | 31.5 | 73 |
| | 4 | 20.6 | 169 | 62.9 | 4.7 | 0.0 | 1.2 | 29.6 | 73 |

TABLE 11F

The effect of cold-storage and incubation at 37° C. on glycolysis phosphorolysis and electrolyte exchanges in citrated blood with 0.005 M I Ac.

| Storage Period | Incub. Period | Plasma | | Cells | | | | | Blood |
|---|---|---|---|---|---|---|---|---|---|
| | | $K^+$ | $Na^+$ | $K^+$ | IP | LP | HP | SP | Glucose |
| (days) | (hours) | meq/l | meq/l | meq/l | Mg% | mg% | mg% | mg% | mg% |
| 0 | 0 | 3.4 | 193 | 94.8 | 1.5 | 8.2 | 4.7 | 30.8 | 79 |
| | 2 | 5.6 | 192 | 91.5 | 6.3 | 1.6 | 5.6 | 20.6 | 72 |
| | 4 | 7.4 | 188 | 87.4 | 8.3 | 2.5 | 2.9 | 24.3 | 79 |
| 2 | 0 | 9.1 | 179 | 84.4 | 5.7 | 5.3 | 0.0 | 34.5 | 77 |
| | 2 | 11.1 | 168 | 80.5 | 5.3 | 3.9 | 2.3 | 25.8 | 79 |
| | 4 | 15.8 | 169 | 71.9 | 5.6 | 2.5 | 1.8 | 18.8 | 84 |
| 5 | 0 | 17.8 | 163 | 68.1 | 5.4 | 5.6 | 4.3 | 26.3 | 78 |
| | 2 | 20.3 | 157 | 63.5 | 4.2 | 3.5 | 3.2 | 27.0 | 82 |
| | 4 | 22.8 | 164 | 58.8 | 4.9 | 3.5 | 1.9 | 29.1 | 81 |
| 9 | 0 | 24.2 | 157 | 56.2 | 7.4 | 1.8 | 1.7 | 23.8 | 82 |
| | 2 | 25.3 | 150 | 54.3 | 5.4 | 6.1 | 0.4 | 28.2 | 82 |
| | 4 | 26.6 | 153 | 51.7 | 11.1 | 7.5 | 1.4 | 22.6 | 82 |

TABLE 11G

The effect of cold-storage and incubation at 37° C. on glycolysis phosphorolysis and electrolyte exchanges in citrated blood with 0.001 M Iac.

| Storage Period | Incub. Period | Plasma | | Cells | | | | | Blood |
|---|---|---|---|---|---|---|---|---|---|
| | | $K^+$ | $N^+$ | $K^+$ | IP | LP | HP | SP | Glucose |
| (days) | (hours) | meq/l | meq/l | meq/l | mg% | mg% | mg% | mg% | mg% |
| 0 | 0 | 2.9 | 190 | 96.6 | 1.8 | 5.6 | 6.0 | 29.2 | 78 |
| | 2 | 4.7 | 192 | 92.6 | 7.0 | 3.5 | 4.6 | 21.3 | 79 |
| | 4 | 6.1 | 191 | 89.8 | 10.7 | 4.9 | 5.2 | 10.7 | 72 |
| 2 | 0 | 7.9 | 177 | 86.5 | 7.4 | 0.0 | 5.7 | 18.0 | 85 |
| | 2 | 9.5 | 174 | 83.6 | 7.4 | 6.8 | 2.9 | 11.7 | 82 |
| | 4 | 11.1 | 179 | 80.5 | 9.3 | 1.4 | 2.1 | 4.7 | 84 |
| 5. | 0 | 12.3 | 162 | 78.4 | 13.9 | 6.8 | 3.7 | 22.9 | 79 |
| | 2 | 14.0 | 156 | 75.2 | 6.3 | 6.3 | 2.4 | 15.2 | 76 |
| | 4 | 16.6 | 154 | 70.3 | 7.9 | 7.4 | 2.7 | 10.5 | 80 |
| 9 | 0 | 18.0 | 160 | 67.8 | 17.0 | 8.4 | 1.6 | 15.4 | 77 |
| | 2 | 18.6 | 162 | 66.2 | 14.1 | 5.6 | 1.0 | 7.2 | 77 |
| | 4 | 21.3 | 160 | 62.0 | 10.9 | 7.4 | 0.0 | 3.1 | 77 |

TABLE 11 H

The effect of cold-storage and incubation at 37° C. on glycolysis phosphorolysis and electrolyte exchanges in citrated blood with 0.004 M NaCN.

| Storage Period | Incub. Period | Plasma | | Cells | | | | | Blood |
|---|---|---|---|---|---|---|---|---|---|
| | | K⁄ | Na⁄ | K⁄ | IP | LP | HP | SP | Clucose |
| (days) | (hours) | meq/l | Meq/l | meq/l | mg% | mg% | mg% | mg% | mg% |
| 0 | 0 | 2.5 | 189 | 96.6 | 0.7 | 4.6 | 4.2 | 32.4 | 76 |
| | 2 | 2.7 | 193 | 96.4 | 4.2 | 7.4 | 4.9 | 27.8 | 38 |
| | 4 | 2.8 | 194 | 96.0 | 5.1 | 6.5 | 4.0 | 23.6 | 18 |
| 2 | 0 | 10.0 | 179 | 82.5 | 1.9 | 8.2 | 2.3 | 26.4 | 52 |
| | 2 | 7.9 | 181 | 86.5 | 6.9 | 6.0 | 2.5 | 25.0 | 31 |
| | 4 | 6.8 | 185 | 88.5 | 7.9 | 9.6 | 4.2 | 10.6 | 7 |
| 5 | 0 | 15.6 | 171 | 72.2 | 1.4 | 2.8 | 3.5 | 31.8 | 18 |
| | 2 | 12.3 | 165 | 78.2 | 6.4 | 4.2 | 3.2 | 26.3 | 0 |
| | 4 | 11.5 | 165 | 79.9 | 9.7 | 4.6 | 2.5 | 11.5 | 0 |
| 9 | 0 | 13.4 | 170 | 67.0 | 17.8 | 4.7 | 0.8 | 27.8 | 2 |
| | 2 | 18.0 | 168 | 67.7 | 11.9 | 5.1 | 0.0 | 23.1 | 0 |
| | 4 | 17.5 | 172 | 68.5 | 13.1 | 2.8 | 1.0 | 12.1 | 0 |

TABLE 11 I

The effect of metabolic inhibitors on spontaneous hemolysis during cold-storage and incubation at 37° C.

| Storage Period (days) | Incub. Period (hours) | % Spontaneous Hemolysis* Citrate Contrd | 0.01M NaF | 0.005M NaF | 0.005M IAc | 0.001M IAc | 0.004M NaCN |
|---|---|---|---|---|---|---|---|
| 0 | 0 | 0.22 | 0.59 | 0.22 | 1.9 | 0.59 | 0.33 |
| | 2 | 0.37 | 0.41 | 0.52 | 2.1 | 0.67 | 0.52 |
| | 4 | 0.37 | 0.44 | 0.33 | 1.9 | 0.67 | 0.37 |
| 2 | 0 | 0.67 | 1.8 | 2.3 | 4.0 | 2.2 | 0.44 |
| | 2 | 0.70 | 3.0 | 2.8 | 5.1 | 2.6 | 0.57 |
| | 4 | 0.78 | 3.7 | 3.1 | 5.4 | 2.7 | 0.59 |
| 5 | 0 | 1.0 | 3.1 | 4.0 | 5.5 | 2.9 | 0.74 |
| | 2 | 1.0 | 5.5 | 6.4 | 7.7 | 4.3 | 0.78 |
| | 4 | 1.5 | 9.7 | 8.8 | 10.0 | 6.6 | 0.74 |
| 9 | 0 | 1.2 | 9.7 | 11.4 | 17.0 | 9.8 | 0.90 |
| | 2 | 1.4 | 13.8 | 17.5 | 24.0 | 12.6 | 1.2 |
| | 4 | 1.5 | 19.0 | 22.0 | 32.1 | 14.8 | 1.1 |

*Percentage of hemolysis = $\frac{\text{Plasma hemoglobin}}{\text{Blood hemoglobin}} \times 100$

TABLE 11 J

The fragility of erythrocytes stored for 10 days with various metabolic inhibitors.

| Percent Isotonic Saline | Citrate Control | 0.01M NaF | 0.005M NaF | 0.005M IAc | 0.001M IAc | 0.004M NaCN |
|---|---|---|---|---|---|---|
| | % hemolysis in buffered saline | | | | | |
| 100 | 6.5 | 25.2 | 25.2 | 24.8 | 20.0 | 2.9 |
| 90 | 7.5 | 58.3 | 50.4 | 39.3 | 33.0 | 9.7 |
| 80 | 10.8 | 65.2 | 72.2 | 65.8 | 67.0 | 16.5 |
| 70 | 19.3 | 78.3 | 83.5 | 88.0 | 81.0 | 33.9 |
| 60 | 31.4 | 86.9 | 85.2 | 93.2 | 84.4 | 43.5 |
| 50 | 83.3 | 91.3 | 92.2 | 100 | 91.7 | 98.3 |
| 40 | 96.6 | 98.3 | 98.7 | 100 | 97.8 | 98.3 |
| 30 | 100 | 100 | 100 | 100 | 100 | 100 |

