SOME ASPECTS OF GLYCOLYSIS

IN STORED BLOOD

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SECTION I

INTRODUCTION

Under present methods of storage, whole blood can be kept satisfactorily for only about three weeks, since after this the glycolytic mechanisms which are needed to keep the cells alive, and thus suitable for transfusion, begin to fail.

Two coenzymes concerned with glycolysis have been reported as being the limiting factors in red cell viability during storage. The first one, diphosphopyridine nucleotide(1), is concerned with the dehydrogenase system in glycolysis. The other reported limiting factor is adenosine triphosphate(24) which is the link between phosphorylations and dephosphorylations.

Recently Leder et al(2) have reported the synthesis of DPN by red cells in vitro in the presence of nicotinamide. The diphosphopyridine nucleotide, it was hoped, could thus be eliminated as a restraint on length of storage. On the other hand it was thought that the adenosine triphosphate concentration could be maintained or even increased during storage by the controlled rate of breakdown of 2,3-diphosphoglyceric acid.

This study is therefore concerned with the factors tending to maintain glycolysis during storage with special reference to the coenzymes adenosine triphosphate, and diphosphopyridine nucleotide. Information thus gained could be used in the technical problem of preserving blood for longer periods of time.

SECTION II

REVIEW OF THE LITERATURE

HISTORICAL

Transfusions of blood prior to Landsteiner's discovery of blood types was indeed a risky undertaking. Thereafter it fared well but the necessity of having the donor present for the transfusion proved to be somewhat of an inconvenience. The urgency of the 1914 war and the then recent discovery of the anticoagulant properties of sodium citrate provided the impetus for great strides in the development of media for the storage of blood. In 1916 Rous and Turner(3) were able to show the protective action of glucose on red cell preservation when it was added to the sodium citrate solution. Several variations of Rous and Turner's original solution have been used. These variations have been quantitative and qualitative with the addition or substitution of saline, sucrose, corn syrup, various salts, buffers, acids, bases, and even plasma protein fractions. The most widely used type of solution at present is that developed by Loutit, Mollison and Young in 1943(4). It is composed of citric acid, sodium citrate and dextrose (ACD).* The advantages of this solution have been upheld by numerous investigators.

*This solution is commonly referred to as ACD, the initials being a short form for acid, citrate, dextrose.

CRITERIA USED FOR THE EVALUATION
OF BLOOD PRESERVATIVE MEDIA

There are two main types of methods for determining whether a given solution is of merit in the preservation of blood. The first is based on _in vitro_ testing in which numerous samples can be tested under easily controllable conditions. Secondly the effectiveness of the preservation may be tested _in vivo_. The latter method is a very laborious procedure but provides the only reliable criterion of satisfactory preservation.

In Vitro Tests

Hemolysis. The value of stored cells is completely lost if the cells hemolyze. Historically the criterion of hemolysis was first used by Rous and Turner(3) when they noticed that the addition of dextrose to the original sodium citrate solution decreased the number of cells that hemolyzed. This criterion like all _in vitro_ tests can only be used safely as a negative test for preservative solutions since it has been shown(31) that certain compounds can destroy vital cell processes without simultaneously inducing hemolysis.

Dimensional changes. During storage red blood cells undergo changes in their shape and size. Rapoport(5) has reported that cell width decreases while cell diameter increases with storage. Due to these changes their maximum attainable volume decreases, thus

rendering them more susceptible to hemolysis in hypotonic media.
Furthermore if the storage media is slightly hypotonic there is a
tendency for the cells to swell. If the volume attained by the cells
is near their critical hemolyzing volume it would prove dangerous to
transfuse the cells. Thus volume changes are also mainly used as
negative criteria.

Other criteria. Other criteria such as changes in the cell
base and organic phosphate levels, have been suggested as criteria.
The thought behind these being that if the cell could be kept in a
condition similar to that when originally drawn from the donor there
would be a greater probability of survival after storage. While
these factors can be important they are not used in the same general
manner as hemolysis and cell dimensional changes.

In Vivo Tests

In vivo tests are based on the determination of survival
of stored cells after transfusion into a recipient. There are two
main techniques used for these determinations.

The oldest technique, that of Ashby(6), involves the trans-
fusion of 'O' type donor cells into a normal 'A' or 'B' type recipient.
Immediately after the transfusion and at selected periods thereafter
blood samples are removed from the recipient's circulatory system.
The 'A' or 'B' recipient's cells are agglutinated from the samples

with anti-A or anti-B serum respectively. From counts of the un-
agglutinated cells and a determination of the recipient's blood
volume it is possible to determine from time to time the percentage
survival, in the recipient's circulation, of the stored cells
originally transfused.

The second technique is the radioactive iron method de-
veloped by Ross et al(7). It involves the use of cells, the hemo-
globin of which has previously been labelled with radioactive iron.
After storage and transfusion, radioactive counts are made on blood
samples taken from the recipient. Because of the constant
reincorporation of radio-iron into newly formed red cells, accurate
determinations of cell survival are limited to the first few post-
transfusion days.

THE DEVELOPMENT OF THE ACID-CITRATE-DEXTROSE MEDIUM

Loutit, Mollison and Young(4) approached the problem of
cell preservation media by finding a citrate-dextrose combination in
which the dextrose was not carmelized on sterilization. They thought
that caramel might have a deleterious effect on the recipient since
Hanzlick and Karsner(8) had shown hemmorhages, distention and con-
gestion of the lungs of guinea pigs injected with caramel solutions.
By autoclaving glucose solutions containing various quantities and

combinations of trisodium citrate, disodium citrate and citric acid they obtained two solutions in which there was no carmelization. Post-transfusion survival of cells stored in the two media was claimed to be not markedly different but the less acid one in routine use revealed a higher incidence of clots in the blood during collection. The solution developed by these workers and now generally adopted is composed of citric acid, sodium citrate and dextrose and is generally designated as ACD.

THE DEVELOPMENT OF THE CITRATE-PHOSPHATE-GLUCOSE MEDIUM

Parpart et al(9) developed a good preservative media for red cells strictly on the basis of in vitro testing. They related the spontaneous hemolysis of stored red cells to a wide variety of factors, included among which were the temperature of storage, dilution by preservative, pH of storage, the percent of glucose added and the amount of phosphate buffer added. On the basis of the conditions that kept hemolysis at a minimum during storage they suggested that 70 parts of blood be collected in 10 parts of a 4% solution of trisodium citrate (hydrated) and 20 parts of a 0.11 molar sodium phosphate buffer (pH 6.85) containing a final concentration of 0.5 gm.% glucose, and that this mixture should be stored at 7°C. As tested in vivo by Gibson et al(10) this solution affords better cell survival than ACD but for routine use it is more difficult

to prepare.

THE DEVELOPMENT OF STORAGE MEDIA
ON A BIOCHEMICAL BASIS

Introduction

It is a generally accepted fact that the viability of red
cells during present methods of storage depends largely on the
ability of the cells to metabolize glucose. The evidence is both
direct and indirect. Sodium fluoride, a glycolytic enzyme poison,
when added to the preserving media causes a marked increase in
hemolysis in vitro. Similarly when glucose is absent from the pre-
servative media, or replaced by sucrose, there is also an increase
in in vitro hemolysis. Mollison and Young(11) noted that, as well
as an increase in in vitro hemolysis in the absence of glucose, there
was a marked decrease in the survival of transfused cells stored
under these conditions. Thus it would seem that glycolysis inhibition
induces damage in the red cell that cannot be corrected in the re-
cipient's circulatory system. It appears that red cells can under-
go a certain amount of rejuvenation when returned into a circulatory
system, as illustrated by Maizels in 1944(12). He showed that the
cell sodium and cell potassium of stored blood was returned to near
normal within two days after transfusion into a recipient. These
changes represented over a 100% decrease and increase in the cell

sodium and potassium respectively, and took place against the normal concentration gradient. In view of the fact that the base shift has been shown to be definitely connected to gly-colysis(13), (14), (15), it seems logical that survival and rejuvenation of stored cells is very intimately connected with glycolysis.

Similarly as the length of time of storage increases the ability of the cells to metabolize glucose decreases. This may be coincidental with other changes in cell composition or may be the causal agent of them.

Thus it was decided to re-examine glycolysis in blood in an effort to gain an insight into the factors which at present limit the length of time of storage.

Glycolysis

The general pattern of glycolysis shown in the follow-ing scheme was derived from several workers. The over-all scheme is that generally attributed to Embden and Meyerhof(16). The more exact details of its operation have been fully discussed by Baldwin(17) so only those portions of the scheme which have a direct bearing on this thesis will be discussed here. Of parti-cular interest in red cell glycolysis is the catabolism of 3-phospho-glyceraldehyde. There are two potential possibilities for its con-version to 3-phosphoglyceric acid.

GLYCOLYTIC SCHEME

GLUCOSE   ATP → ADP

GLUCOSE-6-PHOSPHATE

FRUCTOSE-6-PHOSPHATE   ATP → ADP

FRUCTOSE-1,6-DIPHOSPHATE

3-PHOSPHOGLYCERALDEHYDE ⇌ DIHYDROXYACETONE PHOSPHATE

$+ H_3PO_4$

1,3-DIPHOSPHO-GLYCERALDEHYDE

DPN → DPN-H_2

1,3-DIPHOSPHO-GLYCERIC ACID   Diphosphoglyceric acid mutase   2,3-DIPHOSPHO-GLYCERIC ACID

ADP → ATP

Glyceric Di-phosphatase

I

II

3-PHOSPHO-GLYCERIC ACID   3-PHOSPHO-GLYCERIC ACID + PHOSPHATE

2-PHOSPHOGLYCERIC ACID

PHOSPHO ENOL PYRUVIC ACID

ADP → ATP

DPN-H_2 → DPN

PYRUVIC ACID

LACTIC ACID

The first possibility(I) was elaborated by Warburg and Christian(18) while the second possibility arises as a result of the recent work of Rapoport and Luebering(19,20). The two differ in that the former yields one mole of ATP for each mole of 1,3-diphosphoglyceraldehyde catabolized while in the latter process the 2,3-diphosphoglyceric acid is dephosphorylated to inorganic phosphate and 3-phosphoglyceric acid.

Glycolysis as Applied to Red Cells

The over-all change in the glycolysis of red cells is the formation of two moles of lactic acid for every mole of glucose consumed. Each step below the 3-phosphoglyceraldehyde stage involves double the molar quantities of steps above it.

In red cells there may or may not be a net formation of high energy phosphate in the form of ATP resulting from glycolysis. This point hinges around the fact that red cell glycolysis differs from muscle and yeast glycolysis in that the formation of 2,3-diphosphoglyceric acid may be obligatory. As may be seen from the above scheme, there are two ways in which 3-phosphoglyceraldehyde may be converted to 3-phosphoglyceric acid. The generally accepted pathway(I) for muscle and yeast glycolysis involves the production of molar equivalents of ATP for each mole of 1,3-diphosphoglyceraldehyde oxidized. The second route(II) takes place without ATP formation from the '2' position phosphate of 2,3-diphosphoglyceric acid.

However, the '3' position phosphate of the 2,3-diphosphoglyceric acid accumulated in the red cells is a potential source of

high energy phosphate for the formation of ATP at the phospho-
enol-pyruvic acid stage. If the rate of breakdown of this
accumulated 2,3-diphosphoglyceric acid could be controlled such
that efficient use of its store of phosphate energy were possible,
it might prove valuable to the maintainance of the glycolyzing
ability of the cells.

The Turnover of Adenosine Phosphates

Two moles of ATP are used in the phosphorylations from
glucose to fructose-1,6-diphosphate. These two moles of ATP are
recovered when two moles of phospho-enol-pyruvic acid are dephos-
phorylated to pyruvic acid in the presence of ADP to accept the
phosphate. Since there is only 0.4 mM/1 of adenosine phosphate
available it must be cycled 2.5 times. Meyerhof(21) showed
recently that only a fraction of the total measurable ATP actually
takes part in hexokinase activity. This he attributed to 'bound
inactive complexes' and 'cell organization'. Thus considering his
findings the turnover number of ATP is probably much higher than
stated.

The Oxidation-Reduction Reaction and DPN Turnover

There are two main enzymes coupled by DPN that form an
oxidation-reduction system. When 3-phosphoglyceraldehyde is oxidized
to the corresponding 1,3-diphosphoglyceric acid, one molecule of

hydrogen is released. This molecule of hydrogen is used for the reduction of the carbonyl group of pyruvic acid to form lactic acid. The enzymes involved in this system are 'phosphoglyceraldehyde dehydrogenase' and 'lactic acid dehydrogenase'. These two enzymes require the coupling of DPN and DPN-H₂ respectively to perform their functions. The DPN is apparently bound rather firmly to the phosphoglyceraldehyde dehydrogenase apoenzyme, while with the lactic acid apoenzyme it forms a more highly dissociated complex(22). Reacting in molar equivalents with pyruvic acid, the 0.04 mM/l of DPN usually present in stored blood must be oxidized and reduced $1/0.04 = 25$ times for every millimole of pyruvic acid reduced.

The Relationship of Experiments to Glycolysis

If the turnover of these coenzymes has anything to do with their depletion or 'wearing out' it might be expected that DPN would be more susceptible than ATP. Since these coenzymes are thought to turn over faster than any other factor in glycolysis they may be the more labile links of glycolysis. If the ATP and the DPN levels could be kept high it was thought that glycolysis could be enhanced and possibly the survival of the cells increased.

Since, as a general rule, inorganic phosphate hinders phosphatase action(20) a higher level of ATP might be expected by its inhibition of ATP-ase(23) activity. In ACD stored bloods the ATP level is maintained during storage at the expense of the other phosphate esters(24). Martland(25) had shown that above

pH 7.3 phosphate ester synthesis takes place in glycolysis, while below this pH phosphate esters were decomposed. Denstedt(1) with his McGill-II solution buffered at pH 7.4 confirmed the synthesis of phosphate esters in blood during the first three weeks of storage. However, tests of _in vivo_ survival by Gibson et al(10) have shown that ACD was quite superior to the McGill-II solution. Now if a quantitative relationship between the acidity below pH 7.3 and the degree of ester decomposition exists, it might be possible to strike a happy medium between the two solutions, in which the phosphate ester decomposition could be controlled to keep adenosine triphosphate high. On the premise that phosphate buffered at pH 7.0 might achieve these results, its incorporation into the basal media was tried.

Leder et al(2) have shown that when red cells were incubated with nicotinamide, the pyridine nucleotide content increased. It was decided to add nicotinamide to the preservative media on the premise that the increased pyridine nucleotide levels might enhance glycolysis.

The next section deals with the methods used to investigate the effect of the addition of phosphate buffer and nicotinamide on the ATP and DPN and other factors which may be altered by the proposed coenzyme changes.

SECTION III

METHODS

INTRODUCTION

The general pattern used to investigate the problem
was to analyze serial samples of blood, stored in the various
preservative media, for some of the components of importance
in red cell metabolism which might be affected by the proposed
coenzyme changes.

Basically the blood was stored in the commonly used
acid-citrate-dextrose (ACD) media of Loutit et al(4). In view
of the suggestions already mentioned that the addition of phosphate
or nicotinamide might aid in the maintainance of increased coenzyme
levels, these substances were added together or separately to the
basic preservative medium. Blood was added to these various
media and was kept in a refrigerator between 3 and 6° C. Samples
of the bloods thus stored were analyzed for glucose, lactic acid,
diphosphopyridine nucleotide, and the various phosphate fractions.
To facilitate presentation, the methods used for the individual
analyses are given first, followed by a detailed account of the
preparation of the blood for storage.

PHOSPHATE DETERMINATIONS

Principle

The estimation of phosphate in blood was performed by the method of Fiske and SubbaRow(26). The principle of this method is that orthophosphate (inorganic) combines with a molybdate radicle in the presence of an acid to form phosphomolybdic acid. When this latter acid is reduced by 1-amino-2-naphthol-4-sulphonic acid a mixture of the lower oxides of molbdenum is formed, whose blue colour is directly proportional in intensity to the amount of phosphate present.

Reagents

The reagents necessary for the determination of phosphate by this method are listed in the Appendix.

Procedure

All volumetric tubes, pipettes, and syringes used for the transfer of liquids, in the phosphate determinations were very carefully standardized. All glassware was cleaned in good chromic

acid and thoroughly rinsed with distilled water.

Standards. Four ml. of a standard phosphate solution was pipetted into a thick walled Folin-Wu blood sugar tube. To this was added one ml. of molybdate reagent from a Kroh-Keys syringe and the contents mixed by rotation. Point four ml. of the amino-naphthol-sulphonic acid reducing agent (ANS) was then added and the contents mixed. The solution was then made up to twenty-five ml. with distilled water and mixed by inversion. The contents were transferred to a colorimeter tube and read in a Coleman model 14 Universal Spectrophometer at 660λ against a reagent blank, ten minutes after the addition of ANS. Duplicate standards were run with every set of analyses.

Blanks. The blanks were prepared in a manner analagous to the standards with the exception that four ml. of a nine per cent trichloracetic acid was substituted for the standard phosphate solution.

Blood Filtrates. Blood was deproteinized by adding one volume of blood dropwise to ten to fifty volumes of ice-cold ten per cent trichloracetic acid, the volume of which depended on the phosphate concentration expected. The resultant mixture was then filtered through a Whatman no. 1 filter paper. Aliquots of the filtrate were analyzed for orthophosphate (no hydrolysis), total phosphate(wet ashing in H_2SO_4) and for inorganic phosphate liberated after 7 and 100 minutes of hydrolysis in normal H_2SO_4.

The details of the actual procedures are shown in the 'Flow Sheet for Phosphate Analyses' in the Appendix.

Reliability of the Method

Colour development. A standard orthophosphate solution was treated for the determination of phosphate as mentioned above. The density changes in the blue colour developed were measured in the colorimeter at various times after the addition of the reducing agent. These changes are shown in Figure 1. It may be seen from this graph that the colour develops rapidly for the first two minutes after addition of the reducing agent. For the next eight minutes the rate of development slows down rapidly. From 10 to 25 minutes after the addition the colour remains quite stable.

To compensate for minor colour changes all the tubes of all the sets were read in the same definite order and all samples were read in the interval of 10 to 20 minutes after the addition of the reducing agent.

Standard curves. A twelve-fold range of standards were analyzed for orthophosphate. The results of these analyses are shown in Figure 2. It may be seen from this graph that the colour developed is directly proportional to the amount of orthophosphate present, up to and including 50 micrograms of phosphorus per four ml. of solution.

Variations in standards. From time to time in the phosphate analyses the standards were found to give different densities. It was

found however that the changes in density of unknown solutions from time to time showed the same proportion of variation as that of the standards. Therefore the unknowns were calculated from densities of standards analyzed in the same sets and not from a prepared standard curve.

e.g. Two sets of total phosphate analyses on the same two filtrates gave the following densities. Along with these are shown the mg.% phosphorous in the blood, from which these filtrates were prepared, as calculated from the standards of their sets.