TABLE 12

The effect of storage at 5° C. and of incubation at 37° C. on the oxygen content of whole blood*

| Storage Period (days) | Incub. Period (hours) | Citrate $O_2$ Vol.% | Citrate-glucose | Storage Period (days) | Incub. Period (hours) | Citrate $O_2$ Vol. % | Citrate-Glucose |
|---|---|---|---|---|---|---|---|
| 0 | 0 | 15.6 | 15.4 | 14 | 0 | 9.2 | 9.6 |
| | 2 | - | 15.4 | | 2 | 10.0 | 8.0 |
| | 4 | 15.3 | 13.0 | | 4 | 9.6 | 7.6 |
| 2 | 0 | 14.4 | 12.4 | 21 | 0 | 10.4 | 8.0 |
| | 2 | 13.6 | 12.0 | | 2 | 10.0 | 8.4 |
| | 4 | 15.0 | 9.6 | | 4 | 9.6 | 8.0 |
| 5 | 0 | 12.0 | 12.4 | 28 | 0 | 9.6 | 8.8 |
| | 2 | 11.6 | 13.6 | | 2 | 10.0 | 9.6 |
| | 4 | 12.8 | 12.8 | | 4 | 9.6 | 8.4 |
| 9 | 0 | - | 9.2 | 35 | 0 | 8.4 | 7.6 |
| | 2 | 11.2 | - | | | | |
| | 4 | 11.2 | - | 42 | 0 | 9.2 | 7.2 |

Blood-citrate = 5:1
Blood-citrate-glucose = 5:1:0.5

TABLE 13

The effect of storage at 5° C. of whole citrated blood" on the cholinesterase activity of the blood, the erythrocytes and the plasma.

| Storage Period | Whole Blood | Cell Suspension | Plasma | Whole Blood* |
|---|---|---|---|---|
| (days) | µl./hr./ 0.1 ml. enzyme system | | | |
| 1 | 582 | 416 | 240 | 576 |
| 3 | 580 | 419 | 244 | 581 |
| 6 | 569 | 414 | 252 | 582 |
| 9 | 570 | 409 | 254 | 579 |
| 11 | 569 | 396 | 264 | 572 |
| 13 | 568 | 390 | 258 | 562 |
| 15 | 584 | 382 | 264 | 558 |
| 18 | 580 | 384 | 270 | 564 |
| 21 | 582 | 388 | 276 | 572 |
| 33 | 565 | 276 | 456 | 576 |

*These Whole Blood values are calculated from the values of the cell suspension and the plasma by the formula,

$$WB = CS \neq P.PF$$

where, WB = calculated whole blood value
Cs = cell suspension value
P = plasma value
PF = plasma fraction derived from the hematocrit.

" Blood-citrate-glucose = 5:1:0.5

## Resume.

When blood is stored at 5° C. potassium leaves the erythrocytes and enters the plasma. It is replaced by sodium which diffuses in from the plasma. The rate of exchange of these ions is most rapid during the first week of cold-storage, after which it becomes slower and more constant. For a discussion and mathematical treatment of the kinetics of this diffusion process, the reader is referred to Ponder's recent article (1951).

Studies with radioactive potassium have shown that at 37° C. about 1.5 m.eq. of potassium diffuse from a liter of erythrocytes in one hour, but at 4° C., the rate is only about 0.15 m.eq. of potassium per liter per hour (Raker et al., 1950). During one week of cold-storage, from 25-30 m.eq. of potassium are lost from one liter of red cells, which closely approximates the rate found with the tracer technique. At 37° C., the greater permeability of the cellular membrane to potassium is offset by a mechanism which brings an equal amount of this ion back into the cell. At 4° C., the membrane is much less permeable to potassium (10%), but apparently the potassium accumulating mechanism fails completely, and a net loss of this ion occurs. The same observations may be applied to the entry of sodium into the erythrocytes during the storage of blood in the cold. The permeability of the membrane to this cation is decreased in the cold, but the system that pumps sodium out fails to offset even this low degree of permeation.

The inhibition of glycolysis by the cold has a dual

effect on the sodium and potassium content of the cell. The number of these ions that passively penetrates the membrane along the concentration gradient is diminished. This would tend to hold potassium in the cells, and sodium out. But in the cold, the active transport mechanisms that normally counteract the diffusion process fail completely, and the cells tend, gradually, to assume the ionic composition of the surrounding medium.

That the inhibition of glycolysis actually decreases the rate of diffusion of potassium out of the cell during cold-storage is further shown by the effect of glycolytic inhibitors, such as oxalate, fluoride and iodoacetate, and by the effect of a lowered $p^H$. These factors which diminish the glycolytic rate also decrease the rate of diffusion of potassium from the cells, and of sodium into them, at 5° C.

The enrichment of the blood with glucose, up to 400 mg. per 100 ml., slightly retards the outflow of potassium from the cells during cold-storage by altering the permeability properties of the membrane itself, and not through any effect on the metabolism of the cells. This is further demonstrated by the effect of added glucose in delaying the onset of spontaneous hemolysis and the diffusion of inorganic phosphate from the cells, and improving the stability of the erythrocytes in hypotonic saline, when glycolysis has been inhibited by oxalate.

When stored erythrocytes are warmed to 37° C., they demonstrate a capacity to increase their potassium content and

to decrease their sodium content, both processes occurring against the concentration gradient. This occurs despite the increased rate at which these ions diffuse along their respective concentration gradients at 37° C. The potassium content of a liter of red cells is decreased by 1-2 m.eq. per hour at 37° C. if no active accumulation of this ion is taking place, either through lack of a glycolyzable substrate, or through inhibition of the glycolytic activity. Active accumulation of potassium by glycolyzing preserved erythrocytes more than offsets this diffusion process, and an increase of 2 m.eq. of potassium per liter of cells per hour, actually represents a back-transport of 3-4 m.eq. of potassium per liter of cells per hour.

The capacity of preserved erythrocytes to take up potassium and to excrete sodium is dependent upon the energy metabolism of the cells. Glycolysis is essential for active cation transport in the erythrocyte, and in its absence, only passive diffusion of these ions occurs. The rate of glycolysis, however, cannot be correlated with the number of ions transported. For instance, fresh cells at 37° C., at equilibrium with respect to their potassium content, lose 1-2 m.eq. of potassium per liter per hour, but recapture the same amount by an active transport mechanism. These cells, glycolyzing at their optimum rate, are therefore able to accumulate only 1-2 m.eq. of potassium per liter per hour. In the preceding paragraph we have described how stored cells when incubated at 37° C. are able to accumulate up to 4 m.eq. of potassium per liter per hour. These cells do not

glycolyze as rapidly as do fresh cells, but perform at least twice the amount of osmotic work. In the light of these findings, attempts to link active transport processes directly to glycolysis, and to correlate the transport of cations with the amount of energy derived from this metabolic process, lose their significance.

Fresh erythrocytes are unable to alter their content of sodium and potassium significantly when incubated at 37° C. The ability of preserved erythrocytes to accumulate potassium and to excrete sodium when warmed to 37° C. increases during the first few days of storage, and thereafter gradually decreases as the storage period lengthens.

The behaviour of potassium in blood stored at 5° C. with citrate only, and subsequently incubated at 37° C., is illustrated in Figure 1, page 201. The erythrocytes, on incubation, show a maximum capacity to accumulate potassium between the 3rd and 5th days of storage at 5° C. Thereafter the capacity is decreased and is lost by the 10th day of storage, when all the indigenous glucose has been used up. If glucose is added immediately before returning the 10-day old specimen to 37° C., it is not utilized, nor is there any restoration of the capacity of the cells to accumulate potassium.

If the blood is enriched with glucose prior to the storage period, the capacity of the cells to glycolyze and to accumulate potassium, when returned to body temperature, is preserved for a longer period. The behaviour of potassium in

the plasma of blood stored at 5° C. with added glucose, and subsequently incubated at 37° C. is illustrated in Figure 2, page 202. The behaviour of this cation in the erythrocytes of another blood specimen, under the same conditions of storage and incubation, is illustrated in Figure 3, page 203. The ability of the stored cells to recapture potassium during a two to four hour period of incubation reaches a maximum after the specimen has been stored for about five days. This maximum rate is maintained in specimens stored for as long as ten days at 5° C. The erythrocytes in specimens older than this gradually lose the capacity to recover potassium. After a cold-storage period of more than three weeks, in a glucose-enriched medium, the cells are no longer able to recapture potassium when warmed to 37° C.

When erythrocytes are preserved in the acid-citrate-dextrose medium, the glycolytic capacity and the ability to regain potassium are maintained for a longer period of time, as tested by incubating the cells at 37° C. in a buffered medium at $p^H$ 7.5. These functions are still operative in specimens stored up to at least 28 days at 5° C.

Acidification and a high glucose concentration both appear to favor the preservation of the dynamic processes in the erythrocytes during cold-storage. The preservation of the ability of the cells to recover potassium at 37° C.closely follows the preservation of the ability to consume glucose at this temperature. Despite the lack of a quantitative correlation between the glycolytic rate and the capacity to accumulate potassium, it is obvious, that

when the energy-yielding process fails, cation transport also fails. This points to the existence of a factor in the red blood cell, common to both processes.

A thorough investigation of the phosphate compounds of blood during cold-storage and subsequent incubation has indicated that when the adenosine triphosphate content of the cells is depleted, both glycolysis and active cation transport no longer occur. The other organic phosphate compounds serve merely as glycolyzable substrates, and the inorganic phosphate content of the blood is a reflection of the state of the organic acid-soluble phosphate compounds. Potassium is not accumulated or held in the erythrocytes by the attraction of the anionic phosphate esters. The conclusion arrived at is that cation transport depends on the metabolism of the cell only in so far as it provides the high-energy phosphate bonds of adenosine triphosphate.

When glycolysis is not taking place in the erythrocytes incubated at 37° C., either through the lack of a substrate, or under the influence of a low concentration of a glycolytic inhibitor, active cation transport fails, and potassium leaves the cells at a rate of about 1-2 m.eq. per liter cells per hour, the rate predicted by isotope studies. The loss of potassium from the red cells can be increased to the rate of 3 m.eq. per liter cells per hour by the addition of sodium fluoride to a final concentration of 0.01 M. This concentration of fluoride not only inhibits glycolysis and the active accumulation of potassium, but increases the permeability of the membrane to the passive diffusion of potassium, allowing this ion to move more freely out of the cell, along the concentration

gradient. The rate of the diffusion of sodium into the cells, passively, is practically unaltered by 0.01 M sodium fluoride. The implication of the action of fluoride in increasing the permeation of the erythrocyte membrane by potassium ions has been discussed.

Glycolytic inhibitors (oxalate, fluoride and iodoacetate) increase the fragility of the erythrocyte membrane, and accelerate the onset of spontaneous hemolysis. The damaging effect of these agents probably is due to their interference with the nutrition of the membrane.

The magnesium content of the erythrocytes remains virtually unchanged during the storage period and during subsequent incubation at 37° C. The concentration of magnesium in the cells does not appear to be influenced either by the temperature or by the metabolism of the cells.

Chloride ions tend to leave the cells during the oxygenation of the blood specimens. During prolonged cold-storage, however, this ion tends to enter the cells, along the concentration gradient, possibly in exchange for inorganic phosphate anions that diffuse slowly out of the cells at 5° C. However, when the cells are warmed to 37° C., the inorganic phosphate leaves the cells rapidly, while the concentration of chloride remains unchanged. It is futile to consider Donnan effects in such a rapidly changing system.

Although both insulin and desoxycorticosterone influence

potassium metabolism *in vivo*, no effects of these agents on the electrolyte metabolism of erythrocytes, *in vitro*, was demonstrated. Insulin does not affect the metabolism of glucose or glycogen in red blood cells from healthy donors, either during storage at 5° C., nor during incubation at 37° C.

Erythrocytes depend mainly on aerobic glycolysis for their energy production, but respiratory processes also may contribute to the energetics of the cell. Oxygen is slowly consumed by human red blood cells, and a slight Pasteur effect can be elicited. It is considered that respiratory metabolism may normally contribute to the energizing of the active transport of cations. Glutamic acid, however, does not facilitate the accumulation of potassium by preserved erythrocytes.

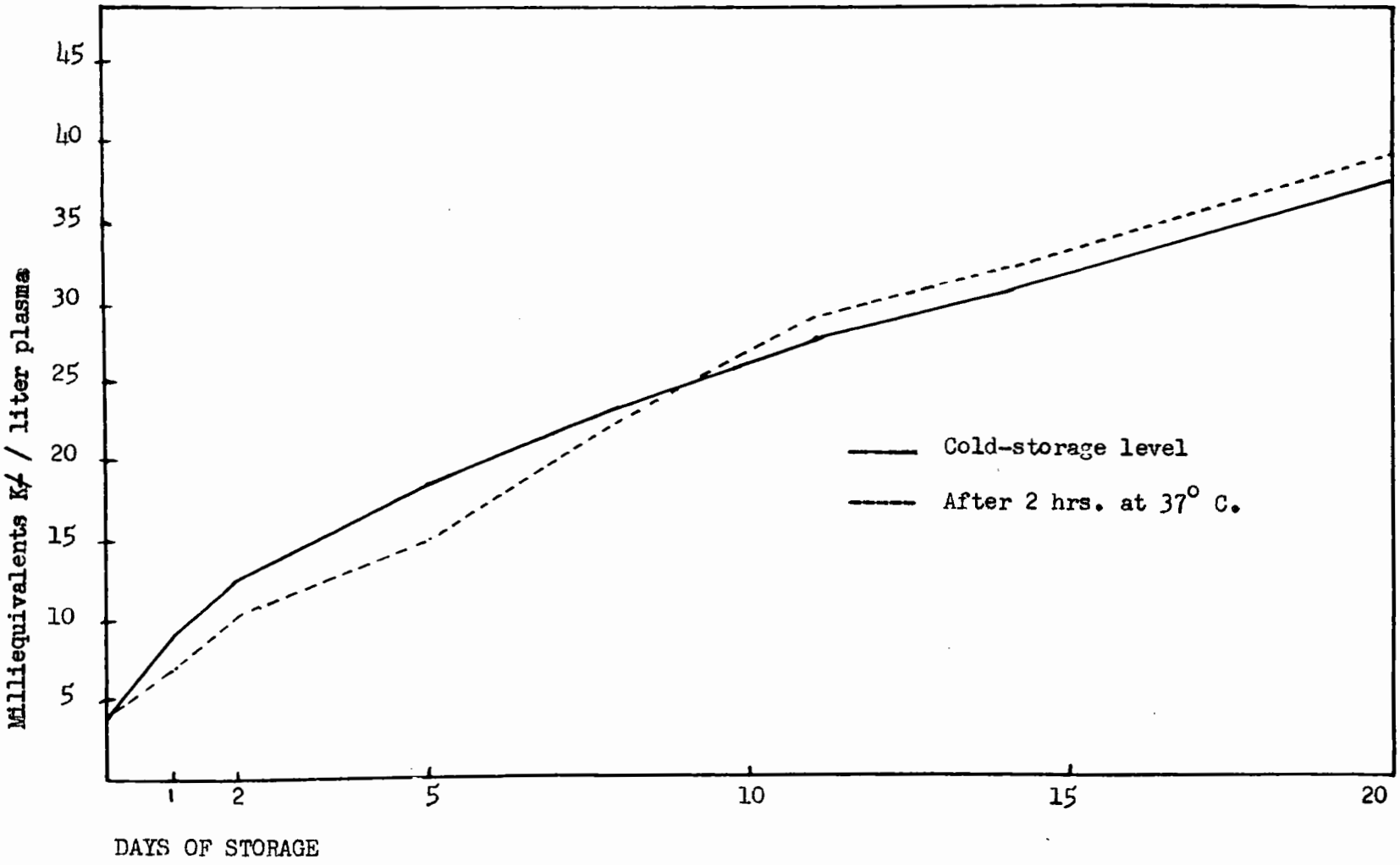
The carbonic anhydrase activity of blood is preserved, under sterile conditions, for an indefinite period. This enzyme does not play a role in the cation transport mechanisms of the red blood cell.

The cholinesterase activity of whole blood is likewise preserved indefinitely under sterile conditions. The erythrocyte cholinesterase is easily detached from the outer surface of the membrane, where it is normally located. It tends to separate from the cell, very slowly, during the first month of cold-storage. After this time, the enzyme leaves the cell very rapidly.

The significance of the activity of the erythrocyte cholinesterase in the maintenance of the cation distribution in the red cell has been discussed.

Figure 1.