	Set I		Set II	
	Density	Mg.% P	Density	Mg.% P
Standard ☆	0.2250		0.2325	
Filtrate A ☆	0.2110	15.75	0.2195	15.85
Filtrate B ☆	0.2200	24.25	0.2280	24.31

☆ All analyses were done in duplicate.

Recovery experiments. In 15 experiments the recovery of added inorganic phosphate, adenosine triphosphate, and hexose diphosphate,☆ measured as orthophosphate before and after partial or total hydrolysis varied from 97.7 to 101.4% with two exceptions of 92.2 and 87.3%. Detailed results of these experiments may be seen in Table 1.

Replicate analyses. The average deviation from the mean in 320 sets of replicate analyses was ±0.285%.

☆ The differential hydrolysis procedure for the determination of ATP and HDP is outlined on page 22.

TABLE 1.   19.

RECOVERY OF ADENOSINE TRIPHOSPHATE, HEXOSE DIPHOSPHATE

AND A MIXTURE OF BOTH IN BLOOD FILTRATES

Duration of hydrolysis	Micrograms of phosphorus in 4 ml. of solution			Per cent ☆ Recovery of phosphorus of:
	Blood Filtrate (A)	ATP Soln. (B)	Equal amounts (A) and (B)	ATP
0 mins.	4.19	0.70	2.44	101.4 ☆☆
7 mins.	11.28	24.55	17.91	100.0
100 mins.	14.87	32.00	23.52	99.5
Total	30.57	40.62	35.91	98.5
	Blood Filtrate (C)	ATP Soln. (D)	Equal amounts (C) and (D)	ATP
0 mins.	4.34	1.03	2.76	87.3 ☆☆
7 mins.	12.63	24.78	18.80	99.2
100 mins.	17.04	32.91	25.00	99.8
Total	37.27	39.93	38.72	99.4
	Blood Filtrate (C)	HDP Soln. (E)	Equal amounts (C) and (E)	HDP
0 mins.	4.34	2.62	3.59	92.2 ☆☆
7 mins.	12.63	12.08	12.36	99.9
100 mins.	17.04	32.23	24.55	100.5
Total	37.27	44.18	40.83	99.5
	Blood Filtrate (C)	Two parts (C), one part (D) and one part (E)		HDP and ATP
7 mins.	12.63	15.75		97.7
100 mins.	17.04	25.00		98.8
Total	37.27	40.14		97.8

☆ Recovery formula: $\dfrac{\frac{1}{2}\text{ Column 2}}{\text{Column 3} - \frac{1}{2}\text{ Column 1}}$

☆☆ When less than 4 micrograms of phosphorus are present in a sample a density difference of 0.0005 can change the recovery by 7%

ACID HYDROLYSIS AND PHOSPHATE ESTERS

Introduction

In 1928 Lohmann(27) noted that two of the three phosphate groups of ATP were released by hydrolysis for 7 minutes in normal HCl at 100°C. Under similar conditions Needham and Pallai(28) noted that 50% of the phosphate of hexose diphosphate was released by hydrolysis for 100 minutes. Several authors including Greenwald(29) have noted the extreme resistance of 2,3-diphosphoglyceric acid to acid hydrolysis under the above conditions. The reported percentage hydrolysis of certain phosphate esters for given periods of hydrolysis have been summarized in Table 2.

Hydrolysis Rates in Normal Sulphuric Acid of the Major phosphate Esters found in Blood Filtrates

A study was made of the rates of hydrolysis in normal H_2SO_4 of three major phosphate esters found in blood. Four ml. of a solution of the ester in question dissolved in 9% trichloracetic acid was placed in a thick walled Folin tube. To this was added one ml. of $5N$ H_2SO_4 and the tubes were placed in a boiling water bath for 7 or 100 minutes. Tubes which were to be boiled for 100 minutes were fitted with water-cooled reflux condensors. After having boiled for the pre-stated intervals these tubes were placed in a large cold water bath for exactly 5 minutes, then analyzed for phosphate. Total phosphate and the

TABLE 2

HYDROLYSIS OF PHOSPHATE ESTERS

REPORTED IN THE LITERATURE

Phosphate ester	Percent hydrolyzed in N HCl for			Reference
	0-7 mins.	7-100 mins.	0-180 mins.	
Adenosine tri-phosphate	66		86	(27)
Adenosine di-phosphate	50		79	(27)
Fructose di-phosphate	28	50	87	(55)
3-phosphoglyc-eric acid	0		2	(56)
2,3-diphospho-glyceric acid	☆1.2	3.6	4.8	(57)
Fructose-6-phosphate	10	70	87	(55)
Adenosine mono-phosphate	---		60	(28)
Diphosphopyrid-ine nucleotide	---		60	(28)
Dihydroxyacetone phosphate	46		100	(56)
3-phosphoglyc-eraldehyde	46		100	(56)
Phospho-enol-pyruvic acid	46		100	(56)

☆ In normal sulphuric acid

orthophosphate originally present were also measured. The results

of the hydrolysis of adenosine triphosphate (Pabst Labs.), fructose-

1,6-diphosphate (General Biochemicals) and 2,3-diphosphoglyceric acid [*]

are shown in Table 3.

Under these conditions it may be seen that 7 minutes of

hydrolysis liberated phosphate mainly from adenosine triphosphate,

a further 93 minutes of hydrolysis liberated phosphate mainly from

hexose diphosphate, while the 2,3-diphosphoglyceric acid was left

mainly unhydrolyzed.

The majority of the organic acid soluble phosphate found

in blood is composed of these three esters. The blood filtrates were

analyzed for inorganic phosphate at the following times: before any

hydrolysis, after seven minutes, after 100 minutes, and after total

hydrolysis. The phosphate which was liberated from blood filtrates

between 0 and 7 minutes of hydrolysis was designated as the 'aden-

osine triphosphate' fraction, that which was liberated between 7 and

100 minutes of hydrolysis was designated as the 'hexose diphosphate'

fraction, and that which was left unhydrolyzed after 100 minutes was

designated as the 'diphosphoglyceric acid' fraction. However since between

22 and 28% of the ATP and HDP fractions are still left unhydrolyzed

after 100 minutes of boiling, and since the 100 minute fraction also

includes about 5% of the DPGA, it is possible to correct the DPGA

fraction by subtracting from it 20% of the phosphate liberated in

the first 100 minutes of hydrolysis. This correction has been applied

wherever the DPGA is given in millimolar concentrations.

TABLE 3

HYDROLYSIS OF ADENOSINE TRIPHOSPHATE, HEXOSE

DIPHOSPHATE, AND 2,3-DIPHOSPHOGLYCERIC

ACID IN NORMAL SULPHURIC ACID AT 100°C.

Ester	Per cent hydrolyzed[*] in	
	7 mins.	100 mins.
ATP		
Prep. 1	59.7	78.4
Prep. 2	58.6	77.6
HDP		
Prep. 3	22.7	71.8
Prep. 4	22.9	71.8
2,3-DPGA		
Prep. 5	0.41	5.06
Prep. 6	0.40	5.02

[*] All analyses in triplicate.

TABLE 4

DEVIATIONS FROM THE MEANS IN THE HEMOGLOBIN VALUES IN

FIVE SETS OF SERIAL ANALYSES ON STORED BLOODS

Bottle no.	No. of analyses	Per cent deviation from the means
1	18	0.91
2	18	1.37
3	18	1.59
4	20	2.22
5	23	1.64
	Average	± 1.55

PYRUVIC ACID DETERMINATIONS

INTRODUCTION

Quantitative determinations of pyruvic acid were considered essential for two reasons. First, it has been reported (36) that accumulated 3-phosphoglyceric acid is metabolized to pyruvic acid while 2,3-diphosphoglyceric acid is not. Confirmation of this in stored blood would be helpful. Secondly any pyruvic acid increase not corresponding to a phosphoglyceric acid decrease might indicate impairment of the dehydrogenase system or outside competition for the reduced diphosphopyridine nucleotide. See Glycolysis Scheme page 9.

Method

The method used was that of Friedemann and Haugen(37). It consists of the colorimetric measurement of the 2,4 dinitrophenylhydrazone derivative of pyruvic acid. Essentially an acid solution of 2,4 dinitrophenylhydrazine was added to a trichloracetic acid filtrate of blood. The hydrazone was extracted from the filtrate with benzene, from which in turn it was extracted with sodium carbonate solution. Strong caustic was added to an aliquot of the sodium carbonate extract to enhance the hydrazone colour. The solutions were then read in an Evelyn Colorimeter using a 520 filter.

The standard curve shown in Figure 3 is a straight line going through the origin of the graph.

In five experiments the amount of pyruvic acid recovered was 100.0, 100.1, 101.3, 101.3 and 103.4 per cent of that added to the blood filtrates. The average deviation from the means in 44 replicate sets of analyses was 1.52%

Stumpf(38) has recently stated that the Friedemann and Haugen method of pyruvic acid does not differentiate between phospho-enol-pyruvic acid and pyruvic acid. However Meyerhof and Oesper(39) have shown that the equilibrium between these compounds in glycolysis was of the order of 2,000 in favor of pyruvic acid. Therefore the contribution of phospho-enol-pyruvic acid to the total 'pyruvate' measurements should be negligible. When checked enzymatically, the pyruvic acid as measured was found to be pyruvic acid.

HEMOGLOBIN AND HEMATOCRIT DETERMINATIONS

Introduction

Hemoglobin and hematocrit values are necessary for the calculation of cell volume changes and the percentage hemolysis.

Hemoglobin Method

Collier's(40) cyanmethemoglobin method was used for the whole blood hemoglobin determinations. Ths colours were read against water in a Coleman Model 14 Universal Spectrophotometer and the absolute

values were calculated from a graph calibrated from iron determinations of hemolysates. The iron vs. cyanmethemoglobin calibration curve was prepared by Dr. W. W. Hawkins using the procedure of Hanzal(41) as modified by Myers and Eddy(42). The largest average deviation from the mean in five sets of serial analyses on 5 bottles of stored blood was 2.22%. Table 4 shows the results of the average deviations from the means in five bottles of stored blood.

Hematocrit Method

The hematocrit (Ht) was determined by measuring the volume of packed cells in relation to the total volume of blood. A Wintrobe tube, a graduated constant bore tube with flat bottom was filled with a volume of blood and centrifuged at an average radius of 14 cm. for 30 minutes at 3,000 r.p.m. The ratio of packed cells to total volume of blood was expressed as a percentage.

The average deviation from the means on twelve duplicate hematoctrit determinations was 0.32%.

DETERMINATION OF THE PERCENTAGE HEMOLYSIS OF
STORED BLOOD

Introduction

Quantitative determinations of the hemoglobin in the supernatant fluid of stored blood were performed. From this data

it would be possible to judge the degree of spontaneous red cell destruction which occurred in the various storage media.

Method of Measuring Supernatant Hemoglobin

A slight modification of the reduced alkaline hematin method of Hunter et al(43) was used. To 0.3 ml. of the supernatant fluid, 1.2 to 4.5 ml. of 10% NaOH was added to convert the Hb. to alkali hematin. After 5.0 minutes a few milligrams of dry sodium hydrosulphite was added to reduce the alkali hematin. The light transmission of the solution at 560 and 580 m.μ. was read in a Beckman DU Spectrophotometer at 1.0 and 2.0 minutes respectively after the addition of hydrosulphite. The 560 m.μ. reading indicated reduced alkali hematin, while the 580 m.μ. reading was stated to be indicative of blank activity of the plasma. A standard curve was prepared from a hemoglobin solution of known Hb. content. The 560/580 transmission ratios were plotted on semilog paper against the hemoglobin content of the sample diluted with NaOH. The standard curve shown in Figure 4 goes through zero and is a straight line up to about 100 mg.%. From 105 to 184 mg.% the standard line is also linear but with a different slope. The majority of the unknown solutions were diluted so as to fall within the 0-100 mg.% range.

The amount of hemoglobin in the supernatant was found by

reading the mg.% hemoglobin from the graph, multiplying this by the dilution with NaOH, multiplying by $\frac{100-Ht.}{100}$ and dividing the product by 1,000 to convert to grams per cent.

The error as originally stated by Hunter was 5%. In the standard curve, five of the points were off the line by 6.3%, 4.8%, 4.6%, 2.2% and 1.8%. The average deviation from the means in 23 sets of replicate analyses was 1.41% with a range of 0.00 to 7.9%. While some of the results from these determinations may contain large errors, the net absolute error was small because the percentage hemolysis was low.

Calculation of the Per Cent Hemolysis

The amount of hemoglobin in the supernatant fluid in grams per cent was divided by the total hemoglobin, also in grams per cent, and the results expressed as a percentage.

CALCULATION OF THE RELATIVE CELL VOLUMES

Rapoport(5) has shown that red cells undergo a decrease in diameter and an increase in thickness during storage. These size variations decrease the maximum possible attainable volume of the cell thus rendering them more susceptible to hemolysis in hypotonic media. The cell volume measurements are also important in connection with the degree of hemolysis in the presence of various additives. If hemolysis takes place without swelling there is a strong indication of a lytic action on the part of the additives.

The hematocrit changes as such are indicative of changes in the red cell volume but corrections must be made for variations in sampling and for cells lost by hemolysis. This was done by dividing the

hematocrit by the cellular hemoglobin concentration. The values were expressed as a percentage of the original Ht - Hb ratio in accordance with the following equation:

$$\dfrac{\dfrac{\text{Ht of sample}}{(100 - \% \text{ hemolysis}) \times \text{Hb conc. of sample}}}{\dfrac{\text{Ht of whole blood}}{\text{Hb of whole blood}}} \times 100 \qquad \begin{array}{l}\text{Cell volume } \% \\ \text{of original}\end{array}$$

The error in this estimation may be calculated from the errors in the hemoglobin, hematocrit and per cent hemolysis as given above. From these values the maximum variation to be expected is about 1.5%.

LACTIC ACID, GLUCOSE, AND PYRIDINE NUCLEOTIDE DETERMINATIONS

Introduction

The changes in the glucose and lactic acid in blood during storage provide the only reliable means of guaging the ability of the cells to glycolyze under varying conditions. The production of lactic acid by red cells provides a reliable means of guaging the over-all ability of the cells to glycolyze. The rate of disappearance of glucose from the media also provides a means of determining the rate of glycolysis, but since large quantities of glucose are present in the media the small changes in the glucose content are subject to larger experimental errors than the lactic acid determinations.

It was considered desirable to determine the changes in the cellular pyridine nucleotide levels to find the effect of storage on

these compounds. Furthermore with the addition of nicotinamide it was considered essential to determine its effect on the cellular pyridine nucleotide levels.

The glucose determinations in Experiments 3 and 4 were performed by Dr. M.C. Blanchaer, while those of Experiment 6 were performed by Miss I. Strautmannis. The lactic acid determinations and the pyridine nucleotide measurements were performed by Miss B.J. Marlatt. The values for these determinations are presented here through the kind permission of Dr. M.C. Blanchaer, and have been discussed only when they have a bearing on the remainder of the thesis.

Lactic Acid Determinations

The lactic acid determinations were done by means of a modification(31) of the method of Barker and Summerson(32). The determinations had a precision of 1%. The error in the method amounted to about 2%.

Glucose Determinations

Glucose was determined by the method of Somogi(33) after treating the filtrates with Nelson's reagent(34) to remove the phosphorylated hexoses. This method was found to give replicate analyses with deviations no greater than 3% from the mean.

Pyridine Nucleotide Determinations

The pyridine nucleotides were analyzed by the fluorimetric procedure of Levitas et al(35). The values for the pyridine nucleotides were

expressed in terms of micrograms of diphosphopyridine nucleotide per gram of cellular hemoglobin. The method was found to be precise within 5%

PREPARATION OF BLOOD SAMPLES FOR STORAGE AND ANALYSES

Collection of Blood

In the past all workers in this field have compared glycolytic changes of blood from one donor in one type of preservative media to the changes observed in blood from a second donor in a different solution. These comparisons may or may not have been valid, but simplicity of preparation of the blood for storage was probably their obvious reason for doing so. In our experiments a blood sample was taken from one donor, and then subdivided into the different media under investigation.

Four salient features had to be kept in mind when designing such a system. These features were homogeneity of the samples, clot prevention, sterile technique and isotonicity. Direct withdrawl of blood from the donor's vein into successive bottles of media was excluded for three reasons: (a) such samples would not be homogeneous due to changes in the donor's hematocrit during the donation; (b) the blood might clot in the collecting tube while transferring the needle from one collecting vessel to the other; (c) maintainance of sterile conditions would be difficult.

The other alternative was to collect the sample of blood in one receptacle, mix thoroughly and then subdivide. Subdivision of the blood by means of a closed system was contemplated, but not used because of difficulties in the sterilization of valves and the lack of sample homogeneity in the final samples.

The open transfer technique for subdivision was the only alternative left. In essence, the blood was collected into a Kelley flask,

containing a modified ACD and fitted with a bell bottom drain, from which the mixture was allocated into flamed bottles of the media under test. By using this method the four obstacles were easily overcome.

Homogeneity. The blood was taken in one donation and mixed thoroughly in the anticoagulant before subdivision. Each bottle contained a homogeneous sample of cells and plasma.

Clot prevention. By collecting the blood in a modified ACD containing predominately sodium citrate it was possible to keep the plasma from clotting.

Sterility. Everything but the blood was autoclaved and kept under cover until used. As an added precaution, the storage bottle mouths and Kelley flask bell were flamed before and after each subdivision. The storage bottles were closed with sterile caps, over which a wad of alcohol soaked cotton was placed. To prevent evaporation this was covered with parafilm.

Tonicity. The modified ACD in the Kelley flask was isosmotic with the modified ACD in the storage bottles. When these two modified ACD's were mixed together in the right proportions the final concentration was the same as that reported by Loutit et al(4). Therefore the solutions exerted a relatively uniform osmotic pressure on the cells during collection and subdivision.

Storage Media Used

As previously mentioned the basic storage medium chosen for the experiments was the acid-citrate-dextrose (ACD) formula of Loutit

et al(4). Each set of experiments was comprised of a blood stored in ACD, a blood stored in ACD containing a phosphate buffer, and a nicotinamide containing ACD blood to which in two cases the phosphate buffer was also added. Three sets of these experiments were performed. 3A, 4A and 6A were the control bloods stored in ACD. 3B, 6B and 4B were samples in which 2.8, 8.2 and 14.4 mM/l respectively of phosphate buffer at pH 7.0 was added. Sample 3C contained 0.164 M nicotinamide and 2.8 mM phsophate buffer. Sample 4C contained 0.164 M nicotinamide and 14.4 mM phosphate buffer. Sample 6C contained only 0.004 M nicotinamide as the additive. Unfortunately Sample 3C suffered from technical difficulties and was analyzed only in the seventh to tenth weeks of storage. A key to these solutions may be found below. The abbreviations of the solutions and the experiment numbers are those used throughout the remainder of the thesis.