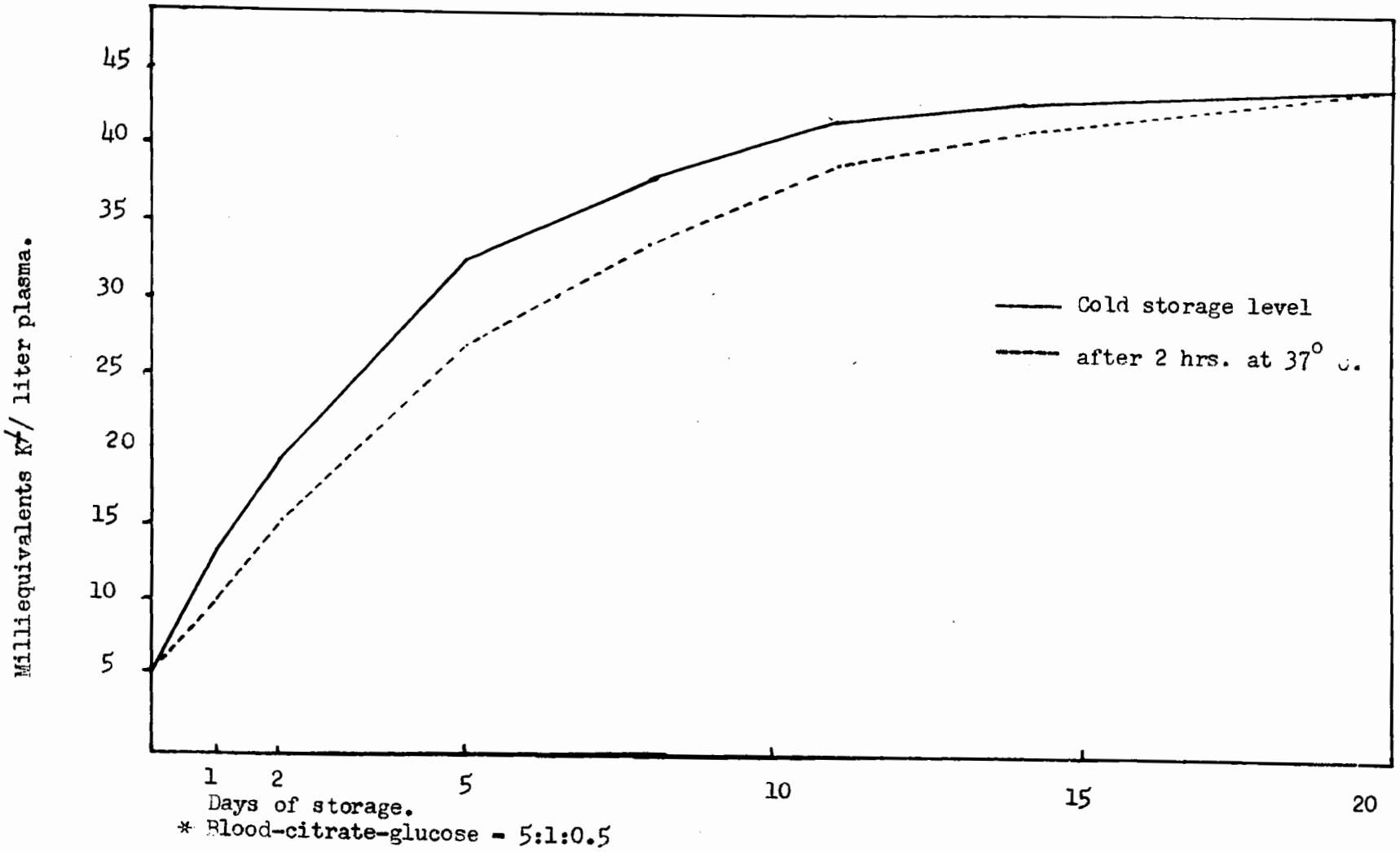
The effect of cold-storage and subsequent incubation at 37° C. on the concentration of potassium in the plasma of citrated blood.*




* Blood-citrate = 5:1

Figure 2.

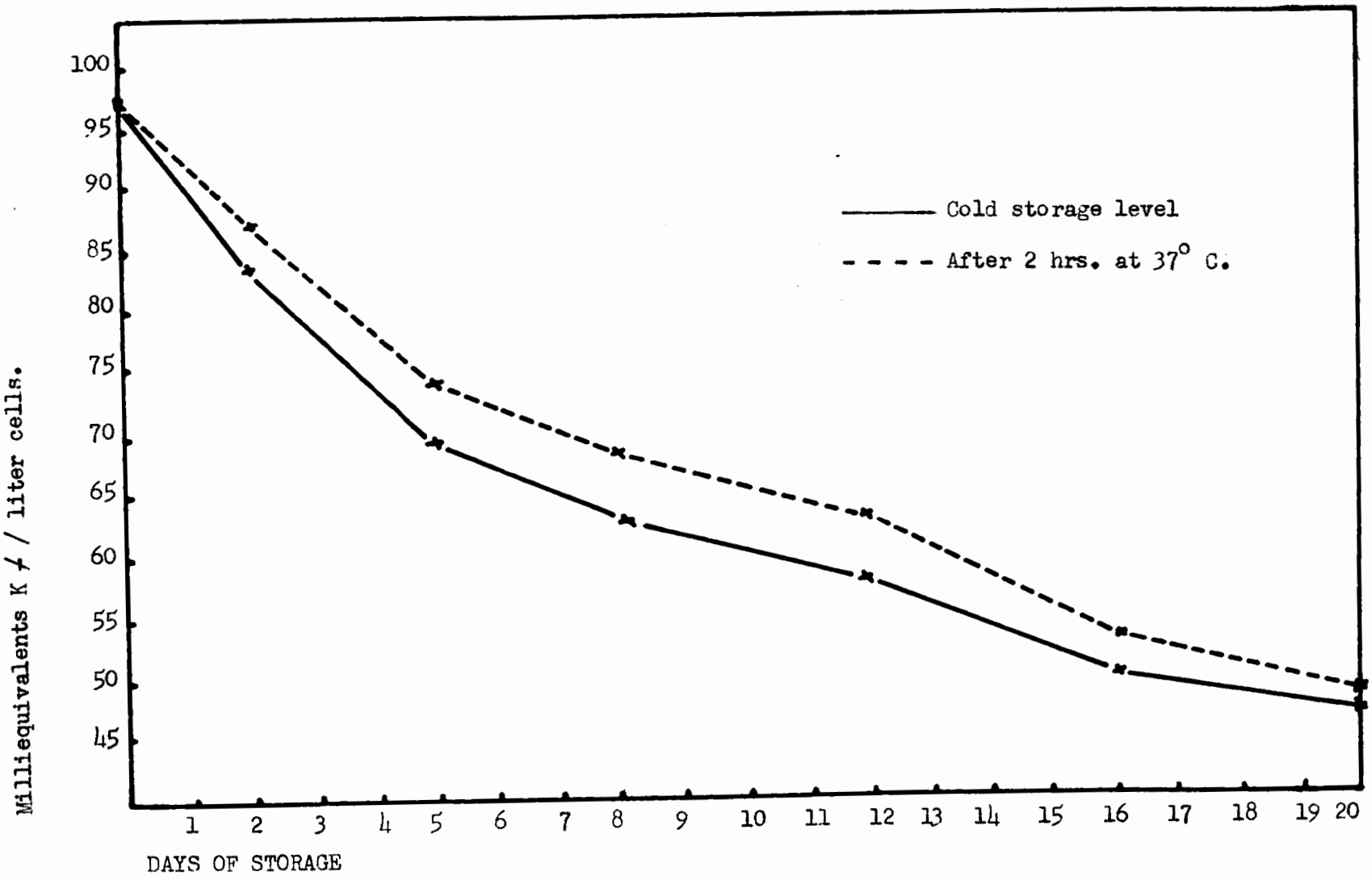
The effect of cold-storage and subsequent incubation at 37° C. on the concentration of potassium in the plasma of citrated blood with added glucose.*




* Blood-citrate-glucose - 5:1:0.5

Figure 3.

The effect of cold-storage and subsequent incubation at 37° C. on the potassium content of the erythrocytes of citrated blood with added glucose.*




*Blood-citrate-glucose - 5:1:0.5

## DISCUSSION

There is no problem more fundamental to biology than that of the passage of materials into and out of living cells. One of the most perplexing aspects of this is the paradoxical distribution of ions between the intracellular and extracellular fluids. Certain ions are held almost exclusively within the cell substance, while others are excluded from it. Knowing that this selectivity exists only while the cell is living, and gives way to a stable distribution on death, and knowing, from isotopic studies, that cell membranes may be permeated by these ions, one naturally wonders what processes are involved in the living cell which maintain the internal ionic concentrations.

Animal cells, and most plant cells, are characterized by having a higher concentration of potassium inside the cell than outside, and a lower internal concentration of sodium and chloride. Uneven distributions of other ions are also found in all living cells.

Living material is, in general, very sensitive to its ionic content, and the ionic concentration of the various body fluids must be maintained within fairly narrow limits to sustain life. The individual roles of the ions in biology are rather ambiguous and complicated (see Lehninger,1950). Of great significance is their thermodynamic role in helping to maintain the proper osmotic pressure across cell membranes. Specific activations of

enzyme systems by electrolyte ions, and their roles as co-enzymes are familiar, and have been thoroughly investigated. We may well wonder at the importance of so much potassium in most cells, or conversely, why sodium is banned from the interior of many cells. In erythrocytes, it is evident that potassium plays no essential role which cannot be served by sodium, for the red cells of Carnivora normally contain much more sodium than potassium.

In attempting an investigation of the reasons behind the electrolyte distributions, and the mechanisms whereby the cell practices its discrimination, all manner of living cells have been examined.

Early botanists, such as Pfeffer, de Vries and Overton, were amongst the first to study permeability and the osmotic forces of living cells, by using Nageli's discovery of plasmolysis and deplasmolysis. Botanists have made good use of the large marine plant cells such as Valonia and Halicystis from sea water, Nitella from fresh water and Chara from brackish water. Single cells of these multinucleate algae are frequently as large as grapes, with a large internal vacuole filled with intracellular sap, which is easily analyzed chemically or bio-electrically. There is a great variation of the internal milieu from one species to another, but of greater interest is how the concentration gradients of ions arise, some up hill, some down, whether they represent a static or dynamic

equilibrium, and if the latter is the case, by what means this equilibrium is maintained.

For many years the concept was that the cells build their ionic pattern in some early stage of development, and then cease to be permeable to the ions that were held in or out against the concentration gradient.

Then, Hoagland and Davis (1923;1926) observed that Nitella cells, which at low temperatures or in the dark become leaky to the inside chloride, recapture it upon warming or during exposure to light. Many other workers, from that time on, have demonstrated the intimacy between ionic concentrations and metabolic activity in plant cells.

In 1937, Steward published a stimulating paper on the role of metabolism and growth in promoting salt accumulation by plants. He realized that the problem was not one of equilibrium, but that the transfer of salt from the external solution to the cell sap tended to increase the total energy of the cell, and work must be done and energy expended. The living cells thus evade the limitations of true thermodynamic equilibrium by means of their own metabolism. Equilibrium is rapidly achieved after the cell is killed, and the second law of thermodynamics is then appeased.

In water plants, such as Nitella, the accumulation of ions takes place only in the light, and this focused attention on the energy relations involved in salt accumulation. The

energy of the light source is not utilized directly, however, but salt accumulation operates through those oxidative reactions in which the products of photosynthesis are the substrates. The light effect, besides supplying carbohydrate, also maintains aeration conditions, which are conducive to active absorption. Steward demonstrated the marked effect of the oxygen concentration on the uptake of both anions and cations by discs of storage tissue, such as potato and artichoke tubers, and by roots. During anaerobic metabolism, carbon dioxide is produced, but no salt is accumulated, suggesting that carbon dioxide production alone does not result in salt accumulation, but a high rate of aerobic respiration is required (see Osterhout's cell 1932, page 224).

Low temperatures cause potato discs to lose potassium comparable to the loss of chloride from single plant cells referred to above, while raising the temperature increases respiration, and an uptake of potassium by the potato discs is elicited.

Blinks (1940), by making bioelectric measurements on the sap of Halycystis, showed that potassium can be accumulated from artifical sea water containing little of this ion. The process is greatly modified by temperature. At 20° C., potassium enters fairly rapidly, but cooling to 5° C. results in a loss of potassium from the cell sap.

Pulver and Verzar (1940) found that yeast cells selectively remove potassium ions from the medium during fermentation, and tend

to release them when fermentation ends. Certain bacteria also show this behaviour.

Conway and his co-workers (1946,1948,1950) postulated that this uptake of potassium by yeast cells during fermentation is due to an exchange for hydrogen ions, which they observed lowered the $p^H$ of the medium from 4 to 1.6. If sodium replaces potassium in the medium, no sodium is accumulated, and few hydrogen ions are formed. Since the $p^H$ of the contents of the fermenting yeast cells is around 6, Conway suggested that there are two regions in the cells, the outer being very acidic and in Donnan equilibrium with the external medium, while the inner compartment is isolated by a cation-impermeable membrane. Potassium would then be accumulated only in the outer compartment by exchange for hydrogen ions. Since this does not account for all of the intracellular potassium, Conway conceded that an active transport of potassium and sodium may occur across the boundary between the acidic region and the rest of the cell. As yet, there is no evidence that the cell has such an area of high acidity.

Ørskov (1950) carefully studied fermentative processes in yeast cells and concluded that the uptake of potassium depends on the general metabolic state of the cells, and not on the acid production, which is merely incidental to the metabolism.

Oxidative and fermentative metabolic processes are clearly related to the absorption of salt by plant cells, but it is difficult to find any quantitative relationship. Perhaps a constant fraction of the total energy produced is directed towards doing this osmotic work. The activity of these plant cells in both salt accumulation and respiration is determined by the amount of carbohydrate available, and it is logical to associate some stage of the oxidative breakdown of sugar with salt transport. Steward (1937) considered that nitrogen metabolism is also involved, as the synthesis of protein from alcohol-soluble nitrogenous compounds is also affected by light, oxygen and temperature, and this synthesis parallels the respiration.

The pattern of the electrolyte distribution in muscle cells and nerve fibres has also been studied intensively for many years. These tissues can be compared with single cells since their fibres are held together loosely, the interspaces being generally open to the surrounding medium. Muscle and nerve fibres contain twenty to thirty times as much potassium as is found in the extrafibral fluid.

It had been supposed for years that cell membranes are impermeable to potassium, but this concept has required radical alterations. The rapid disappearance from the blood stream of injected potassium demonstrated that tissue cells could admit this ion. A more direct proof is afforded by the injection of radio-

active isotopes of potassium (Fenn et al. 1941; Hevesy,1948). Evidence is not lacking for the permeability of cellular membranes to sodium as well as to potassium (Cohn and Cohn, 1939; Eisenman et al.,1940).

Numerous workers have shown that sodium may exchange for potassium under various conditions. Heppel (1939;1940) raised rats on a potassium deficient diet, and nearly half of the normal potassium content of the muscle was replaced by sodium. Injected radioactive sodium readily exchanged with the intracellular sodium of these muscles. When potassium was restored to the diet of these animals, a redistribution to the normal electrolyte pattern resulted. Steinbach (1940) did analagous experiments by soaking isolated frog muscles in potassium-free Ringer solution, whereby 40% of the intracellular potassium was replaced by sodium. On returning these muscles to potassium-containing Ringer solution, they were able to reconcentrate this ion in the fibres against a concentration gradient of 10:1.

Over a period of many years, it has been well established that during activity, muscle cells lose potassium and gain sodium. Fenn and Cobb (1936) showed that artificial stimulation caused rat muscle to lose about 15% of its potassium, which is replaced by sodium. During recovery, potassium is regained by the fibres, and sodium is expelled, each transfer taking place against a steep concentration gradient.

It is difficult to see how the muscle can get rid of sodium by any equilibrium process, but must expend energy and

perform work to do so (see Steinbach,1952).

Nerve fibres possess an inorganic ion composition similar to that of muscle. The fibres of the giant axons of the squid have a ratio of intracellular potassium to potassium in the extrafibral space of about 30:1 (Baer and Schmitt,1939; Webb and Young,1940). Gerard (1929) and Furusawa(1929) were the first to show that the normal polarized state of nerves is maintained by an energy expenditure related to the utilization of oxygen. The resting potential of the nerve falls during anoxia and is restored after oxygen is returned to it. During anoxia, potassium escapes from the axons, as it does during excitation. Certain poisons also cause depolarization and a loss of potassium. The metabolic activity that maintains the polarized state, and presumably maintains the potassium concentration, appears to be related to the oxidation of the intermediary products of glycolysis, such as lactate and pyruvate, and only when oxygen is present can the fibre maintain the normal electrolyte composition (Shanes and Browne,1942; van Harreveld,1950). That the isolated axon loses potassium on stimulation and rapidly regains it at rest has been well established (see Shanes 1951; Keynes,1951; Hodgkin,1951).

Hodgkin subscribes, in general, to the theories of of Conway (Boyle and Conway,1941), with Dean's (1941) modification, whereby sodium is continuously extruded from the axon by some

"pumping" device, and potassium diffuses in passively, to maintain electrostatic and osmotic equilibrium. Obviously it makes little difference in this theory if sodium is held out by an impermeable barrier, as in Conway's original hypothesis, or is actively transported out.

During the passage of an impulse along a nerve, the membrane becomes more permeable to sodium, or the process of sodium extrusion is diminished. In any case, sodium enters the fibre, with a resultant leakage of potassium. The resting axon rapidly readjusts its ionic composition, either by accumulating potassium or excreting sodium or both. Such processes must require work, and energy must be derived from the metabolic activity of the tissue.