KEY TO THE SOLUTIONS USED FOR PRESERVATION

Expt. no.	Abbrev. used for solutions	Citric acid, sodium citrate and dextrose	Phosphate buffer at pH 7.0	Nicotinamide
3A	ACD	X	–	–
3B	ACD-P	X	2.8 mM/l	–
3C	ACD-PN	X	2.8 mM/l	0.164 M
4A	ACD	X	–	–
4B	ACD-P	X	14.4 mM/l	–
4C	ACD-PN	X	14.4 mM/l	0.164 M
6A	ACD	X	–	–
6B	ACD-P	X	8.2 mM/l	–
6C	ACD-N	X	–	0.004 M

A more elaborate table showing solution composition and osmolarity may be found in the appendix. Table 12 page 104.

Storage and Sampling

As soon as possible after collection and subdivision, the bottles of blood were placed in the refrigerator which was maintained between 3 and 6°C. The actual temperature variations were recorded with a thermograph and a typical thermogram is shown in Figure 4A below.

Just before sampling, the blood was mixed by rotation and inversion of the storage bottles. The parafilm and the underlying alcohol soaked cotton were then removed from the top of the amber rubber capped storage bottles. The cap was swabbed with 70% alcohol and a sterile needle vent was placed through the cap. The blood sample was removed with a sterile needle and syringe by passing the needle through the cap and withdrawing the plunger. The sample was placed in a capped centrifuge tube in an ice bath. The storage bottle cap was again swabbed and covered with the usual alcohol soaked cotton and parafilm, thence returned to the refrigerator.

FIGURE 4A
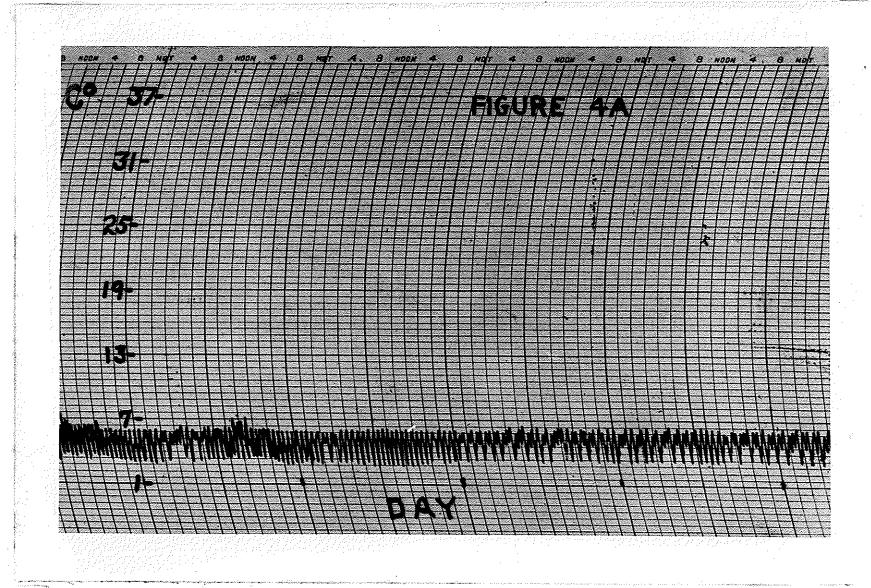
SECTION IV

RESULTS - PHYSICAL CHANGES

Among the changes in blood cells listed as physical
are those dealing with osmotic forces. Cell volume changes
and percentage hemolysis are discussed separately.

CELL VOLUME CHANGES

Initial changes. The osmotic pressure of plasma is
said to be 310 milli-osmoles per liter(5). The effective milli-
osmolarities of the solutions used in these experiments may be
seen in Table 5. Shown also in the same table is the ratio of
diluent to plasma, and the milli-osmolarities of the plasma-diluent
mixtures. Table 6 shows the milli-osmolarities after mixing,
the observed swelling, and the theoretical swelling(44) of the
cells on the basis that the cells behave as perfect osmometers.
It may be seen from Table 6 that the actual swelling of the blood
cells in each of the solutions was greater than the calculated
theoretical swelling. The relationship of the actual to the
theoretical swelling held approximately for those solutions
which were hypotonic or isotonic. When the hypertonicity was due
in part to ions which penetrate the red cell membrane such as
phosphate ions [*] as was the case in Expts 4B and 4C, one would
expect a new equalibrium to be set up in which the phosphate

[*] See Table 12 in the Appendix.

TABLE 5

OSMOLARITIES OF THE PRESERVATIVE MEDIA

BEFORE AND AFTER MIXING

Preservative media	Expt. no.	Effective milli-osmolarity	Ratio of diluent to plasma	Milli-osmolarity on mixing
ACD	3A	203.2	15.4 / 52.8	286
ACD	4A	203.2	22.2 / 55.3	279
ACD	6A	203.2	16.6 / 55.2	285
ACD-P	3B	256.3	15.9 / 55.8	298
ACD-P	4B	491.4	23.1 / 55.3	363
ACD-P	6B	313.1	17.7 / 55.2	311
ACD-PN	4C	491.4	24.3 / 55.3	365
ACD-N	6C	203.2	16.9 / 55.2	285

Milli-osmolarities of solutions = milli-molarity of solutions multiplied by their number of osmotically active particles.

$$\text{Amount of diluent} = 100 - \frac{\text{Hb. of mixed blood}}{\text{Hb. of whole blood}} \times 100$$

$$\text{Osmolarity on mixing} = \frac{\text{Plasma volume} \times 310 + \text{Diluent volume} \times \text{osmolarity}}{\text{Plasma volume} + \text{Diluent volume}}$$

TABLE 6   37.

PERCENTAGE OSMOLARITY ON MIXING, THEORETICAL

SWELLING AND ACTUAL SWELLING OF BLOODS

Expt. no.	Percent osmolarity on mixing	Theoretical percentage swelling	Observed swelling on mixing
4A	90.1	111.0	116.8
6C	91.9	108.8	111.1
6A	92.0	108.7	111.0
3A	92.2	108.5	110.2
3B	96.0	104.2	105.3
6B	100.2	99.8	103.6
4B	117.2	85.3	103.8
4C	117.9	84.8	105.0

Theoretical swelling = reciprocal of tonicity.

TABLE 7

pH[*] AFTER MIXING BLOOD IN THREE DILUENTS

Time after[**] mixing	ACD pH	ACD-P pH	ACD-PN pH
9 mins.	6.91	6.91	6.87
40 mins.	7.08	7.05	7.00
65 mins.	7.20	7.18	7.15
19 hrs.	7.25	7.18	7.11

[*] All pH measurements at room temperature.

[**] Samples left at 4°C. for 18 hours, otherwise bloods at room temperature.

distributed itself equally in the plasma and cell water. By
cooling the blood as rapidly as possible and spinning the hema-
tocrits in cold centrifuge cups every effort was made to hinder
the transfer of phosphate, the rate of transfer of which is very
slow at low temperatures(45). Assuming however that a new equili-
brium was set up, other factors than phosphate must be responsible
for the increase in cell size of these samples.

Cell volume changes with time. Figures 5, 6, and 7,
show the time vs. cell volume relationship of eight experiments.
As may be seen, the cell volume increases in these experiments
tended to follow the typical biological sigmoid curves. The fact
that the curves of each series tended to reach a common end point
would seem to indicate that the original differences between the
plasma and cellular osmolarities can be overcome. In each instance
the maximum cell volumes achieved were 10 to 13% higher than their
initial volumes just after mixing, and were in all cases less than
130% of their corresponding normal volume.

THE RELATIONSHIP BETWEEN CELL VOLUME

AND WATER CONTENT

As would be expected when cells swell, the increase in
volume is largely due to the intake of water. Figure 8 shows the

relationship of cell swelling to water content. The points on the graph represent blood stored up to four weeks in acid-citrate-dextrose. The cell water was determined by drying at 70°C. for twenty-four hours.

HEMOLYSIS IN STORED BLOOD

Three factors are thought to have an influence on spontaneous hemolysis in stored blood. It has been related to lysins(46) swelling(11) and cell metabolism. Guest(44) has shown that freshly drawn blood cells can expand to 160% of their original volume before lysis. Rapoport's data(5) on changes in cell width and thickness during storage in ACD show that there is a steady decrease in the maximum attainable volume during storage. Over a forty day period the maximum attainable volume decreased 10%, thus cells which previously were able to swell to 160% of their normal volume were then able to swell to only 145% of original, thus increasing the susceptibility of the cells to osmotic hemolysis.

Figure 9 shows the amount of hemolysis in the various media as a function of time. With the exception of Expt. 4C the amount of hemolysis in the samples within each series was approximately the same. With the same exception the degree of hemolysis was the same in all samples up to about day 15. For the next

two weeks of storage some divergence in the solutions were apparent.
Thereafter those of Expt. 3 showed more hemolysis with time than
those of Expt. 6. The undue amount of hemolysis in Expt. 4C is
attributable to the effects of large concentration of nicotinamide.
The main point of interest is that the additions of three levels of
phosphate buffer or a small amount of nicotinamide did not influence
the degree of hemolysis.

RESULTS - CHEMICAL

GLUCOSE AND LACTIC ACID RESULTS

Introduction

Glycolysis in red cells as in other tissues consists of
the catabolism of glucose to form lactic acid. The rate of glucose
consumption by red cells depends on many factors, among which are
pH, buffering capacity, and temperature. In these experiments the
only differences in metabolism expected in the various bottles
were those which would be caused by the addition of either phosphate
or nicotinamide or both to the preservative media. The rates of
glycolysis can be measured by determining either the glucose con-
sumed or the lactic acid produced.

The glucose values are less reliable than the lactic

acid values since measurements of small differences in large amounts of glucose are less accurate than comparatively large differences in small amounts of lactic acid. Besides representing the decrease in glucose the lactic acid values may also be augmented by the small amounts of 2,3-diphosphoglyceric acid catabolized. Furthermore it is possible, but not probable that the lactic acid may back up to produce pyruvic acid.

General Changes in Glucose and
Lactic Acid During Storage

The results of the lactic acid and glucose determinations of blood stored in the various media are shown in Figures 10 to 18. These plots are on two scales. The left hand scale is for glucose changes while the doubled right hand scale is indicative of the theoretical and actual lactic acid production. The theoretical lactic acid production was calculated by doubling the decreases in glucose for the corresponding intervals.

Near the end of the period of storage in Experiments 6A, 6B, and 6C there were consistent discrepancies between the theoretical and actual amounts of lactic acid produced. In all other experiments, however, the amount of glucose which disappeared from the various media was rather closely associated with the corresponding lactic acid increases.

The glycolytic rates in general were found to vary slightly from one experiment to another, but were observed to follow similar patterns. The glycolytic rates were quite constant for the first three to five weeks of storage, but thereafter they became slower.

The Influence of Phosphate Buffer
on Lactic Acid Production

In the presence of more than 2.8 mM/l of phosphate buffer, i.e. in Experiments 4B and 6B, the rates of lactic acid production were consistantly greater than their controls. Moreover, in Experiment 4B, where 14.4 mM/l of phosphate buffer was added, this rate difference was nearly twice as large as in Experiment 6B where only 8.2 mM/l of phosphate buffer was added. These differences may be due to the differences in pH caused by the phosphate buffer or may be a direct phosphate effect on glycolysis.

The Influence of Nicotinamide
on Lactic Acid Production

In the presence of small concentrations of nicotinamide (Expt.6C) there was little or no difference in the formation of lactic acid as compared to the control. In the presence of large amounts of both nicotinamide and phosphate buffer, (Expt.4C) lactic acid production was found to be at a very much slower rate than in its phosphate control.

THE EFFECT OF ADDED PHOSPHATE BUFFER ON THE

TOTAL ORGANIC ACID SOLUBLE PHOSPHATE

Introduction

Martland(25) has shown that organic phosphate accumulated
in glycolysis when the pH was above 7.3. The accumulation of phos-
phate esters during storage was shown by Denstedt(1) in McGill-II
solution, which was buffered at pH 7.4. It was thought, as outlined
on page 13, that the addition of phosphate buffer might delay ester
decomposition.

Results

When three levels of phosphate buffer at pH 7.0 were added
to blood, it was found that the organic acid soluble phosphate esters
were
in these media higher than in their corresponding controls. These
differences in the organic acid soluble phosphate between the phos-
phate containing bloods and their controls were larger with increased
amounts of phosphate buffer as may be seen in Figure 19. The buffer
kept the initial pH lower, as may be seen in Table 7, and it had a
temdency to check the rapid drop in pH during the first week of
storage recorded by Rapoport(5). This was not unexpected in view of
the increased buffering capacity in the phosphate medium as may be
seen from the titration curves shown in Figure 20.

Rapoport and Guest(47) have shown that in blood below pH 7.3 at 37°C. there was a decrease in all the organic acid soluble fractions with the largest breakdown occurring in the diphosphoglyceric acid fraction. The organic acid soluble phosphate differences noted above were found in all fractions with the most prominent differences noted in the diphosphoglyceric acid fraction, as may be seen in Table 8.

THE EFFECT OF NICOTINAMIDE ON THE TOTAL ORGANIC ACID SOLUBLE PHOSPHATE

Introduction

When nicotinamide was added to the preservative media the organic acid soluble phosphate was found to be lower than the controls for at least two weeks. Thereafter the values of the organic acid soluble phosphate in both cases had a tendency to approach their controls. This may be seen in Figure 19. From this graph it may be noticed that the higher the concentration of nicotinamide the lower the organic acid soluble phosphate value in comparison to the ACD bloods. This effect was noticed both in the presence of, (Expt.4C) and in the absence of (Expt.6C) phosphate, and can thus be attributed directly to the influence of nicotinamide.

TABLE 8   45.

DIFFERENCES IN PHOSPHATE FRACTIONS BETWEEN BLOOD OF

PHOSPHATE CONTAINING MEDIA AND THEIR A.C.D. CONTROLS

Expt. no. and day	Organic acid soluble phos-phate diff.	'ATP' fraction diff.	'HDP' fraction diff.	'2,3-DPGA fraction diff.
4. 0	-0.36	-1.91	+2.72	-1.17
3	+1.82	+0.45	+0.50	+0.87
10	+2.16	-1.09	-0.16	+3.41
16	+4.94	+0.85	-0.76	+4.85
23	+5.80	+0.82	+0.61	+4.41
31	+5.49	+1.87	-0.48	+3.76
6. 0	+0.05	+0.51	0.0	-0.46
3	+0.21	+0.33	-0.33	+0.21
7	+0.67	+0.81	-0.42	+0.28
9	+0.62	+0.14	+0.23	+0.25
13	+1.39	+0.40	+0.11	+0.88
16	+1.28	+0.22	+0.23	+0.85
23	+0.72	+0.18	+0.02	+0.52
35	+1.60	+0.75	+0.11	+0.76
50	+1.93	+0.35	-0.16	+1.73
70	+1.74	-0.08	+0.39	+1.43
3. 0	-0.90	-0.02	+0.53	-1.41
1	-0.59	+0.10	+0.16	+0.85
2	+0.69	-0.01	+0.21	+0.42
5	+0.02	-0.22	+0.49	-0.25
8	+0.34	+0.17	-0.16	+0.33
12	+0.78	+0.29	+0.29	+0.20
15	+0.65	-0.21	+0.57	+0.29
21	+0.64	+0.17	-0.06	+0.52
40	+0.74	+0.28	+0.33	+0.13
54	+0.23	-0.26	+0.42	+0.07
71	+0.91	---	---	---

ADENOSINE TRIPHOSPHATE AND HEXOSE DIPHOSPHATE

CHANGES IN STORED BLOOD

Introduction

Variations in the hexose phosphate fractions are usually closely allied with changes in adenosine phosphate. The Harden-Young effect, where excess hexose phosphate is formed by excess ATP has been shown in yeast extracts to be an indirect effect due to lack of ATP-ase(21). In red cells this may or may not occur. It is necessary first to consider the alternative schemes for the catabolism of 3-phosphoglyceraldehyde as outlined on page 10. If the scheme yielding 2 net moles of ATP per mole of glucose is considered, the excess ATP may either induce the Harden-Young effect or be dephosphorylated by ATP-ase activity. If the variations due to the presence of the Rapoport and Luebering enzymes are considered,[*] there could be no Harden-Young effect.

The diminution or lack of ATP would in all probability result in the slowing down or complete cessation of glycolysis. The lack of hexose phosphates could indicate two things; the cessation of glycolysis or more highly active aldolase than hexokinase activities.

[*] As the reader will recall, the role of the Rapoport and Luebering enzymes, 'diphosphoglyceric acid mutase' and 'glyceric diphosphatase', is the formation and dephosphorylation of 2,3-diphosphoglyceric acid without the transfer of phosphate to ADP.

Changes in Adenosine Triphosphate Fractions

in the Various Media

The changes observed in the blood 'ATP' fractions in the three types of preservative media are shown graphically in Figs. 21, 22 and 23.

ATP in ACD blood. The ATP fraction in ACD bloods remained somewhat constant for about the first two weeks of storage. Thereafter they dropped at a slow uniform rate for the remainder of the period of storage.

ATP in ACD-P bloods. The ATP fractions in the ACD-P bloods showed a tendency to be higher than their corresponding controls. In the presence of the larger quantities of phosphate buffer the ATP fraction remained higher for periods up to 5 weeks of storage.

ATP in ACD-N and ACD-PN bloods. The ATP fraction in the presence of a low concentration of nicotinamide (Expt.6C) decreased at a rate similar to its control for the first sixteen days of storage. Thereafter it decreased at a slightly more rapid rate. Where large amounts of nicotinamide and phosphate buffer were added (Expt.4C) the ATP fell rapidly and remained low for the duration of the experiment. The ATP was probably used in the formation of diphosphopyridine nucleotide (48) in accordance with the following reaction:

Adenosine triphosphate $+$ nicotinamide mononucleotide $\longleftrightarrow$

diphosphopyridine nucleotide $+$ inorganic pyrophosphate
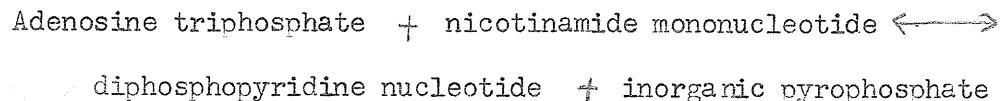
Changes in the Hexose Diphosphate fractions

in the Various Media

Changes in the hexose diphosphate fractions in the various media may be seen in Figures 24, 25 and 26.

HDP in ACD bloods. In three experiments the HDP fraction dropped at a rather slow uniform rate for at least 5 weeks of storage, then levelled off.

HDP in ACD-P bloods. The HDP fraction in ACD-P bloods were more erratic than in their controls. The tendency to level off after the fifth week was also apparent in these experiments.