Dixon (1949) has demonstrated that anaerobic glycolysis, as well as the oxidative processes, may be instrumental in energizing the transport of potassium into brain cells. Krebs and his collaborators (1950) have suggested that l-glutamic acid has an important role in ion transport mechanisms in brain and retinal cells. Glutamine does not promote potassium transport, so they proposed that the ionized Y-carboxyl group may serve as an anion, carrying in the cationic potassium equivalently. This would maintain electrical neutrality, but an active outward transport of sodium, with an equivalent anion, must be hypothesized in order to maintain osmotic equilibrium. Krebs, in contrast to Dixon, was unable to demonstrate

potassium transport under anaerobic conditions. The requirement of an oxidative process utilizing glucose, pyruvate and lactate to energize sodium and potassium transport is well recognized. The role of glutamate is more obscure. It will facilitate the metabolism of pyruvate by the formation of a-ketoglutarate, but also may have a more specific function. As early as 1943, Nachmansohn had shown that glutamate facilitates, and may participate in, the synthesis of acetyl chdine in the brain.

Despite the increasing literature on the subject, the role played by the enzymes capable of synthesizing and hydrolyzing acetyl choline, in the maintenance of ionic constitution of cells, is obscure. This subject is briefly discussed with regard to erythrocytes in a preceding section. Results: L, page 153 (see Nachmansohn with regard to nerves).

Flink, Hastings and Lowry (1950) studied the inorganic composition of liver slices in relation to their metabolic activity. Such tissue cells lose potassium and gain sodium if their aerobic metabolism is interfered with, but are able to recapture potassium and expel sodium if returned to a favourable environment, before the irreversible effects of oxygen deprivation occur. The reader is referred to the extensive studies of Mudge (1951) on the maintenance of the sodium and potassium content of kidney slices by metabolic processes. These cells also lose potassium in unfavourable conditions and take up sodium; this process may be reversed under the stimulus of active aerobic metabolism. These

studies show the need of an active oxidative metabolism, as a source of energy, for such tissues to maintain their ionic composition, or to restore it, if it has been previously disrupted. On the other hand, tissues and cells that normally derive energy from aerobic glycolysis, or are able to revert to an active glycolysis in the absence of oxygen, do not depend on aerobic conditions to maintain or restore, their ionic composition.

Regarding erythrocytes, the groundwork was laid in the late 19th century by Hamburger, Gruber and Koepe, that the membrane of these cells is impermeable to cations, while demonstrating a high degree of permeability to small anions. Erythrocytes invariably contain more potassium and less sodium than the extracellular fluid, the plasma, they are bathed in, but this varies a great deal with the species. The erythrocytes of man, and most other species, contain about 100 m.eq. of potassium and about 10 m.eq. of sodium per liter. Those of the Carnivora, such as the dog and cat, have about 5-10 m.eq. of potassium and about 100 m.eq. of sodium per liter. The ox is intermediate, with about 20 m.eq. of potassium and 80 m.eq. of sodium per liter of red cells (Kerr,1937). This selectivity is independent of the cation concentration of the surrounding medium, as the ionic composition of the plasma is identical in all species of mammals, with sodium far in excess of potassium.

The characteristic imbalance of sodium and potassium is easily upset under circumstances that alter the normal environment of the erythrocyte , and a movement towards a more equal distributinn begins.

Before discussing the penetration of the cellular membrane by sodium and potassium, an examination of this membrane may be in order.# Human erythrocytes stromata contain, by chemical analysis, 4.7 mg. of lipid per ml., or 430 x $10^{-12}$ mg. per cell, or 2.6 x $10^{-12}$ mg. per $u^2$ of cell surface. Assuming a density of 0.85, this quantity of lipid could form a continuous layer 31 $A^o$ thick. About 40% of the total lipid is cephalin, 21% lecithin, 25% cholesterol,5% cholesterol ester and 9%"neutral fat", now recognized as consisting of glycolipids and referred to as "the cerebroside fraction". The total amount of lipid, and the proportion of each, varies from one species to the next.

The protein content of the red cell stroma is about 8.6 mg. per ml. or 780 x $10^{-12}$ mg. per cell. Taking 1.3 as the density of the protein, it could form a layer 37 $A^o$ thick over the surface of the erythrocyte. The washed and extracted protein,

#The writer has made liberal use of the discussion of Ponder (1949a) on the ultrastructure of the erythrocyte, and of the articles appearing in the Annals of the New York Academy of Science, 50, 1950, for the following remarks.

called stromatin, belongs to a class of proteins similar to, but not identical with, the keratins and collagens (Ballantine, 1944). By physical means, Boehm (1935) described the stromatin as having a very high assymetry number, and as seen in the electron microscope (Wolpers,1941), the red cell surface has a fibrous structure. The erythrocyte envelope is visualized as being composed of a scaffolding or felt-work of protein fibrils, in which lipid molecules are interspersed. Other proteins exist in the ultrastructure, but have not yet been characterized. A globulin is found in the supernatant fluid in the course of the preparation of stromatin. Ponder and Furchgott(1940;1941)described an anti-sphering albumin that covers the erythrocytes loosely to a depth of 50 $A^{o}$. Enzymes and immune bodies are also known to be present in or on the red cell ultrastructure.

By weighing the stromata or post-hemolytic residues of erythrocytes, and by calculation from the phospholipid content of red cells and hemoglobin-free residues, it has been established that the cell stroma composes 3.4% of the dry weight of the human erythrocyte. The process of washing the post-hemolytic residues to rid them of hemoglobin, causes a loss of lipids and proteins, especially in electrolyte-poor media. Erickson and her collaborators (1938) report that they were able to obtain stromata with 90% of the original lipid content, while Waugh and Schmitt (1940) admitted to losses of up to 60%, by allowing the cell residues to stand in the hemolyzing medium. At the same time 25% of the original protein may be lost to the medium.

The question as to how much of the material that makes up the stroma or fixed framework of the red cell can be regarded as composing the surface ultrastructure is not easily disposed of. As yet we do not know if the cell is a balloon, filled with a solution of hemoglobin, or has an internal framework supporting the hemoglobin, and surrounded by denser surface layers. By assuming that all the material of the fixed framework goes into the construction of the surface ultrastructure, or membrane, a value for its thickness may be deduced, which will have to be scaled down when, and if, an internal fixed architecture is established. If 3.4% of the dry weight of the intact human red cell goes into the surface ultrastructure, the thickness of such a membrane would be 190 $A^{o}$ (cell volume = 90 $u^{3}$, cell area = 163 $u^{2}$; assumed density = 1.0). This calculated (Ponder, 1949) thickness does not take into account the contribution of water, which is appreciable. Waugh and Schmitt (1940), with the aid of the leptoscope, have found rabbit red cells to be 220 $A^{o}$ thick at $p^{H}$ 6.0, as compared to a thickness of 135 $A^{o}$ calculated from the weight of the fixed framework. They estimated that water contributes about 25% of the thickness of the ultrastructure. Ponder (1949a) believes that water makes up about 80% of the natural thickness of the erythrocyte membrane. Electron microscope measurements of the thickness of the red cell stroma of humans range from 230-500 $A^{o}$, and by allowing for a 25% contribution of water, the total thickness becomes 300-600 $A^{o}$ (Zwickau,1941: Rebuck 1948;1949).

Polarization studies of the residues of rabbit erythrocytes show that the surface ultrastructure is optically similar to that of the sheath of invertebrate nerve. The protein component has its long axis oriented tangentially, while the interspaced lipids are radially oriented. The lipids, cephalin, lecithin and sphingomyelin, are visualized to be lying in the ultrastructure, with their fatty acid ends, bearing serine and choline, protruding into the surrounding medium. It is these ionized polar groups which dominate the surface from an electrophoretic standpoint. From electrophoretic data, it can be deduced that the surface of the red cell is dominated by strong acidic groups, the isoelectric point being about 1.7. The extracted lipid has an isoelectric point of 2.6, and the protein 4.7. The phosphoric acid groups of cephalin and lecithin are probably responsible for the electrophoretic behavior of the cell residues, although such charged groups are not necessarily limited to the region occupied by the outermost molecules.

Electron microscope micrographs show that the red cell surface is not a homogeneous film, but is folded, and holes may appear after lipid extraction. Wolpers (1941) has visualized the surface ultrastructure as being similar to a matting of protein fibres, in which lens-shaped lipid bodies are embedded. Such a protein-lipid mosaic, with the protein axis tangentially arranged and the lipid molecules radially oriented, fits well into the observations with polarization optics.

What is referred to as the red cell membrane, and held responsible for the permeability properties, is suggested to be a specially oriented region in the ultrastructure. The restraints on diffusing ions may be thought of as due to charges on the oriented molecules in this region. Fricke and his colleagues (1939) estimated this region, by impedance measurements, to be 30 $A^{o}$ thick.