HDP in ACD-N and ACD-PN bloods. In the presence of a low concentration of nicotinamide (Expt.6C) the HDP fraction fell at a slightly faster rate than the control. In the presence of large quantities of nicotinamide and phosphate buffer (Expt. 4C) the HDP fraction dropped rapidly within two weeks and remained low. The lack of HDP was probably due to the ATP lack.

2,3-DIPHOSPHOGLYCERIC ACID CHANGES

Introduction

Twenty-seven years ago Greenwald(29) isolated 2,3-DPGA from blood, and while the factors which influence its metabolism have been characterized, its presence in red cells in large quantities has never been fully explained. It has been postulated to act as a store of phosphate and as a buffer(47).

Rapoport and Guest(47) have shown that in an acid media one-half of the cellular 2,3-DPGA can be catabolized at 37°C. in four hours. Under otherwise similar conditions, a shift towards an alkaline pH produces an increase in the amount of 2,3-DPGA. Lennerstrand(49) has shown that this compound may be catabolized in hemolysates at pH 7.4 in the presence of large quantities of phosphate buffer. He also showed that its lability increased in the presence of adenylic acid and magnesium ions--conditions which also favor the catabolism of 3-PGA. Furthermore, he noted that this breakdown is virtually stopped by the presence of NaF.

(1) 2,3-Diphosphoglyceric acid $\longleftrightarrow$ 2 phosphate + glyceric acid

(2) 2,3-DPGA $\longleftrightarrow$ 3 Phosphoglyceric + phosphate

(3) 3-PGA $\longleftrightarrow$ 2 Phosphoglyceric

(4) 2-PGA $\xrightarrow[\text{by NaF}]{\text{inhibited}}$ Phospho-enol pyruvic acid

(5) PEPA $\xrightarrow[\text{ADP}\leftrightarrow\text{ATP}]{\text{Mg}^{++}}$ Pyruvic acid
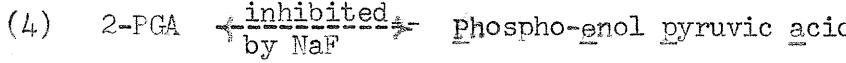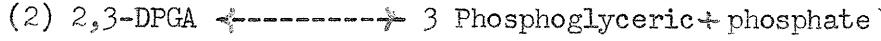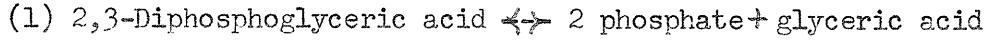
c.f.
Glycolysis
scheme page 9

Lennerstrand's evidence described above would indicate that 2,3-diphosphoglyceric acid was catabolized to pyruvic acid. However he also showed that the reaction was slowed greatly by the presence of iodoacetate, and that 2 moles of orthophosphate were released for each mole of DPGA catabolized. The 2 moles of phosphate are not released by means of reaction 1 above, since the 2,3-diphosphoglyceric acid changes were measured as decreases in the glyceric acid moiety by a modification of the method of Rapoport(50). However, it is possible to explain the phosphate increase on the basis of other known reactions. More probably the first mole of phosphate was released during reaction 2, while the second mole of phosphate appeared as a result of ATP-ase action(23) on the ATP formed at reaction 5. Thus, except for the iodoacetate inhibition, all Lennerstrand's evidence points to the catabolism of 2,3-diphosphoglyceric acid to pyruvic acid. In contrast to this, Schuchardt and Vercellone(36) reported that 2,3-DPGA was not metabolized to pyruvic acid. What happens to the 3-PGA formed from 2,3-DPGA is still open to question. The only explanation consistent with all the available evidence is that if pyruvic acid is formed from 2,3-DPGA it is rapidly reduced to lactic acid. However if this explanation is valid there must be an outside coupling system linked via DPN to lactic dehydrogenase that is specific for the metabolism of 2,3-diphosphoglyceric acid.

Recently Rapoport and Luebering(19,20) isolated two enzymes which are associated with the formation and breakdown of 2,3-DPGA. One enzyme, 'diphosphoglycerate mutase', was said to catalyze the transfer of the carboxyl phosphate of 1,3-DPGA to the secondary ester position forming the stable 2,3-DPGA. The second enzyme 'glycerate diphosphatase' is said to catalyze the breakdown of 2,3-DPGA to form 3-PGA and orthophosphate. Surprisingly enough, 3-PGA inhibits glycerate diphosphatase activity, but orthophosphate does not. On the other hand 2-PGA accelerates the reaction. The whole problem of 2,3-diphosphoglyceric acid catabolism has recently been more confused by the discovery that it acts as a coenzyme(51) for the conversion of 3-phosphoglyceric acid to 2-phosphoglyceric acid.

Storage Media Effect on 2,3-
Diphosphoglyceric Acid

The results of the changes in the 2,3-diphosphoglyceric acid fractions may be seen in Figures 27, 28, and 29. From these graphs it is evident that within each series, the 2,3-diphosphoglyceric acid decreased fastest in the nicotinamide bloods, not quite as fast in the control ACD-bloods, while it decreased the slowest in the phosphate containing bloods.

On comparing one series with another, it may be seen that the effect of phosphate in slowing the rate of decrease of 2,3-

diphosphoglyceric acid was cumulative. Thus the bloods in Experiments 3B, 6B, and 4B, which contained increasingly higher amounts of phosphate buffer, were found to contain proportionately higher amounts of 2,3-diphosphoglyceric acid than their controls. This effect was found throughout the duration of storage.

In converse to this, the effect of nicotinamide on promoting the catabolism of 2,3-diphosphoglyceric acid was found only for the first two and one half weeks of storage. But as in the case of phosphate, this effect was also cumulative. The higher the concentration of nicotinamide, the greater the degree of promotion of 2,3-diphosphoglyceric acid catabolism. The increase in the diphosphoglyceric acid fraction in 4C during the third week of storage was not fully understood. It may be attributable to effects on glycolysis of either the nicotinamide or the phosphate or even both.

Fate of 2,3-Diphosphoglyceric Acid

As may be seen from Figures 27, 28 and 29, the changes in the DPGA were associated with slightly larger than equivalent changes in the inorganic phosphate. Since DPGA contains two phosphate groups, its complete catabolism would result in two moles of phosphate released for each mole of DPGA broken down. Since the DPGA and orthophosphate curves shown in the figures are

essentially mirror images of one another, it may be expected that the DPGA was completely catabolized.

However, this is not necessarily the case since the difficulty hydrolyzable phosphate may also, in part, represent 3-PGA. But according to Lohmann and Meyerhof(52) and Meyerhof and Kiessling(53) the dephosphorylation of 3-PGA to pyruvic acid takes place at as high a rate, in the presence of acceptors, as that of its formation. Therefore it is unlikely that in the presence of phosphate acceptors there would be any 3-PGA. That 3-PGA does not normally accumulate in blood to any extent was also shown by the following experiment. No pyruvic acid was formed at 37°C. for $\frac{1}{2}$ hour in red cell hemolysate (1 part cells, 7 parts water) containing 4mM/l of $MgCl_2$, 3mM/l of AMP, and 4mM/l of iodoacetate. When 3-PGA was added, 75% of it appeared as pyruvic acid in less than 15 minutes.

Having fairly well established the absence of 3-PGA it is therefore logical to assume that the DPGA has been completely metabolized leading to the release of inorganic phosphate. If DPGA is catabolized in the manner shown in equations 2 to 5 (page 49) a quantity of pyruvic acid equivalent to the decrease in DPGA should have been formed.

PYRUVIC ACID RESULTS

Introduction

Pyruvic acid determinations were done throughout Experiments 4 and 6, while in Experiment 3 the analyses were performed only on days 40, 54 and 71. The pyruvic acid (PA) changes with time are recorded on Figures 30 and 31.

On inspection of these figures it may be seen that the trends in nearly all cases were the same. Usually there was a small rise in pyruvic acid for the first two weeks followed by a decline in the latter half of the first month of storage. Finally in all cases, a marked rise in the PA concentration may be observed starting around the end of the first month of storage and continuing for at least six weeks.

The initial phase (rise and decline) and the second phase (terminal rise in PA) will be discussed under separate sections.

The Initial Phase of Pyruvic Acid Changes During Storage

It may be seen in the following simplified scheme of glycolysis that three factors may influence the pyruvic acid changes in the initial phase.

```
                         GLUCOSE
                            ⇅
                    TRIOSE PHOSPHATES ⎞
                            ⇅         ⎟
2,3-DIPHOSPHO  →  3-PHOSPHOGLYCERIC ACID
GLYCERIC ACID               ⇅
                                        DPN-H₂    DPN ⟷ XH₂ ⟷ X
                         PYRUVIC ACID ⎞
                            ⇅         ⎟
                         LACTIC ACID  ⎠
```
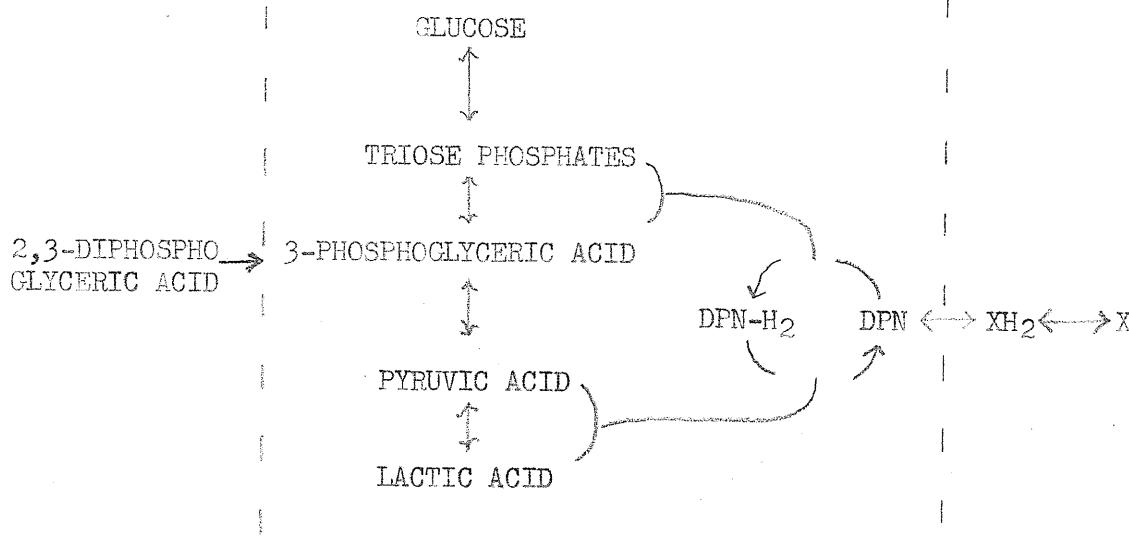
<u>Normal pyruvic acid changes</u>. Glycolysis ordinarily func-

tions as a closed system as indicated by the vertical lines of the

above scheme. Under these conditions the triose phosphates are

oxidized to 3-phosphoglyceric acid (3-PGA) by triose phosphate de-

hydrogenase (TPD). The 3-PGA formed is converted to pyruvic acid,

which in turn is reduced to lactic acid (LA) by lactic acid de-

hydrogenase activity (LDA). This, in turn, is coupled to TPD by

catalytic amounts of diphosphopyridine nucleotide (DPN). In the

closed system one would expect no increase in PA since the formation

of 3-PGA, its precursor, is limited, mole for mole by the amount of

oxidized DPN made available by the action of LDA on PA. Thus in the

closed system the amount of pyruvic acid should remain relatively

constant. However, there may be minor variations in PA in the closed

system which are limited to 0.04 mM/l, the usual concentration of

DPN present.

Changes in the pyruvic acid as related to the 2,3-diphos-
phoglyceric acid breakdown. The second factor influencing PA con-
centration is the side reaction involving the breakdown of 2,3-
DPGA (shown to the left of the closed system). It may be seen from
the scheme that its catabolism would result in an equimolar increase
in PA. However such was not the case since the PA changes of figures
30 and 31 are not mirror images of the 2,3-DPGA changes of Figures
28 and 29. But there was a distinct correlation between DPGA changes
and PA changes. Thus it may be seen in Table 9 that the decreases
in DPGA per day, for Experiments 4A, 4C, 6A, 6B, and 6C bear a close
relationship to the corresponding changes in the PA concentrations.
From Table 9 it may be seen that most large daily decreases of DPGA
over 0.056 mM/l/day were accompanied by increases in pyruvic acid
concentration. Decreases in DPGA less than 0.056 mM/l/day were
usually accompanied by decreases in pyruvic acid concentration. It
may also be noted that the pyruvic acid increases and larger DPGA
decreases both usually occurred within the first two weeks of storage.
Thus the early appearance of the transient peak in pyruvic acid appears
to be closely related to the rate of breakdown of DPGA. Once the
rapid rate of DPGA breakdown ceased the pyruvic acid concentration
decreased. In Experiment 4B where there was no transitory peak in

TABLE 9

THE RELATIONSHIP OF DAILY AVERAGE PYRUVIC ACID

CHANGES TO THE CORRESPONDING AVERAGE DAILY

DECREASES IN 2,3-DIPHOSPHOGLYCERIC ACID [x]

Expt. no.	Interval for which the rates were calculated (days)	Average daily decreases in 2,3-diphospho-glyceric acid mM/1/day	Corresponding pyruvic acid changes mM/1/day
4C	0-3	0.139	+0.156
6C	3-7	0.114	+0.050
6A	7-13	0.106	+0.033
6C	7-13	0.098	+0.037
6B	3-7	0.095	+0.038
6A	3-7	0.095	+0.031
6B	7-13	0.091	+0.022
4A	3-10	0.083	+0.013
4A	10-16	0.059	+0.003
6B	13-16	0.056	+0.007
6A	13-16	0.056	-0.007
4C	3-10	0.050	-0.024
6C	0-3	0.049	-0.008
4A	16-23	0.034	-0.030
6B	16-23	0.029	-0.013
6C	13-16	0.026	-0.017
6A	16-23	0.022	-0.021
6C	16-23	0.014	-0.023

[x] These values, listed in order of magnitude, were calculated as indicated on page 22.

pyruvic acid, it may be noted that the DPGA fell at a very slow rate, its maximum rate of fall being 0.051 mM/l.

The preservative solution therefore has an indirect effect in the initial phase of pyruvic acid formation. The heighth of the pyruvic acid peak appears to be dependent on the over-all rate of breakdown of DPGA. The more rapid the DPGA decrease, the larger the pyruvic acid temporary increase. In both sets of Experiments 4 and 6 we notice that in the transitory peaks of pyruvic acid $C \rangle A \rangle B$. In the corresponding rates and quantities of DPGA breakdown $C \rangle A \rangle B$.

Pyruvic acid conversion to lactic acid. Assuming that the pyruvic acid was converted to lactic acid there must be a third factor which influences pyruvic acid formation. This factor would theoretically provide hydrogen via DPN for the reduction of pyruvic acid. This source of hydrogen may be the original acceptor of hydrogen from DPN during the formation of the stores of DPGA. This source, depicted on the right hand side of the above scheme may be the rate limiting factor in the reduction of pyruvic acid thus leading to its temporary increase. Once the rate of this externally coupled reduction of pyruvic acid exceeded the rate at which pyruvic acid accumulated, the pyruvic acid concentration went down. The rate of fall of pyruvic acid from its transitory peak may give some indication of its activity. It was found that this rate of fall of pyruvic

acid from its transitory peak varied directly in proportion to the maximum transitory pyruvic acid peak. These results as shown in Table 10 would appear to indicate that the rate of decline of pyruvic acid from its transitory peak was independent of the media in which the cells were stored.

One exception to complete proportionality was Expt. 4A where the pyruvic acid peak was only 0.25 mM/l. An error of 0.01 mM/l in the pyruvic acid determinations could cause this discrepancy. In Expt. 4B no peak in pyruvic acid was noticed. In its place there was a small but steady increase in the pyruvic acid concentration. It could be then that the fall in pyruvic acid from the peak in the other samples was to a point achieved by a slow but steady increase in pyruvic acid. This would infer that the two processes were superimposed. A slow increase in pyruvic acid independent of DPGA and possibly due to an inefficient use of DPN-H_2 by LDA, and the secondary process resulting from DPGA catabolism. This would account for the large differences in the pyruvic acid concentration between the nicotinamide containing bloods and their controls.

The Terminal Rise in Pyruvic acid

Introduction. Shown in Figures 30 and 31, the pyruvic acid

TABLE 10

THE RELATIONSHIP BETWEEN THE TRANSITORY
PEAK CONCENTRATION OF PYRUVIC ACID
AND THE RATE OF DECLINE THEREFROM

Preservation medium	Expt. no.	Pyruvic acid peak. mM/1 (X)	Rate of pyruvic acid decline. mM/1/day (Y)	$\frac{(X)}{(Y)}$
ACD-PN	4C	0.71	0.0271	26.2
ACD-N	6C	0.56	0.0210	26.7
ACD	6A	0.43	0.0170	25.2
ACD-P	6B	0.39	0.0142	27.5
ACD	4A	0.25	0.0055	46.4

TABLE 11

PHOSPHATE CHANGES ASSOCIATED WITH THE TERMINAL
RISE IN PYRUVIC ACID

Expt. no.	Interval days.	Organic phosphate decrease mM/1	Pyruvic acid increase mM/1	'3-Phosphoglyceric acid' at start of time interval mM/1 [*]
3A	40-71	0.55	0.89	0.36
3B	40-71	0.50	≑ 0.9	0.38
3C	40-71	0.14	1.28	0.36
6A	35-70	0.67	0.95	0.51
6B	35-70	0.63	0.80	0.72
6C	35-70	0.63	1.36	0.58

[*] This value was calculated for the purpose of argument, on the basis that the difficultly hydrolyzable phosphate was a mono-phospho-glyceric acid.

concentration commences to increase around the end of the first month of storage. The rate of increase accelerates for the next six weeks. It was thought that this pyruvic acid might come from any of three sources. It might have come from 3-phosphoglyceric acid left over from the incomplete catabolism of 2,3-diphosphoglyceric acid, it may have accumulated as a result of cintinued externally coupled triose activity, or it may originate from lactic acid backing up. These possibilities are discussed below.

Formation of pyruvic acid via 3-phosphoglyceric acid. As discussed in a previous section (page 53), the 2,3-diphosphoglyceric acid could theoretically be catabolized to 3-phosphoglyceric acid or some phosphorylated intermediate and remain there, later to be converted to pyruvic acid. However if such were the case there must have been an amount of difficultly hydrolyzable phosphate, e.g. 3-phosphoglyceric acid or 2-phosphoglyceric acid, available at days 35 or 40, equal in molarity to the net terminal rise in pyruvic acid. Furthermore in the interval in which the pyruvic acid increased, there should have been a release of inorganic phosphate, equal in molarity to the pyruvic acid increase. The data in connection with this theoretical conversion is shown in Table 11. Thus it may be seen that with the exception of Expt. 6B it might have been possible for at most 50% of the pyruvic acid increases to arise from this source. Since this is obviously not the source of the pyruvic acid terminal rise it follows that 3-phosphoglyceric acid does not accumulate in the red cell.

Formation of pyruvic acid from glucose. From the glycolytic
scheme on page 9 , it may be seen that glucose can be metabolized to
pyruvic acid if oxidized diphosphopyridine nucleotide is continually
being supplied for the triose phosphate dehydrogenase step. The
accumulation of pyruvic acid may, if accompanied by a decrease in
glucose, then have been a result of an impairment of the coupled
lactic-triose dehydrogenase system. This may have come about as a
result of an outside system taking precedence over lactic dehydrogenase
for the reduced DPN available. The system whereby methemoglobin is
reduced by DPN-H$_2$ might be able to cause the increases in pyruvic
acid.[*] Another possibility is based on the degree of dissociation of
the apoenzyme-coenzyme complexes if the coenzyme level goes down.
The triose dehydrogenase has its DPN bound quite firmly and might
function more efficiently than the lactic acid dehydrogenase where
the DPN is not bound as firmly(22).

Formation of pyruvic acid from lactic acid. The terminal
rise in pyruvic acid increase may also be the result of the backing
up of the lactic acid to form pyruvic acid and reduced DPN. The
only logic for this reaction taking place would be that another
system such as MHb reduction had a more negative redox potential
than the pyruvic acid to lactic acid system.

[*] Gibson(58) has shown that MHb can be reduced to Hb by DPN-H$_2$
and that the reaction can be coupled either with triose reduction or
lactic acid oxidation. Calculations based on his data, assuming simi-
lar Q_{10}'s of activity, indicate that as high as 2 mM/l of pyruvic
acid/day or storage might accumulate by this process.

PYRIDINE NUCLEOTIDE CHANGES

Introduction

As mentioned earlier, Leder et al(2) have shown that red cells in vitro can synthesize pyridine nucleotides from nicotinamide. They were able to show a ten-fold increase in the pyridine nucleotides at 37°C. in 20 hours by suspending washed cells in a phosphate medium containing 2 gm.% nicotinamide. More recently, these workers(54) showed that this synthesis could also take place with smaller amounts of nicotinamide than originally used but that the amount of pyridine nucleotides formed was less per unit time. They also characterized 10 to 25% of the pyridine nucleotide increase as diphosphopyridine nucleotide while the remainder was found to be nicotinamide mononucleotide, which had been shown by Kornberg(48) to be a precursor of diphosphopyridine nucleotide.

Since no specific method for the routine determination of DPN was available, the pyridine nucleotide method of Levitas(35) was used. This fluorimetric procedure measures the following quaternary nitrogen compounds:

    N-methyl nicotinamide
    nicotinamide riboside
    nicotinamide mononucleotide
    diphosphopyridine nucleotide
    triphosphopyridine nucleotide.

In view of the characterization of the pyridine nucleotide changes in red cells the results reported below are probably indicative of

changes in the diphosphopyridine nucleotide and nicotinamide mono-nucleotide fractions.

Pyridine Nucleotide Changes in ACD
and ACD-P Bloods

The results of the changes in the cellular pyridine nucleotide content of the red cells during storage are shown in Figures 32 and 33. The pyridine nucleotides are expressed in micrograms per gram of cellular hemoglobin, thus avoiding apparent differences due to cell volume changes.

The changes in the cellular pyridine nucleotides in the controls were not consistent from one experiment to another. In Experiments 3 and 4, the cellular pyridine nucleotide content remained fairly constant for the first two or three weeks of storage. Thereafter in these experiments the pyridine nucleotides decreased to about 70% of their original content. In Experiment 6, the control levels of cellular pyridine nucleotides remained quite constant throughout the period of storage.

Pyridine Nucleotide Changes in the
Presence of Nicotinamide

The pyridine nucleotide changes in blood stored in nicotinamide containing preservative media are also shown in Figures 32 and 33.

In Expt. 4C, where 0.164 molar nicotinamide was added, the pyridine nucleotide content rose rapidly but irregularly during the first few days. The highest point reached was double that in the control. In Expt. 6C, with only 0.004 molar nicotinamide present, the pyridine nucleotide content was found to increase at a much slower rate, but the net increase was similar to that in 4C. Thus one-fortieth of the concentration of nicotinamide achieved the same net result but took longer to do it.

The effect desired from the addition of nicotinamide was the maintainance of the diphosphopyridine nucleotide content at a high level. If we assume that the pyridine nucleotide changes in the cold are similar to those at 37°C. then between 10 and 25% of the increases would represent diphosphopyridine nucleotide. However the rest of the increase which was presumably nicotinamide mononucleotide (NMN), might also aid in the maintainance of high diphosphopyridine nucleotide (DPN) levels. Because of the equilibrium between nicotinamide mononucleotide and diphosphopyridine nucleotide in the reaction NMN + ATP $\longleftrightarrow$ DPN + P-P, and increase in NMN from outside of this reaction might help to keep the DPN high.

SECTION V

A DISCUSSION OF THE RESULTS

Generally speaking the glucose that disappeared from the preservative medium was accounted for by the appearance of equivalent amounts of lactic acid. The glucose changes were relatively large, and it appears that it, as in most other tissues, is the main source of energy for the red cells. The lactic acid thus produced from the glucose was not metabolized further. The results of the analyses of the other components in glycolysis give some idea as to their connection to the glycolytic scheme in general, and how they have a bearing on the rates of glycolysis.

The red cells differ in their glycolysis from most other tissues in that they build up stores of 2,3-diphosphoglyceric acid. This stored 2,3-diphosphoglyceric acid, when broken down during storage was found to yield two things: (a) an equimolar amount of inorganic phosphate, and (b) a less than equimolar amount of pyruvic acid which soon after disappeared.

The adenosine triphosphate levels in blood during storage were found to start dropping before the rates of lactic acid production slowed down.

As measured in these experiments, the diphosphopyridine nucleotide content of blood during storage remained fairly constant and therefore does not appear to be a limiting factor in red cell glycolysis.

The terminal rise in pyruvic acid might be an indication of glycolytic malfunctioning on the part of the lactic dehydrogenase apoenzyme. However, it does not necessarily indicate this because in all cases in which there was a terminal increase in pyruvic acid, this increase was small in comparison to the amount of lactic acid being produced in the same interval. It is more likely to indicate that other reduction systems are competing for the reduced diphospho-pyridine nucleotide available.

The changes in the components of glycolysis per se in blood during storage in acid-citrate-dextrose are very important to know. However, when glycolysis in blood during storage was altered by the addition of other compounds to the basic preservative media, a much better insight was gained into the factors which appear to regulate glycolysis during storage.

A Discussion of the Changes in Glycolysis
Due to the Presence of Phosphate Buffer or
Nicotinamide in the Preservative Media

The ATP fractions were kept higher during storage by the presence of phosphate buffer in the media. It may also be noted in these cases that the rates of lactic acid production were also higher. Furthermore, the 2,3-diphosphoglyceric acid (DPGA) broke down at a slower rate. This may indicate that this store of DPGA

is a stand-by source of energy that is not usually used except when
glycolysis is slow. In contrast to this belief the glycolytic rate
in the first portion of storage in the presence of small amounts
of nicotinamide was the same as the control, while the DPGA fell at
a faster rate. This would partially seem to counteract the theory
that it is a stand-by source of energy in the cell.

Furthermore, the pyruvic acid transient peak in the
first month of storage in phosphate containing bloods was found to
be less than the controls or not to occur at all. This might indicate
that the phosphate has a protective influence on the lactic de-
hydrogenase apoenzyme or that the rate of pyruvic acid reduction by
an externally coupled source of DPN-H_2 was able to keep nearly abreast
with, or ahead of, the pyruvic acid rate of formation from DPGA.
If phosphate has a protective influence on lactic dehydrogenase
activity then it showed this protection only in the first month of
storage.

On the other hand the presence of nicotinamide induces
a much higher rate of decrease of DPGA. The pyruvic acid peaks in
these cases were found to be much higher than their controls. Simi-
larly, this might indicate that nicotinamide has an inhibitory effect
on LDA, or that the externally coupled pyruvic acid reduction is
not able to keep abreast with the pyruvic acid formed from DPGA

However the rates of disappearance of pyruvic acid from
the transient peak were found to be independent of the medium in

which the cells were stored. Thus the alternative explanation that the peak was dependent on the rates of fall of DPGA appears to be the more logical answer to the problem.

The ATP fractions are decreased in the presence of large amounts of nicotinamide. This may be the cause of the decreased rate of glycolysis in this sample, and it may be the result of the ATP being used to form DPN, but is not the result of the increased breakdown of DPGA, since it is believed to have transferred its high energy phosphate to ATP to be used in the early large formation of DPN.

In the presence of small amounts of nicotinamide the ATP fraction decreased at a rate faster than its control after 16 days of storage. In the first 16 days the high energy phosphate for the formation of DPN probably came via ATP from the DPGA which broke down rapidly in the first two weeks of storage in this medium. Thereafter the high energy phosphate for DPN formation probably came from the ATP normally used for hexokinase and phosphohexo-kinase activity.

This would account for the decrease in the hexose diphos-phate fractions, noted early in storage in the case where large amounts of nicotinamide were present, and noted after the sixteenth day of storage in the case where only a small amount of nicotinamide was present.

In the presence of large amounts of nicotinamide, glycolysis is slowed greatly. The DPN was apparently there, but the ATP was not. If one adheres to the stand-by theory of the role of DPGA then its fast breakdown may have been to provide useful energy for the cell. Despite the apparent lack of ATP for hexokinase and phosphohexokinase activities in this experiment, the glucose seemed to disappear after day 16 of storage. Furthermore, the hexose phosphates which were measurably low, appeared to be going through to form the increase in DPGA noted in the latter part of this experiment. Therefore it might seem that triose dehydrogenase was still functioning, but was coupled to an external reducing system and so producing 2,3-diphosphoglyceric acid.

In the presence of small amounts of nicotinamide, however, the rate of lactic acid production was slowed to a slight extent and only after 5 weeks of storage. This is consistant with the finding of lower levels of ATP during the latter half of storage, and a further indication that ATP and glycolysis are quite interdependent.

Thus the beneficial effects achieved by the addition of phosphate are completely nullified by the addition of nicotinamide.

SECTION VI

SUMMARY

INTRODUCTION

This study was undertaken to re-open the problem of
what happens to the cells during storage under the present methods
of preservation. The object of this work was three-fold.

(A) To find out what happens in glycolysis during
storage of cells in the prevalently used acid-citrate-dextrose
(ACD) preservative medium.

(B) To determine if any of the changes taking place in
the measurable components of glycolysis during storage can be
beneficially modified by the addition of other substances to the
ACD medium.

(C) To find the labile factors in glycolysis, if any,
which might render blood unsatisfactory for transfusion after
storage for three weeks.

The blood donations were taken and subdivided into 3
bottles. Two of these bottles contained, in addition to ACD, a
phosphate buffer, or a nicotinamide solution, or both. Three differ-
ent levels of phosphate buffer were used for three different ex-

periments. These were 2.8 mM/l, 8.2 mM/l, and 14.4 mM/l of sodium
phosphate buffer all at pH 7.0. Two levels of nicotinamide were
used in three different experiments. In one experiment a final con-
centration of 0.004 molar nicotinamide was added to the preserva-
tive medium. In another experiment 0.164 molar nicotinamide and
14.4 mM phosphate buffer were added to the preservative medium.
In still another experiment 0.164 M nicotinamide and 2.8 mM phosphate
buffer were added. Thus in each series there was an ACD control,
a phosphate containing ACD experimental solution, and one contain-
ing nicotinamide, as well as ACD.

Phosphate was chosen as an additive with the object of
helping to maintain organic phosphate esters, particularly the
ATP fraction, at a high level. Phosphate was chosen partly because
of its inhibitory effect on phosphatases such as ATP-ase. It was
also used in an attempt to slow the rate of breakdown of 2,3-di-
phosphoglyceric acid which comprises over half of the organic acid
soluble phosphate of red cells. If its rate of decrease could be
slowed, the potential high energy phosphate in 2,3-diphosphoglyceric
acid might be kept available to be passed onto phosphate acceptors
at a time when the ATP would otherwise be down. The three levels of
phosphate were chosen to see if differences in the degree of change
of the glycolytic components could be effected.

The second additive chosen was nicotinamide. In red cells
it had been shown that the coenzyme diphosphopyridine nucleotide
could be synthesized from nicotinamide via nicotinamide mononucleotide.

The bloods were stored in a refrigerator between 3 and $6^{\circ}C$.
for periods of up to 10 weeks. Samples were removed aseptically from
time to time and analyzed for the following:

Inorganic phosphate

Adenosine triphosphate

Hexose diphosphate

2,3-Diphosphoglyceric acid

Pyruvic acid

Glucose

Lactic acid

Pyridine nucleotides

Hemoglobin content

Supernatant hemolysis

Hematocrit and cell volumes

THE SUMMARY OF THE RESULTS OF THE CHANGES DURING
STORAGE OF CERTAIN CHEMICAL AND PHYSICAL CHARACT-
ERISTICS OF BLOOD PRESERVED IN THE VARIOUS MEDIA
UNDER INVESTIGATION

The changes in the chemical and physical characteristics
of cells stored in acid-citrate-dextrose are enumerated below. An
enumeration is also given of the general changes in the glycolytic
picture during storage which were found to be common to all the
preservative solutions under investigation. To avoid all unnecessary
repetition, only major metabolic differences, directly attributable
to the ACD solution additives, were listed under separate headings.

Typical Changes in Certain Physical and Chemical
Characteristics of Blood Cells During Storage in
the Acid-Citrate-Dextrose Medium

(1) When first placed in the ACD medium the cells swelled
to between 110 and 115 per cent of their normal size. During storage
the cells swelled even further to attain volumes between 125 and 128
per cent of their normal size.

(2) The cells hemolyzed at a very slow rate for the first
four to five weeks of storage. Hemolysis at this time being limited
to a maximum of one per cent. Thereafter the cells began to hemolyze
at a much faster rate, with a minimum hemolysis of six per cent at

the end of ten weeks of storage.

(3) The glycolytic rates of the blood cells, as measured by the amount of lactic acid produced, varied somewhat but remained fairly constant for the first 20 to 35 days of storage. Thereafter the rates fell off as the length of storage increased.

(4) The adenosine triphosphate fraction remained constant for the first two weeks of storage, then dropped slowly but steadily throughout the duration of storage to about 30% of its original at the end of ten weeks.

(5) The hexose diphosphate fraction dropped rather steadily to 50% of its original for the first five weeks of storage, then had a tendency to level off.

(6) The 2,3-diphosphoglyceric acid fell rapidly in a typical sigmoid fashion, the fastest rates of decrease being in the periods between days 2 and 12. The majority of the 2,3-diphosphoglyceric acid was gone by days 15 to 20.

(7) The inorganic phosphate rose rapidly in an amount equimolar to the simultaneous decrease in the 2,3-diphosphoglyceric acid. The ATP and HDP fractions contributed slightly to the inorganic phosphate levels near the end of the experiments.

(8) The pyruvic acid rose to a temporary peak about the end of the first two weeks of storage. For the next two weeks the pyruvic acid concentration decreased. Thereafter it rose rapidly for the next six weeks.

(9) The pyridine nucleotides varied somewhat from sample to sample. In one case the level dropped to 70% of its original in 71 days while in the other case the levels remained the same throughout storage.

Thus when blood was stored in an acid-citrate-dextrose medium the glycolytic rates remained constant for the first 20 to 35 days, then the rates slowed down. The 2,3-diphosphoglyceric acid fell rapidly for the first two weeks of storage and the ATP fraction remained high for the same time interval. The pyruvic acid rose and fell during the first month and then increased rather rapidly during the remainder of the storage period. The pyridine nucleotides either remained constant throughout storage or dropped 30% in ten weeks.

General Changes in Certain Chemical and Physical Characteristics that were Found to be Common to All the Bloods During Storage in All the Preservative Media Under Investigation

(1) The amount of swelling that the cells initially undergo when placed in the various preservative media was found to agree rather closely with the osmolarity of the solutions after mixing with the plasma. In no event did shrinkage occur when the preservative media was made hypertonic due to the inclusion of the phosphate buffer, or the nicotinamide.

(2) The amount of swelling that the cells underwent during storage varied slightly but in no case did it exceed 20% of the swelling of the cells when they were initially mixed. Oddly enough, the largest degree of swelling during storage occurred in the bloods stored in the hypertonic media.

(3) The amount of hemolysis of the cells stored in the various media under investigation was, with one exception, no more than 1% during the first month of storage.

(4) The decrease in the glucose content of the media during storage was found to be approximately equivalent to the amount of lactic acid produced.

(5) The increase in the inorganic phosphate during storage was found to be a mirror image of the decrease in 2,3-diphosphoglyceric acid up to a point where the other esters started to decompose.

(6) The pyruvic acid, during storage, increased in concentration for the first two weeks, then decreased for the next two weeks. The pyruvic acid concentration then increased rather rapidly for the next six weeks. There was one exception to this general pattern in which just the transient peak did not occur.

(7) The magnitude of the pyruvic acid transient increase during the first two weeks of storage was found to be dependent on the magnitude of the over-all rates of decrease of 2,3-diphosphoglyceric acid. Thus in the exception where there was no transient increase in pyruvic acid, the 2,3-diphosphoglyceric acid decrease was very slow.

(8) The molar transient increase in pyruvic acid where
it occurred was found to be much less than the decrease in 2,3-di-
phosphoglyceric acid. One exception to this rule occurred in which
the temporary increase in pyruvic acid was equivalent to the decrease
in 2,3-diphosphoglyceric acid.

(9) The decrease in pyruvic acid concentration during the
second two weeks of storage was found to be a function of the
height of the peak achieved during the first two weeks of storage.

(10) The terminal rise in pyruvic acid during the latter
six weeks of storage was found not to come as a result of the de-
creases in phosphoglyceric acid or its intermediary metabolites.

Cumulative Changes in Certain Physical and Chemical Characteristics of Blood During Storage in Media Containing Increasingly Higher Levels of Phosphate Buffer as Compared to the Corresponding Changes in Their Controls

(1) There were no distinctly discernable changes in the
physical characteristics of blood during storage in phosphate con-
taining media.

(2) The organic acid soluble phosphate esters in bloods
remained higher during storage in phosphate containing media in
comparison to the controls, and proportionately higher when higher
levels of phosphate buffer were added to the media.

(3) The rate of fall of 2,3-diphosphoglyceric during storage was slower in comparison to the controls when increasing amounts of phosphate buffer were added to the preservative media.

(4) The glycolytic rates were proportionately slightly higher during storage in comparison to the controls, when increasingly higher amounts of phosphate buffer were added, except where the medium was supplemented with only 2.8 mM/l of buffer.

(5) The ATP fraction showed a tendency to be higher during storage and remain that way for longer periods of time, except where only 2.8 mM/l of buffer was added.