It cannot be expected that the penetration of substances through such a complex surface ultrastructure can be explained adequately by the classical theories of membrane permeability. These theories were of a purely physical nature, depending on a lipid layer in one version, and on a sieve-like membrane (Collander) in another. Such theories possessed many discrepancies, but by fusing them into a "lipoid-sieve" theory, some of the failings were overcome. The theory was built around model experiments with sieve-like membranes, and living structures were supposed to be enveloped in such sheets (see Hober, 1945). All permeability studies on erythrocytes were interpreted in the light of this theory (Davson and Danielli, 1943). It had long been known that certain anions move readily in and out of the erythrocyte, as seen in the "chloride-shift". Experiments of short duration contributed to the idea that cations could not penetrate the intact cell membrane. It is probable that in these experiments a steady state existed, in which the passive diffusion of cations was balanced by the active processes which are now known to occur.

Until quite recently it was thought that the cation distribution in red cells (and all body cells for that matter) is established in some inexplicable manner during the active stage of cell development and is perpetuated in maturity by the cell becoming impermeable to at least one of the cations, sodium or potassium. That the cation content of the erythrocyte can be altered very readily had been shown as early as 1924 by Ashby, and by Kerr in 1929, but because of the currently accepted theories, these findings were either ignored, or at best, explained away.

Studies on the changes occuring in stored blood called attention to the fact that the red cell membrane does permit the diffusion of cations. Proponents of the classical theory suggested that the normally impermeable membrane was injured in some way, permitting cation diffusion. Among the conditions which allow the leakage of potassium from the red cell, and presumably damage the membrane, are suspension in slightly hyper-or hypotonic media, a slight imbalance of cations in the suspending salt medium, a rise or fall in the temperature, centrifugation, or even standing in serum or Ringer solution. When Wilbrandt demonstrated that glycolytic poisons caused massive cation shifts, attention was focused on the metabolic factor involved. The supporters of the classical theory of cation impermeability proposed that normal glycolysis was necessary to provide energy to maintain a structural situation which is cation impermeable (Wilbrandt, 1937; 1940).

Harris' findings in 1941 made this concept untenable. Not only does active glycolysis prevent the loss of potassium from

the red cells, but it will energize a slow uptake of this ion by previously depleted cells. Such a phenomenon cannot possibly be explained on the basis of surface properties alone, but implies the existence of a metabolic "pump", or an accumulating mechanism, of some sort.

The conclusive evidence that cation gradients in cells are not maintained by membrane impermeability came from experiments with radioactive isotopes of these ions (see Hevesy, 1948, for references to pioneering work). The early experiments with radioactive potassium showed that about 12-15% of the potassium in human red cells, kept in vitro at 37° C., is exchanged for plasma potassium in ten hours. Later experiments, from different laboratories (Sheppard and Martin, 1950; Raker et al., 1950; Solomon, 1951) have achieved remarkable agreement in demonstrating that 1.6-1.8% of the red cell potassium is exchanged for potassium in the extracellular medium per hour at 37° C. All the intracellular potassium is free to participate in the exchange. The rate of exchange of potassium is slow in some respects, but is fast when compared with the 120 day life span of the circulating erythrocyte. Red cells are even more permeable to sodium than to potassium , as has been demonstrated by radioactive tracer studies (Sheppard and Beyl, 1951; Solomon, 1952). The cells normally excrete over 3 m.eq. of sodium per hour per liter of cells, or, in other words, a complete turnover of the intracellular sodium can occur every three hours.

It is impossible, then, to retain the idea that the human red cell possesses a membrane that does not normally permit the entry or exit of cations. The question still is, what maintains the cation gradients between the intra- and extracellular fluids, or in other words, what prevents the cells from assuming an ion composition identical to the medium surrounding them?

The classical point of view is that the ionic gradient between the red cell interior and the plasma is maintained by the permeability properties of the membrane. Undoubtedly this plays a major role, presumably due to the insulating layer of Fricke (1939), which will restrain positively charged ions. Despite this, potassium does escape from the cell and sodium enters it, and to offset these shifts, potassium must be transported back and sodium transported out, against their respective concentration gradients.

That the active transport of cations across the boundaries of living cells does occur has been recognized for almost a quarter of a century. Netter (1928; 1934; Mond and Netter, 1930) demonstrated a model cell that was able to concentrate potassium. He separated with a collodion membrane, a solution, "I", consisting of M/1400 potassium sulfate and M/10 sulphuric acid, from a solution "O", consisting of the same amount of potassium sulfate made isotonic with respect to "I" by the addition of the proper amount of glucose, to avoid water shifts. The collodion membrane used to separate "I" from "O" was permeable to hydrogen and potassium ions, but not to

sodium ions, glucose or anions. Hydrogen ions tend to pass through the membrane, following the steep concentration gradient from $p^H$ 1 to $p^H$ 7, and for electrostatic reasons, such a passage is only possible by the exchange of an equivalent amount of potassium. This model is capable of eventually creating a potassium gradient of 25:1 between "I" and "O". Thus, in living cells, acid production could result in potassium accumulation, if the cell membrane possessed the same permeability properties as does a dried collodion membrane.

Osterhout and Stanley (1932) recognized that cells are enclosed in a structure that does not permit a ready inflow of ions. They devised an artificial "cell" that could accumulate potassium by the use of a carrier in the "membrane". In their model, a guaiacol solution serves as the cellular "membrane", separating potassium hydroxide outside the "cell" from potassium hydroxide inside. On bubbling carbon dioxide into the solution inside the cell, potassium bicarbonate forms. Potassium ions in the outer compartment form a complex with the guaiacol, which diffuses inward and the potassium is released by combining with the carbon dioxide. Hence the guaiacol is free to transport more potassium, and the concentration of potassium may become much greater inside the cell than outside. The production of carbon dioxide is not essential for potassium accumulation by living cells, but anything that will preferentially take this ion from its carrier would serve equally well. Similarly, the destruction of the carrier, in this case the guaiacol, on the inside

of the cell, would result in a net uptake of potassium by the cell. Guaiacol apparently demonstrates a much lower affinity for sodium than for potassium. Osterhout's (1952) timely review, which summarizes his earlier ideas, coincides with the advent of several modern models to explain the transport of cations through living membranes.

The failure to explain the cation distribution in muscles by physical forces alone, led Steinbach (1940) and Dean (1941) to accept an active transport of sodium across the cellular membrane. Dean recognized that the concentration of ions by cells requires work and hypothesized a "pump" mechanism, whereby sodium is excreted by the muscle fibre and potassium diffuses in, to maintain osmotic and electrostatic equilibrium. He recognized that such a transport mechanism must be motivated by metabolic energy. Muscle can maintain its high potassium content under anaerobic conditions, or in the presence of glycolytic poisons, but not when both oxidative and anaerobic metabolism was stopped. Muscle is well known for its ability to function normally anaerobically and aerobically.

As yet, the exact nature of the forces bringing about the transport of ions across cell boundaries is unknown, but, in many cases at least, it must involve chemical reactions between cell constituents and the transported ion. The accumulation of ions in response to an electrical potential (Lundegardh, 1945; Conway, 1951) is not sufficiently specific to perform, for instance, the separation of sodium and potassium ions from a mixture of the two ions.

In the case of phosphate transport, it is easy to visualize the formation of a phosphate ester at one boundary of a cell membrane and the splitting of the ester at another point in the cell. Gourley (1952) has, in fact, shown that inorganic phosphate ions enter the erythrocyte through the formation of adenosine triphosphate in the cell membrane.

The search for chemical constituents of cells capable of transporting sodium or potassium by complexing with either cation preferentially in the cell membrane has met with little success. Steinbach (1950) isolated a fraction of a muscle homogenate that appeared to bind sodium. This must be sodium in the process of being transported through the cellular membrane. Proteins apparently do not selectively bind either sodium or potassium, although the ability of certain ones to take up alkaline earth metals is well known (Denstedt; Klotz, 1952). Lamm and Malmgren (1940) describe the selective binding of sodium, in preference to potassium, by polyphosphates. The binding of these cations by the polyphosphates in cellular membranes warrants further study. The possible participation of the cholinesterase and choline acetylase systems, in the maintenance of ionic gradients, points to a possible role for the choline containing phospholipids in the cellular membrane. Investigations of purified lipid components of brain homogenates, however, show no selective binding of potassium as compared to

sodium (Christensen and Hastings, 1940).

Ussing (1949) warns that too much emphasis should not be placed on the chemical similarity between sodium and potassium. The artificial cell of Osterhout described above, accumulates potassium in preference to sodium, due to the higher affinity of guaiacol for potassium. Hodgkin (1947) and Steinbach (1952) point out that cations with small atonic numbers generally have a greater tendency to form complexes, and sodium should have a greater affinity for a lipoid carrier than potassium.