(6) The transient pyruvic acid peak during the first two weeks of storage was less than the control when 8.2 mM/l of phosphate buffer was added. When the largest amount of buffer (14.4 mM/l) was added, no peak in the pyruvic acid was discernable.

Changes in Certain Physical and Chemical Characteristics
of Blood During Storage in the Presence of Large Amounts
of Nicotinamide and Phosphate Buffer as Compared to the
Corresponding Changes in the Control

(1) The swelling of the cells during storage was about the same as its control but the per cent hemolysis was very much greater.

(2) The glycolytic rate was found to be only about 25%

of that of the control and furthermore the lactic acid production practically ceased after 16 days of storage. It appeared, however, that some glucose was being utilized after the sixteenth day of storage.

(3) The transient peak and terminal rise in the pyruvic acid concentration was found to be much higher than the controls.

(4) All the organic phosphate ester fractions were much lower than the controls. The 2,3-diphosphoglyceric acid decrease was much more rapid than the control but there was an increase in this fraction from the sixteenth to the thirty-first day of storage.

(5) The pyridine nucleotide level was found to be double that of the control within two weeks of storage.

Changes in Certain Physical and Chemical Characteristics of Blood During Storage in the Presence of Small Amounts of Nicotinamide as Compared to the Corresponding Changes in the Control

(1) There were no consistant differences between the physical changes noted in this experiment and those noted in the control.

(2) The 2,3-diphosphoglyceric acid fell during storage at a slightly faster rate than its control.

(3) The adenosine triphosphate fraction remained the same as the control for the first sixteen days of storage, thereafter it was consistantly lower.

(4) The hexose diphosphate fraction was consistantly slightly lower for the first two weeks of storage, while thereafter it was consistantly much lower than the control.

(5) The temporary peak and terminal rise in pyruvic acid during storage were greater than the controls.

(6) There appeared to be two rates of increase in the pyridine nucleotides. The first rate was quite fast and lasted until day 16. The second slower rate started at that time and continued for the duration of storage.

SECTION VII

CONCLUSIONS

(1) When blood is stored in an acid-citrate-dextrose
medium, the glycolytic rates remain constant for the first twenty to
thirty-five days. From then till the end of the tenth week of
storage the glycolytic rate is slower. The stored 2,3-diphospho-
glyceric falls rapidly for the first two weeks of storage and the
adenosine triphosphate remains high for the same interval. The
pyruvic acid rises to a temporary peak in the first two weeks of
storage then declines for the next two weeks of storage. During
the last six weeks of storage the pyruvic acid increases again.
The pyridine nucleotides either remain constant throughout storage or
decline to 30% of their original level in ten weeks.

(2) When an inorganic phosphate buffer at pH 7.0 is added
to the basic ACD medium, it maintains the blood adenosine triph-
osphate during storage at higher levels for longer periods of
time than when it is not added. This increased adenosine triphos-
phate level permits a slightly faster glycolytic rate during storage.

(3) When a small amount of nicotinamide is added to the
basic ACD medium, it induces a higher blood pyridine nucleotide
level during storage. The adenosine triphosphate in this event
is used to synthesize the diphosphopyridine nucleotide formed. The

glycolytic rate in the latter half of storage is thus slightly
slowed, not by the excess DPN, but by the slight shortage of adenosine
triphosphate.

(4) When large amounts of nicotinamide and phosphate buffer
at pH 7.0 are added to blood in ACD, a rapid increase in the cellular
pyridine nucleotides is induced during storage. The adenosine
triphosphate is rapidly decreased during this pyridine nucleotide
formation. The glycolytic rate under these conditions is decreased
markedly, again not by the excess DPN, but by the marked lack of
adenosine triphosphate.

(5) The temporary increase in pyruvic acid in the first
two weeks of storage is a result of the rapid breakdown of 2,3-
diphosphoglyceric acid. This pyruvic acid, which disappears during
the third and fourth week, may be reduced to lactic acid by an
externally coupled reducing mechanism supplying reduced coenzyme.

SECTION VIII

FIGURES

Figure 1.   The relationship of the density of the colour developed in the phosphate method to the lime after addition of the reducing agent.

Figure 2.   The relationship of the density of the colour produced to the amount of phosphate present in the sample tested.

Figure 3.   The relationship of the density of the colour produced to the amount of sodium pyruvate present in the sample tested.

Figure 4.   The relationship between the absorption ratio at 260 mµ and 280 mµ to the amount of reduced alkaline soluble protein present in the sample tested.

Figure 1.   The relationship of the density of the colour
            developed in the phosphate method to the time after
            addition of the reducing agent.

Figure 2.   The relationship of the density of the colour
            produced to the amount of phosphorus present in
            the sample tested.

Figure 3.   The relationship of the density of the colour
            produced to the amount of sodium pyruvate present
            in the sample tested.

Figure 4.   The relationship between the transmission
            ratio at 560 mu and 580 mu to the amount of re-
            duced alkaline hematin present in the sample tested.

FIGURE 1

FIGURE 2
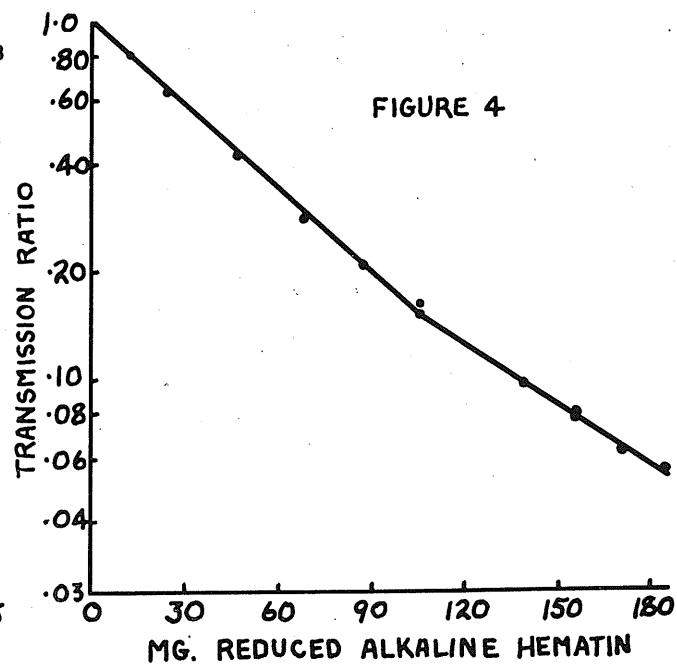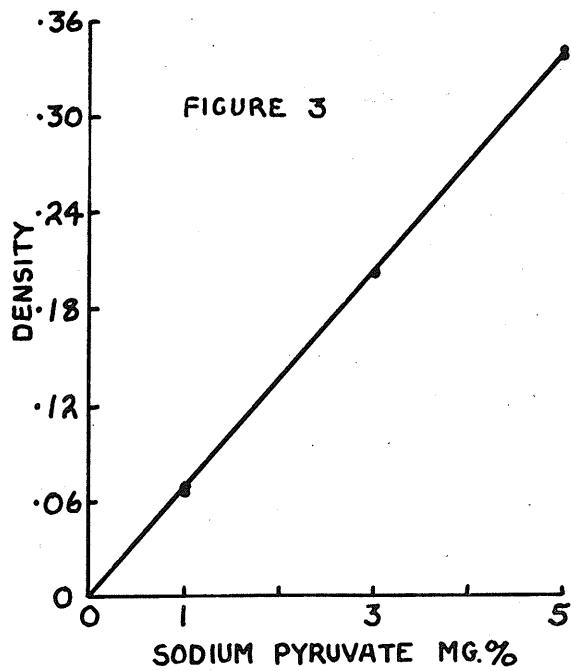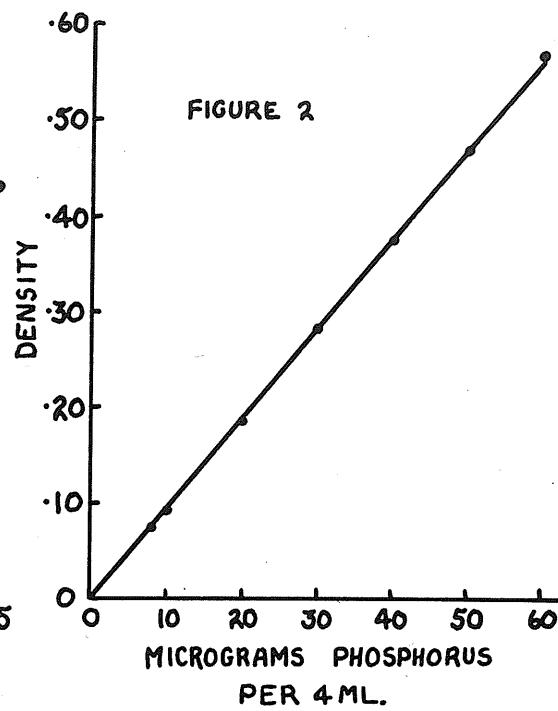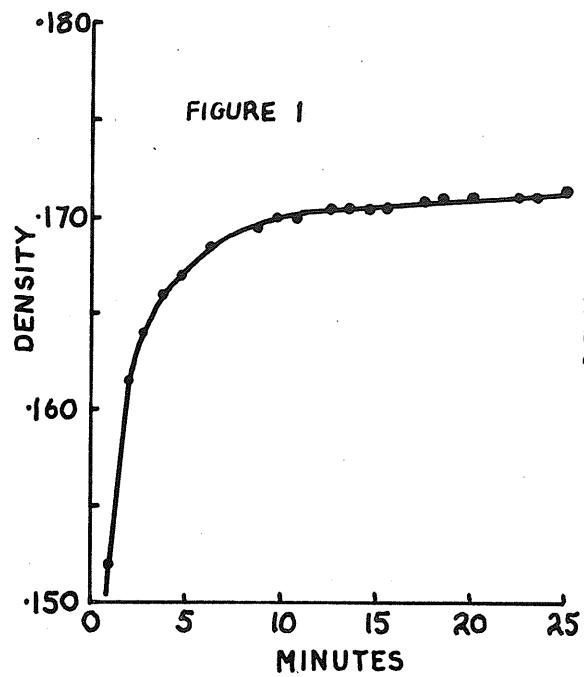
FIGURE 3

FIGURE 4

Figure 5.   The relationship of the per cent swelling

of blood cells in Experiments 3A[*] and 3B to

the length of time of storage.

Figure 6.   The relationship of the per cent swelling

of blood cells in Experiments 4A, 4B and 4C,

to the length of time of storage.

Figure 7.   The relationship of the per cent swelling

of blood cells in Experiments 6A, 6B and 6C,

to the length of time of storage.

[*]   The key to the contents of the preservative solutions
used in these and subsequent experiments may be found
on page 33.

FIGURE 5
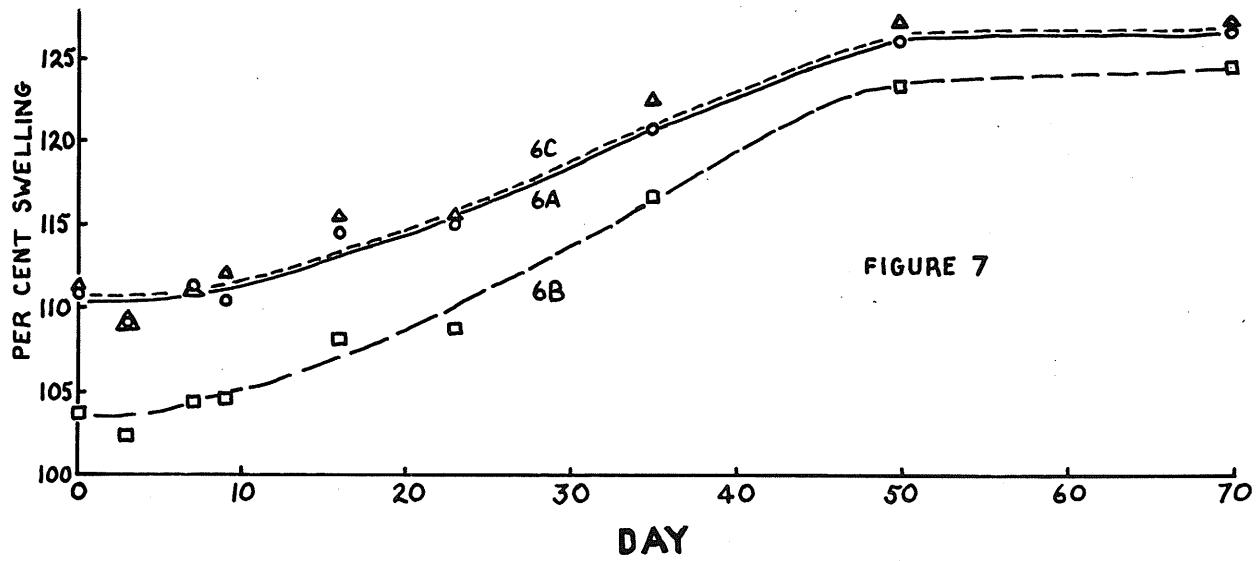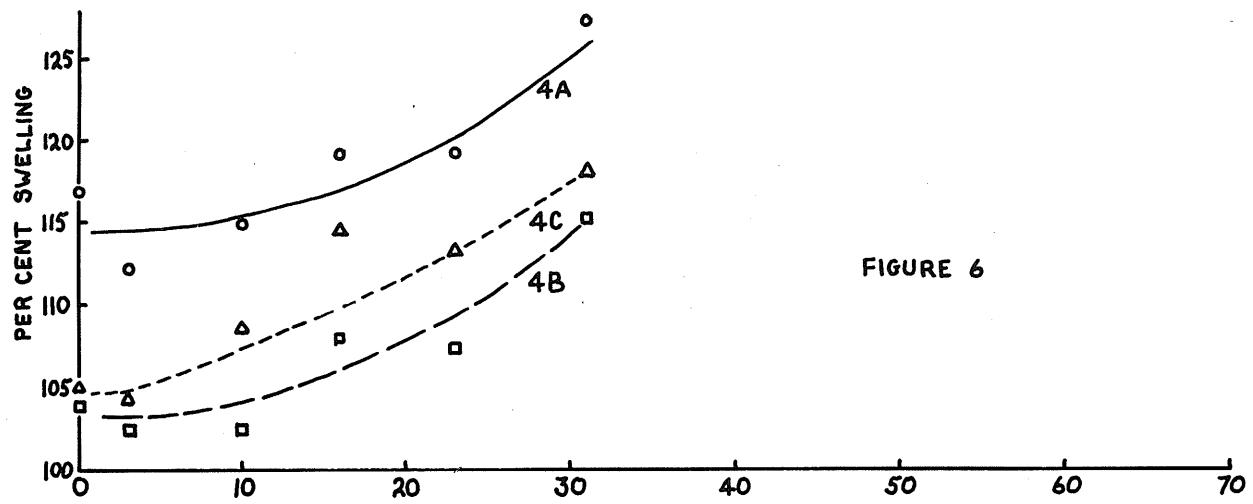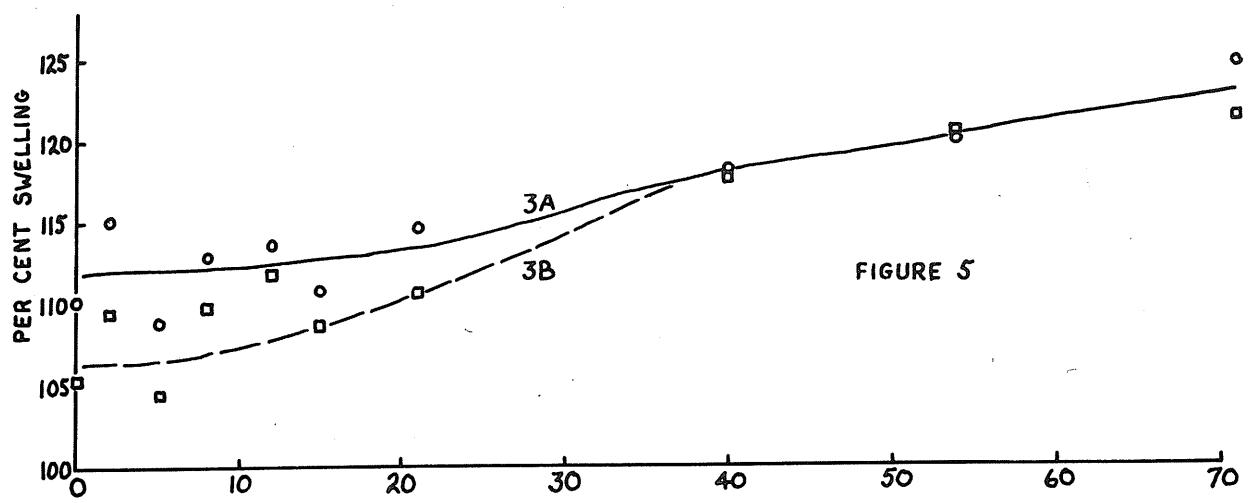
FIGURE 6

FIGURE 7

Figure 8.   The relationship of the cell volume of the

cells during storage to the cell water. Both

factors are expressed as percentages of the

original measurements of the cells in heparin.

Figure 9.   The relationship of the per cent hemolysis

in all the experiments to the length of time

of storage.

FIGURE 8

$Y = 2.50\,X - 152.1$

CELL VOLUME
PER CENT OF ORIGINAL

CELL WATER
PER CENT OF ORIGINAL

FIGURE 9

PER CENT HEMOLYSIS

DAY
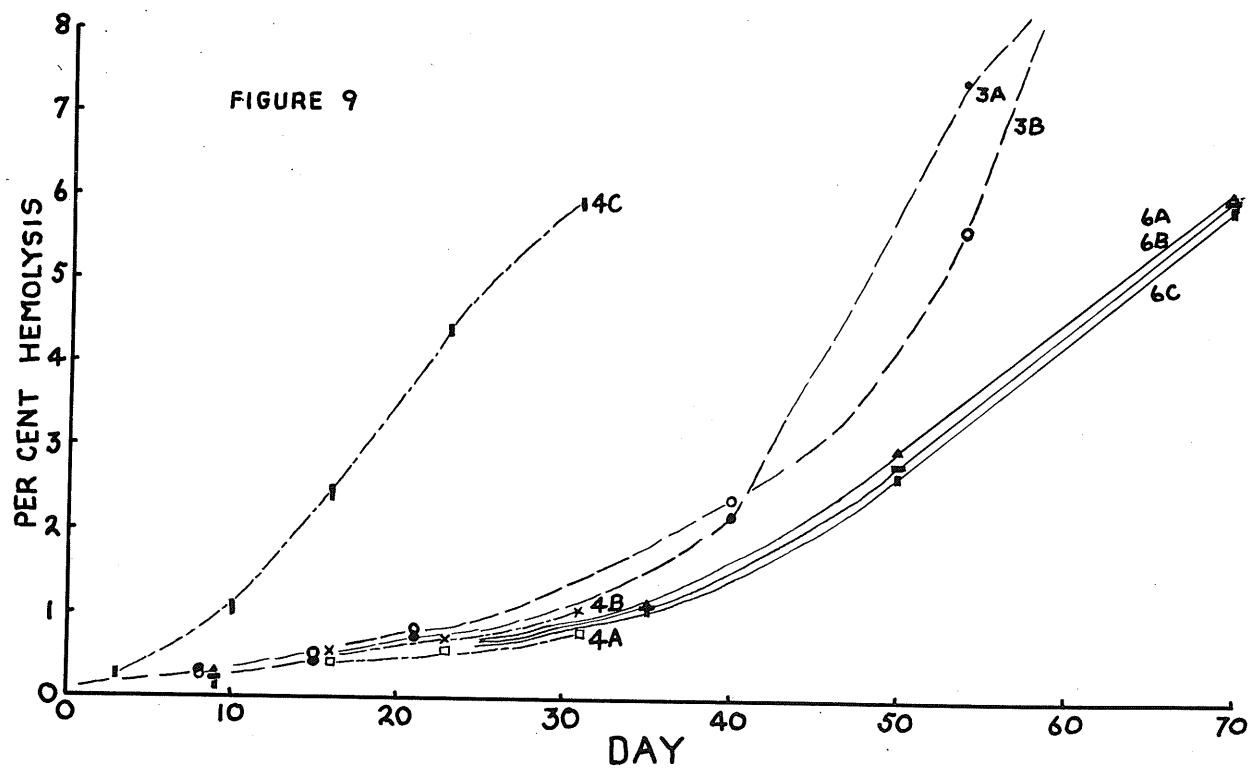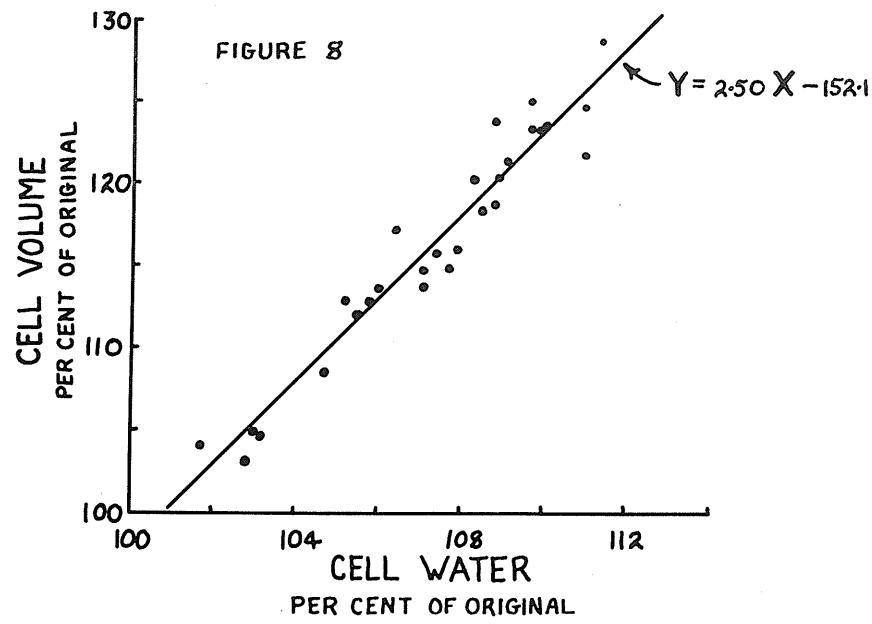
Figures 10 and 11.   The relationship between glucose concentration, theoretical and actual lactic acid production in Experiments 3A and 3B to the length of time of storage.

Figure 12.   Key to the figures in this photo.

Figures 13, 14 and 15.   The relationship between glucose concentration, theoretical and actual lactic acid production in Experiments 4A, 4B and 4C to the length of time of storage.

Figures 16, 17 and 18.   The relationship between glucose concentration, theoretical and actual lactic acid production in Experiments 6A, 6B and 6C to the length of time of storage.

FIG. 10 — 3A
FIG. 11 — 3B

KEY
FIG. 12
THEORETICAL LACTIC ACID CURVE →
GLUCOSE CURVE
LACTIC ACID CURVE
MM/l GLUCOSE
MM/l LACTIC ACID
DAY

FIG. 13 — 4A
FIG. 14 — 4B
FIG. 15 — 4C

FIG. 16 — 6A
FIG. 17 — 6B
FIG. 18 — 6C
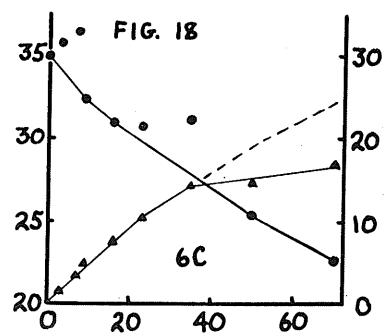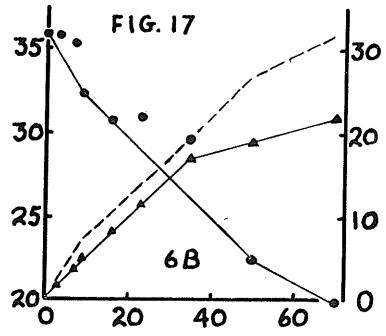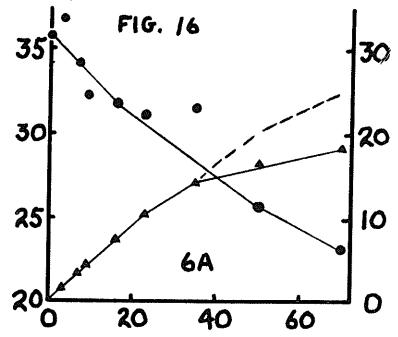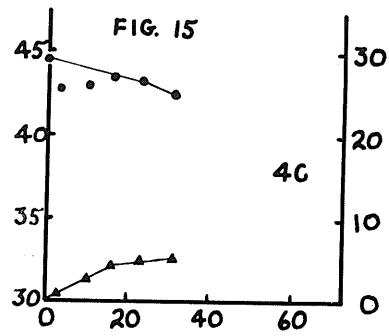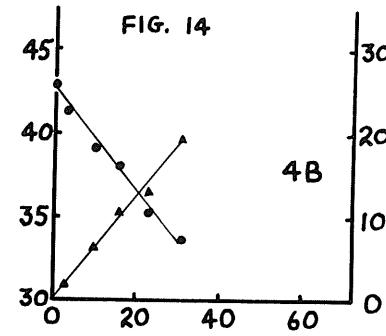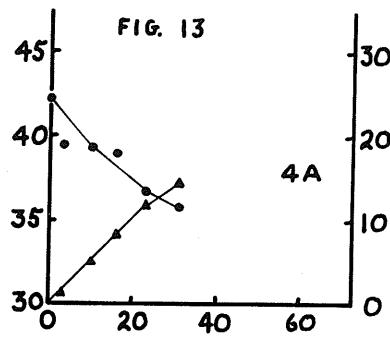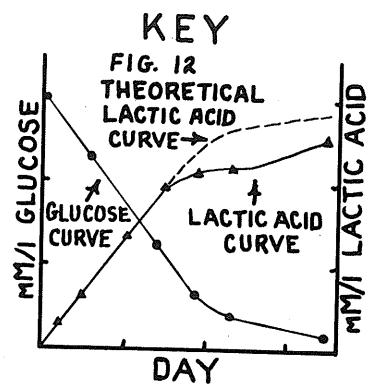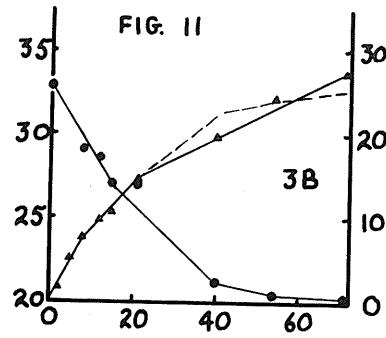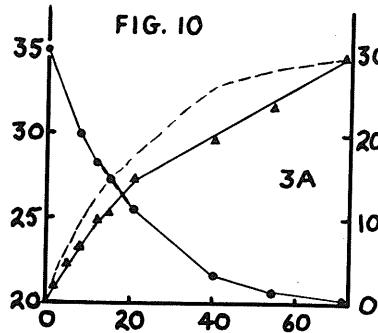
Figure 19.   The relationship of the differences in

organic acid soluble phosphate between phosphate

containing bloods (solid lines) and their controls,

and between nicotinamide containing bloods (dotted

lines) and their controls, to the length of time of

storage.

Figure 20.   The titration curves of the three solutions

used in Experiments 3A, 3B and 3C.

FIGURE 19
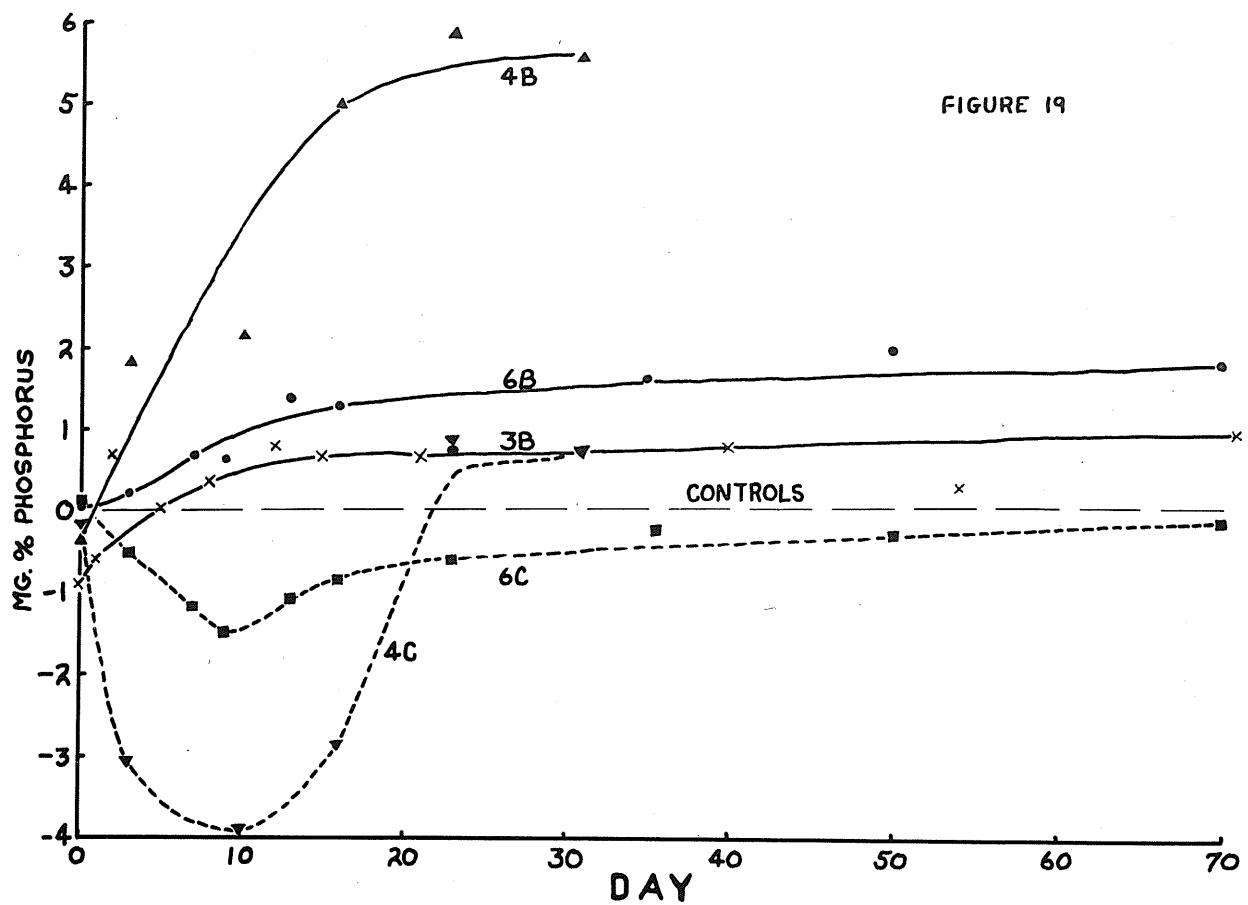
FIGURE 20
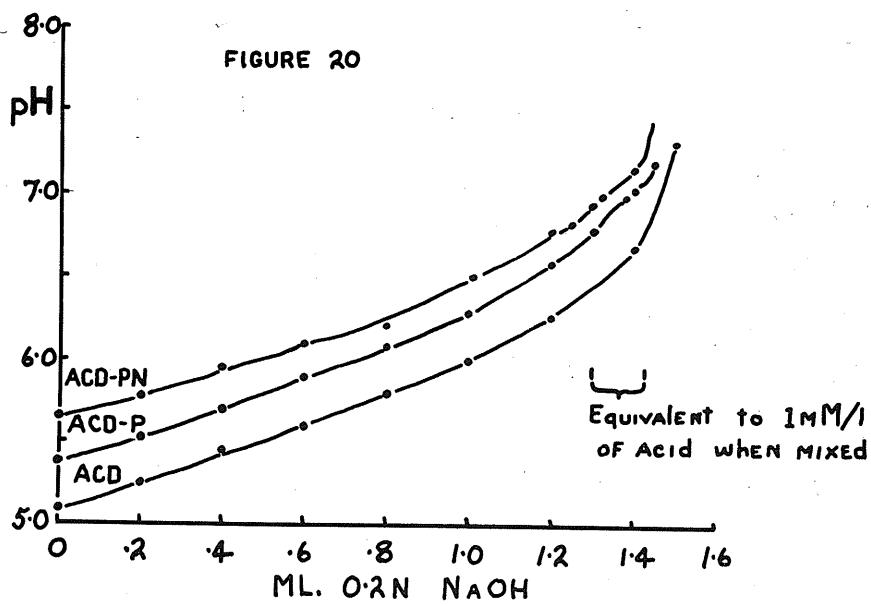
Figure 21.   The relationship of the amounts of adenosine

triphosphate phosphorus in nicotinamide contain-

ing bloods, to the length of time of storage.

Figure 22.   The relationship of the amounts of adenosine

triphosphate phosphorus in phosphate containing

bloods, to the length of time of storage.

Figure 23.   The relationship of the amounts of adenosine

triphosphate phosphorus in bloods stored in

only ACD, to the length of time of storage.

FIGURE 21

NICOTINAMIDE
BLOODS
6C ●
4C ■

FIGURE 22

PHOSPHATE
BLOODS
6B ●
4B ■
3B ▲

FIGURE 23

CONTROL
BLOODS
6A ●
4A ■
3A ▲
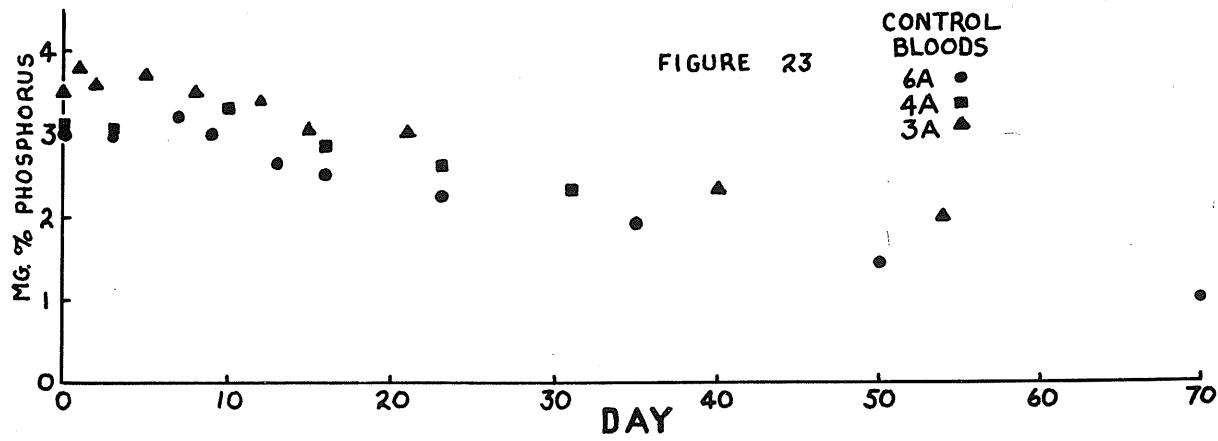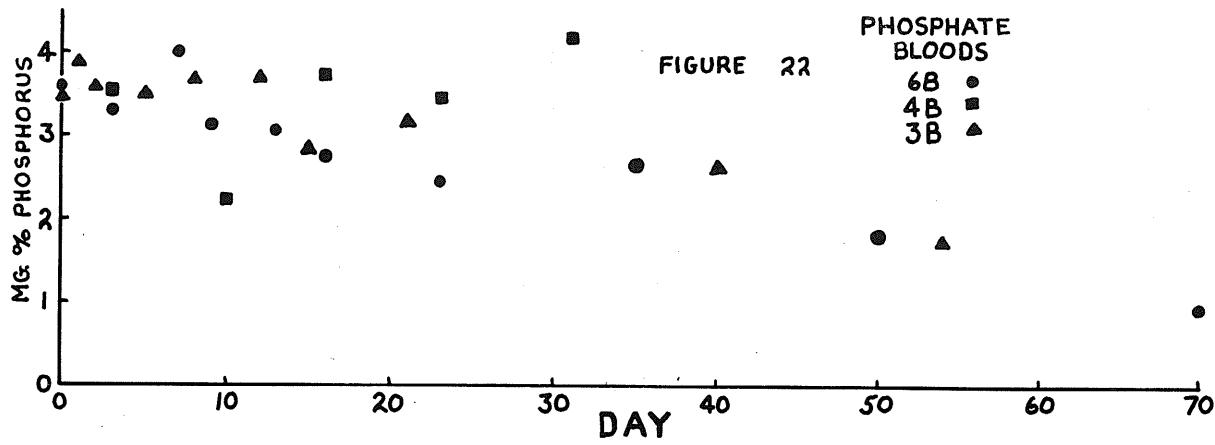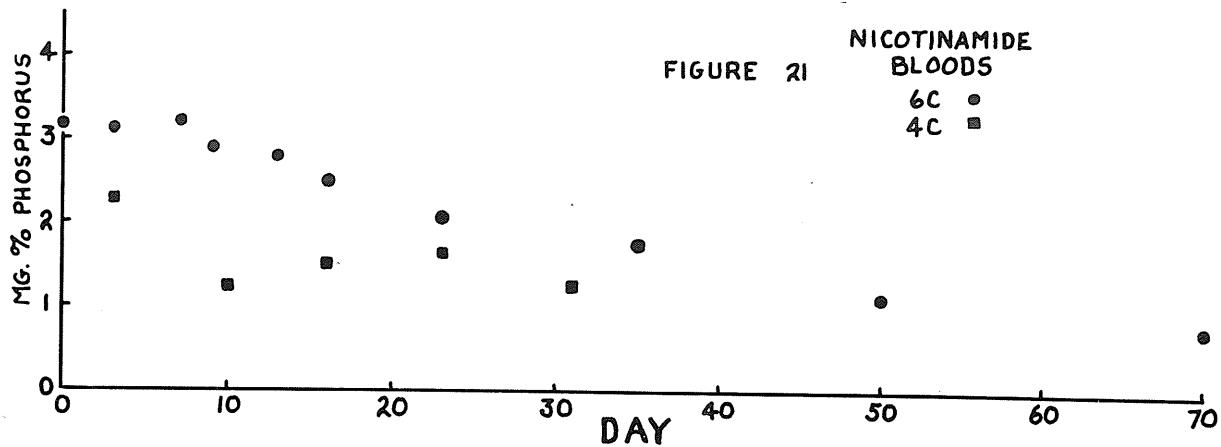
Figure 24.   The relationship of the amount of

hexose diphosphate phosphorus in nicotin-

amide containing bloods, to the length of

time of storage.