Krogh (1946), Ussing (1949), Steinbach (1952), and others, subscribe to the view that ion transport must entail a chemical reaction between some cell constituent and the specific species of ion to be transported. Such a system requires that the carrier form a relatively stable complex with the ion, and be free to diffuse, or be rotated, through the membrane. At the other boundary of the membrane the ion-carrier complex must be broken, either by altering the carrier chemically, or by supplying another ion which can replace the transported ion in the complex. Ussing concedes that pore size and electrical potentials resulting from metabolic processes may be involved in the transport of ions, but believes that a specific chemical reaction between the ion and some organic membrane constituent is of decisive importance.

Steinbach (1952) has provided a model to explain the cation distribution in muscle and nerve fibres. He postulated that the membrane of the cell has "holes" which permit the slow penetration

of sodium into and of potassium out of the fiber. To remove the sodium which tends to accumulate in the cell, a transport or carrier is provided. This carrier-ion complex is supposed to diffuse through the membrane and be released at the external boundary, or perhaps the sodium is released and the organic carrier salvaged or destroyed. Such an active removal of sodium should create an electrical gradient which would induce potassium to diffuse in passively. During excitation, the complexing of sodium ceases and the ions move passively in reponse to the chemical gradients. Steinbach realized that sodium transport must be linked with the energy-yielding reactions in the cell, either to synthesize the carrier, or to synthesize the ion-carrier complex, or both.

Conway (1947) and Ussing (1949) advanced reasons for believing that the potassium movements in nerve and muscle are purely passive along an electrical gradient and against the chemical gradient.

Solomon (1952) has recently advanced an hypothesis to account for cation transport in human erythrocytes which is similar, in some respects, to that suggested by Osterhout (1952) for plant cells, and by Steinbach (1952) for nerve and muscle cells. Solomon postulated that separate carrier mechanisms operate in the red cell membrane for the transport of sodium and potassium.

Moreover, in this hypothesis, sodium and potassium are not only transported through the membrane against their respective chemical gradients by the organic carriers, but are transported, by different carriers, along their respective chemical gradients. Solomon suggested that simple diffusion of cations through the membranes, even along the concentration gradient, cannot occur for thermodynamic reasons. He reinforced this argument by showing that within certain limits at least, potassium transport is unaffected by the external concentration of potassium ions. With the inward transport of potassium proceeding at the maximum rate, the outflow of potassium is unaffected by the external potassium concentration, (up to 14 m.eq. per liter) implying that the outflow is not due simply to free diffusion in response to the concentration gradient. Solomon suggested, therefore, that the outflow of potassium must also be mediated by a carrier, working maximally. However, if free diffusion were to take place through "holes" in the membrane, the rate could be limited by the number and size of the "holes", and not by the number of carriers available. Such movements in response to the chemical gradient are generally assumed to be passive, and Ussing (1949) has defined active transport as the transfer of a substance against a chemical potential or gradient. This definition is really too limited. As Shannon (1938) has pointed out, an active transport system can limit the rate of transfer in the direction

of a gradient, as well as facilitate transport against it.

At the time of writing, there are two divergent schools of thought with regard to active cation movements through the membranes of animal cells. Steinbach's (1952) theory exemplifies the view generally accepted for nerve and muscle cells, while Solomon (1952) has advanced an hypothesis for application to erythrocytes. Both theories propose an active extrusion of sodium through the cell membrane, energized in some way by metabolic processes in the cell, but they are at variance with regard to the mode of potassium accumulation.

Steinbach's explanation for the uptake of potassium is based largely on the Boyle-Conway theory, of which the fundamental concept is that as sodium is being actively carried out of the cell, potassium diffuses in passively, in response to the large amount of fixed anion in the cell, in order to maintain electrical neutrality. There is no allowance for an active transport of potassium, and such a mechanism need not be postulated if the amount of non-penetrating anion, the phosphate esters and the protein, remains unchanged during the expulsion of sodium and the accumulation of potassium.

However, in the erythrocyte, the active extrusion of sodium, by stored cells at least, is usually accompanied by a decrease in the amount of fixed anions in the cell, and electro-neutrality could be maintained by the outflow of inorganic phosphate,

which actually does occur. The osmotic equilibrium would be maintained by a simultaneous outflow of water, and the cell would shrink, but would not take up potassium. It follows, therefore, that the accumulation of potassium must also be an active process, and Solomon has established this by radioactive tracer studies (see Results, G and L for further evidence of an active transport mechanism for potassium).

Maizels (1949-1951) has pointed out that all living cells must be able to eliminate sodium, to offset its inflow, accompanied by water, which would eventually cause the cell to burst. He argued for an active output of sodium, and since he considered that there was no evidence for an active uptake of potassium, was of the opinion that potassium could be attracted in, and accumulate passively.

As described above, the concept that potassium is accumulated passively by nerve and muscle cells depends primarily on the belief that no anionic change occurs during the exchange of sodium for potassium. However, there is no evidence against the existence of a mechamism for the transport of potassium into these cells. In the light of the accumulated evidence for an active transport of potassium, as well as of sodium, in the human red cell, it is suggested that all living cells possess similar mechanisms for cation transport in order to maintain their chemical integrity. It is now well recognized that the capacity to regulate the internal

concentration of sodium and potassium is a property common to all cells. Hence, phylogenetically, these mechanisms must be very old, and it is possible that the extrusion of sodium and the accumulation of potassium, energized by metabolic processes, may have acquired an extra and later significance in highly differentiated tissues.

## SUMMARY

1. The exchange of sodium and potassium, glycolysis and phosphorolysis in human blood during prolonged storage at 5° C. has been discussed, both from the point of view of the existing literature on the subject, and from the writer's own experiments.

2. During the storage of human blood at 5° C., the rate of diffusion of potassium from, and of sodium into, the erythrocytes is decreased.

3. The inhibition of the glycolytic process in the red cells by the cold is held responsible for the decreased permeability of the membrane to sodium and potassium.

4. The further inhibition of glycolysis by low concentrations of glycolytic inhibitors, or by a lowered $p^H$, delays the loss of potassium from the erythrocytes kept at 5° C.

5. The transport mechanisms in the red cell that normally accumulate potassium and expel sodium are completely inhibited in the cold. This results in a net decrease in the potassium content and a net increase in the sodium content of the cells.

6. The failure of these transport mechanisms is attributed to the inhibition of the energy metabolism of the erythrocytes in the cold.

7. Added glucose does not affect the glycolytic rate of blood stored at 5° C., but nevertheless tends to delay the diffusion of potassium from the erythrocytes. This is attributed to a specific effect of glucose on the permeability properties of the membrane.
8. The ability of preserved erythrocytes to accumulate potassium and to expel sodium when incubated at 37° C. was thoroughly investigated. A liter of preserved erythrocytes is able to accumulate up to 4 m. eq. of potassium per hour, and to expel a similar amount of sodium. This activity is indirectly related to the glycolytic activity of the cells. Both processes fail within ten days if the blood is stored without additional glucose. If the blood is enriched with glucose during the cold-storage period, the capacity of the cells to glycolyze and to accumulate potassium and excrete sodium when incubated at 37° C. is preserved for three weeks. When the blood is stored in a slightly acidified medium (ACD) these functions are preserved for at least four weeks.
9. The metabolism of the red blood cell is related to the active transport of cations only in so far as the former process provides high-energy phosphate bonds for the latter processes.
10. The ability of preserved erythrocytes both to glycolyze and to effect the active transport of cations fails when their

adenosine triphosphate content is depleted.

11. Glycolytic inhibitors, at low concentrations, allow the red cells to lose potassium and to gain sodium at 37° C. by abolishing the active transport mechanisms.

12. At 37° C. a concentration of 0.01 M sodium fluoride increases the permeability of the membrane to potassium twofold, while the permeability to sodium is relatively unaffected.

13. Aerobic glycolysis is the main source of energy in the mature human red blood cell, but oxidative processes may contribute. Erythrocytes slowly consume oxygen and a Pasteur effect can be elicited.

14. Glutamic acid does not play a role in the transport of cations in preserved erythrocytes.

15. Neither insulin nor desoxycorticosterone affect the electrolyte or carbohydrate metabolism of erythrocytes from normal donors.

16. The distribution of magnesium in human blood is unaffected by the glycolytic rate of the cells.

17. Chloride ions tend to diffuse into the red cells during a prolonged period of storage of blood at 5° C.

18. Carbonic anhydrase is preserved indefinitely in human blood stored under sterile, refrigerated conditions. This enzyme does not play a role in the transport of cations in the erythrocyte.

19. The cholinesterase activity of human blood remains unchanged over extended periods of storage at $5^{o}$ C. The cholinesterase on the erythrocyte membrane tends to slowly leave and to enter the plasma during the first three weeks of cold-storage. This process is greatly accelerated during the following week.

20. The active transport mechanisms for cations in several widely divergent tissues have been discussed and an attempt has been made to compare these with the active cation transport mechanisms existing in the human erythrocyte.

The statements found in the Summary briefly describe original contributions by the writer to scientific knowledge.

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