Figure 25.   The relationship of the amount of

hexose diphosphate phosphorus in phosph-

ate containing bloods, to the length of

time of storage.

Figure 26.   The relationship of the amount of

hexose diphosphate phosphorus in bloods

stored in only ACD, to the length of time

of storage.

FIGURE 24

NICOTINAMIDE
BLOODS
6C ●
4C ■

FIGURE 25

PHOSPHATE
BLOODS
6B ●
4B ■
3B ▲

FIGURE 26

CONTROL
BLOODS
6A ●
4A ■
3A ▲

DAY
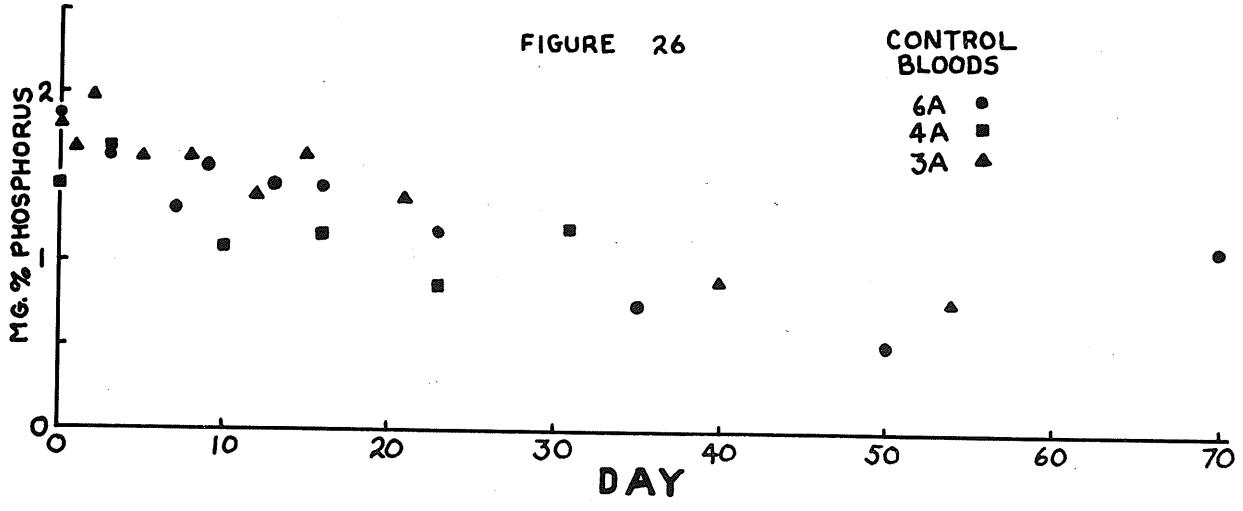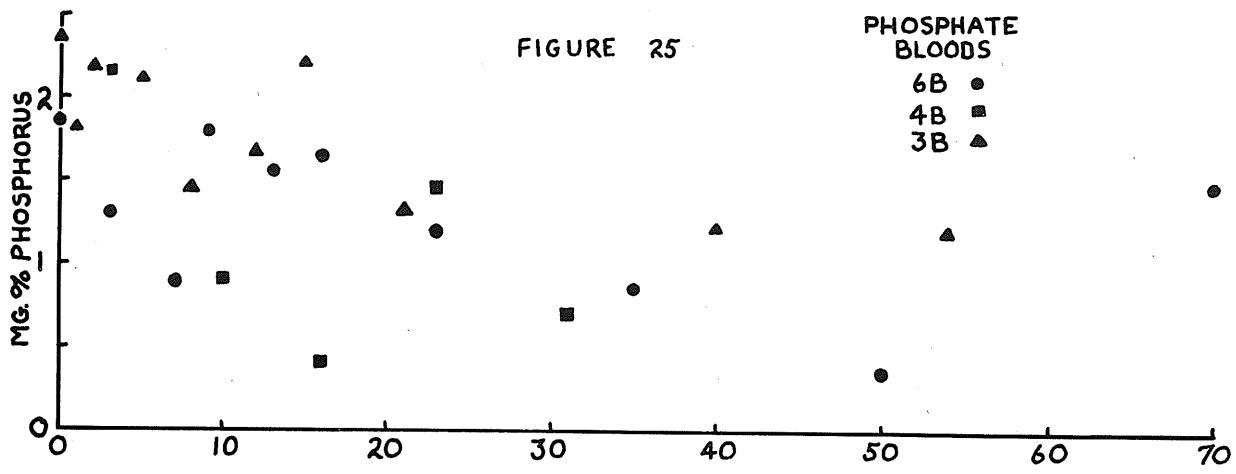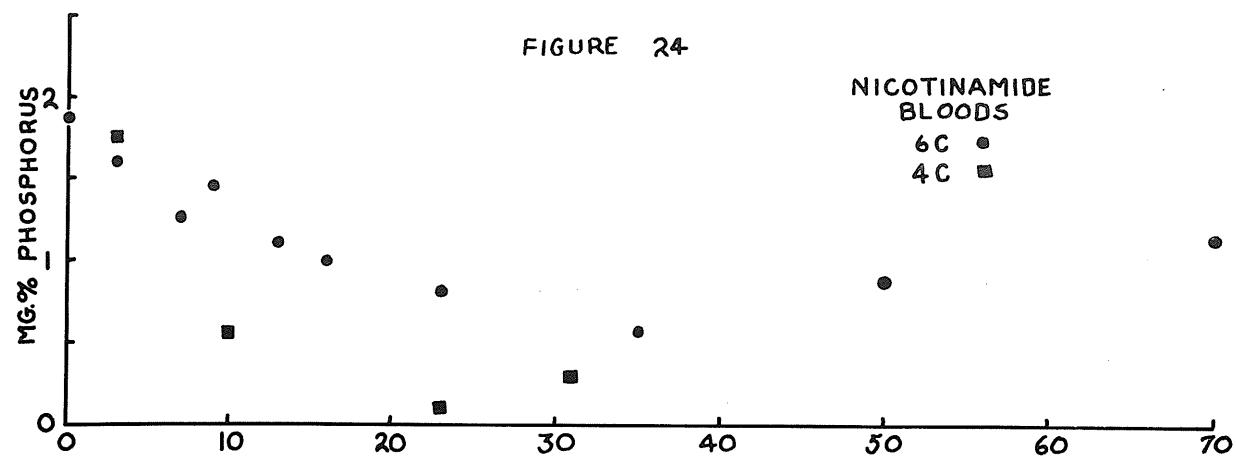
Figure 27.   The relationship of the inorganic phosphate

phosphorus and the diphosphoglyceric acid

phosphorus in Experiments 3A and 3B, to the

length of time of storage.

Figure 28.   The relationship of the inorganic phosphate

phosphorus and the diphosphoglyceric acid

phosphorus in Experiments 4A, 4B and 4C, to the

length of time of storage.

Figure 29.   The relationship of the inorganic phosphate

phosphorus and the diphosphoglyceric acid

phosphorus in Experiments 6A, 6B and 6C, to the
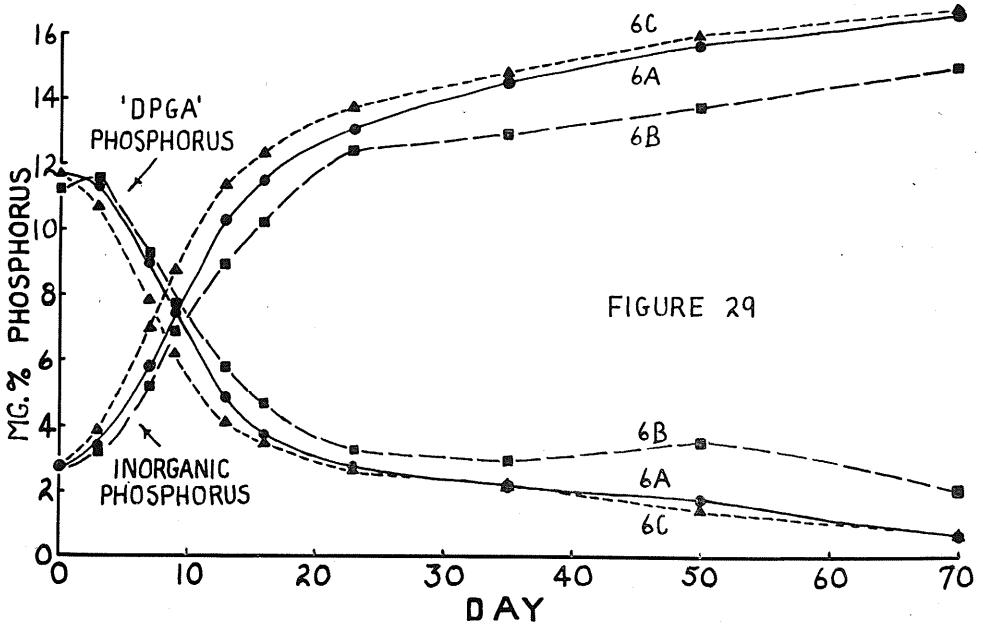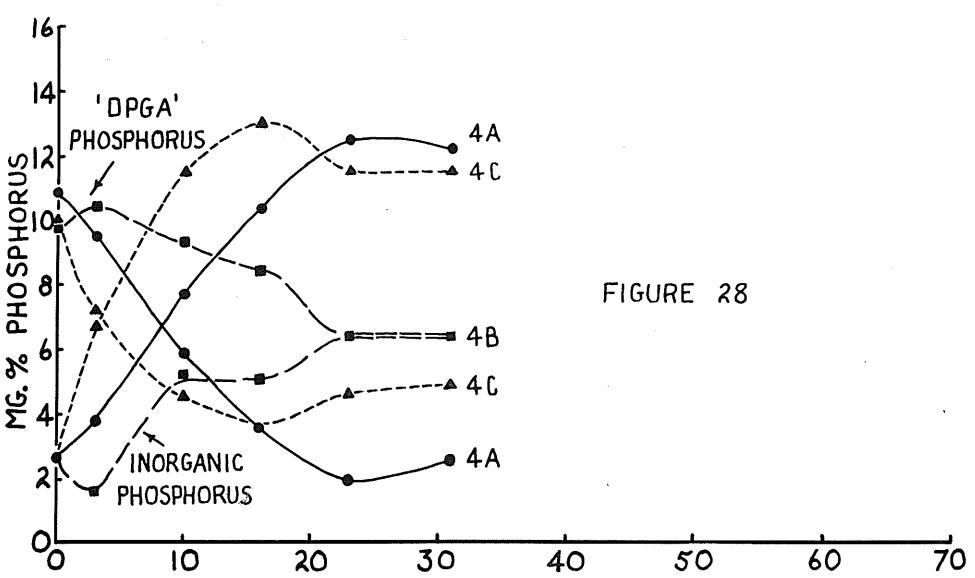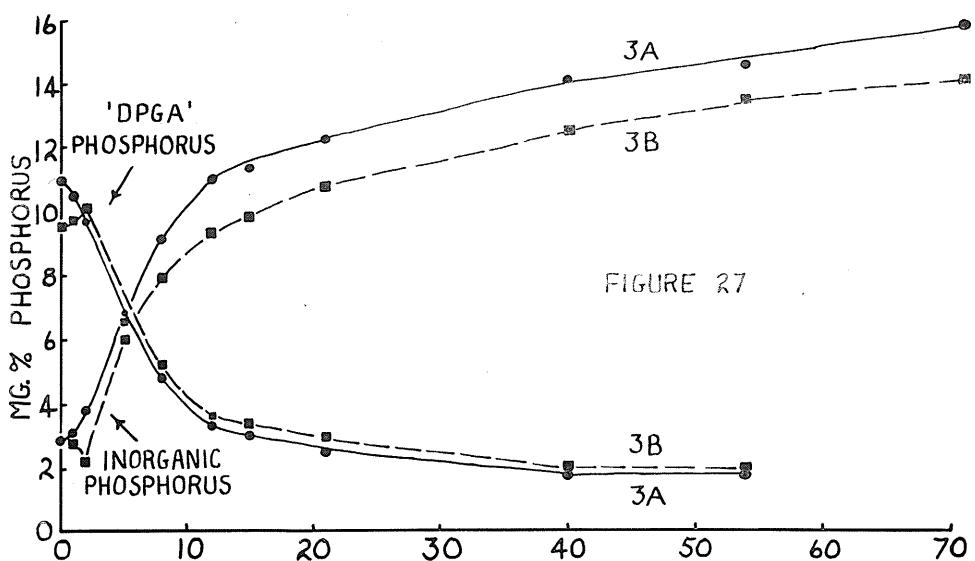
length of time of storage.

FIGURE 27

FIGURE 28

FIGURE 29

FIGURE 30

FIGURE 31

FIGURE 32

FIGURE 33

REAGENTS NECESSARY FOR THE DETERMINATION OF PHOSPHATE

BY THE METHOD OF FISKE AND SUBBAROW

(1) Ten Normal Sulphuric Acid. Five hundred twenty grams

of 95% H_2SO_4 were diluted to a liter with distilled water. Ten-ml.

samples of this were diluted to a liter and titrated with 0.10 N

sodium hydroxide. From the results of duplicate titrations, the

sulphuric acid solution was adjusted to 10.0 N.

(2) Molybdate Reagent. Twenty-five grams of reagent grade

ammonium molybdate was dissolved in 250 ml. of distilled water. To

this was added 300 ml. of 10 N H_2SO_4 and subsequently diluted to 1

liter with distilled water.

(3) Five Normal Sulphuric Acid. Five hundred ml. of 10 N H_2SO_4

was diluted to one liter with distilled water.

(4) Reducing Agent. One-half gram of 1-amino-2-naphthol-4-

sulphonic acid was ground in a mortar with successive quantities of

15% sodium bisulphite solution until the acid was suspended finely in

195 ml. of the bisulphite solution. To this was added small amounts

of 20% sodium sulphite solution until the mixture had all but dissolved.

Usually this stage was accomplished with 3 ml. of the sodium sulphite

solution. The resulting mixture was then shaken mechanically in a glass

stoppered flask for about twenty minutes, allowed to stand overnight and

filtered into a brown glass stoppered bottle. This reagent was made up

fresh every three weeks.

(5) Ammonium Molybdate Solution. Twenty-five grams of reagent grade ammonium molybdate was dissolved in a liter of distilled water.

(6) Ten per cent Trichloracetic Acid. One hundred grams of trichloracetic acid was weighed with a plastic spatula, dissolved in distilled water, and made up to one liter with distilled water.

(7) Standard Solution. 0.4393 grams of dried monobasic potassium phosphate (Merck ACS specific.) was dissolved in distilled water in a liter volumetric flask. To this was added 10 ml. of 10 N H_2SO_4, then made up to volume and mixed. This solution contains one hundred micrograms of phosphorous per ml. and was diluted with 10% trichloracetic acid for use.

TABLE 12

COMPOSITION AND OSMOLARITY OF MEDIA

USED FOR PRESERVATION

Compound	Formula and molecular wt.	Expts. 3A 4A and 6A		Expt. 3B		Expt. 4B		Expt. 6B		Expt. 3C		Expt. 4C		Expt. 6C	
		gms.%	milli-osmolarity	gms.%	milli-osmolarity	gms.%	milli-osmolarity	gms.%	milli-osmolarity	gms.%	milli-osmolarity	gms.%	milli-osmolarity	gms.%	milli-osmolarity
Citric acid $C_6H_8O_7 \cdot H_2O$ 210.14		.47	22.4	.47	22.4	.47	22.4	.47	22.4	.47	22.4	.47	22.4	.47	22.4
Trisodium citrate $Na_3C_6H_5O_7 \cdot 2H_2O$ 294.12		1.33	180.8	1.33	180.8	1.33	180.8	1.33	180.8	1.33	180.8	1.33	180.8	1.33	180.8
Dextrose $C_6H_{12}O_6$ 180.09		3.00	166.6	3.00	166.6	3.00	166.6	3.00	166.6	3.00	166.6	3.00	166.6	3.00	166.6
Disodium phosphate Na_2HPO_4 141.98				.1887	39.9	1.0237	216.3	.3903	82.5	.1887	39.9	1.0237	216.3		
Monosodium phosphate $NaH_2PO_4 \cdot 2H_2O$ 156.03				.1034	13.2	.5611	71.9	.2144	27.4	.1034	13.2	.5611	71.9		
Nicotinamide $C_5H_4NCONH_2$ 122.12										10.00	818.9	10.00	818.9	.25	20.5
Total milli-osmolarity		369.8		422.9		658.0		479.7		1241.8		1476.9		390.3	
Effective milli-osmolarity		203.2		256.3		491.4		313.1		256.3		491.4		203.2	

Blank   Standard   Blood Filtrate

4 ml.   4 ml.

4 ml.   4ml.   4 ml.   4 ml.

Add 0.4 ml. of 10.0
normal H_2SO_4

Digest at 130°C.
for 60 mins.

Cool

Add 0.4 ml. 3% H_2O_2

Add 1.0 ml. of 5.0
normal H_2SO_4

Digest at 130°C.
for 20 mins.

Boil at 100°C.
for

7.0   100.0
mins.   mins.

Cool and add
4 ml. of H_2O

Cool in water bath   Boil 10 mins. at
at room temp. for   100°C.
5 mins.

Cool in water bath
to room temp.

Add 1.0 ml. of molybdate-   Add 1.0 ml. of 2.5% ammonium
H_2SO_4 reagent   molybdate solution

Add 0.4 ml. of A.N.S. reagent and make up to volume 25 ml.

Read in colorimeter at 660 mu

Reagent   Phosphate   Bld. Filt.   Bld. Filt.   Bld. Filt.   Bld. Filt.
BLANK   STANDARD   INORGANIC   7 MINUTE   100 MINUTE   TOTAL
phosphate   phosphate   phosphate   phosphate

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