THE INFLUENCE OF VARIOUS FACTORS ON GLYCOLYSIS IN HUMAN BLOOD DURING STORAGE

by

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Thesis

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INTRODUCTION

General

The investigation to be described in this thesis forms part of a larger study which has been carried on in the Department of Biochemistry at McGill University since January 1940. The work was initiated at the beginning of the second World War under the sponsorship of the National Research Council of Canada with the object of improving the methods of preserving and administering human blood and with a view to the application of improvements in the use of blood in the treatment of the wounded.

During the years of the war rapid advances were made in blood preservation as a result of the concerted efforts of numerous groups of investigators particularly in America and Great Britain. By 1946 it became apparent to many that methods of preservation had reached what appeared to be the limit of perfection and that any further advancement would be achieved only by more fundamental studies on the enzymology of the red blood cells, with a view to obtaining a chemical or physical interpretation of the process of aging of the cells and the loss of viability. This phase of our study was undertaken in 1945 and attention was directed to an investigation of the process of glycolysis in the red corpuscles during storage since this process is prominent in the metabolism of these cells and presumably is intimately connected with their function and viability. The writer was preceeded in this study in the Department of Biochemistry by Mrs. Shirley Andreae, who

under the direction of Dr. O. F. Denstedt, prepared the way for the present study which has to do with the influence of various factors on the glycolytic process.

The studies on enzymology have been supported financially by a grant from the John and Mary Markle Foundation of New York.

REVIEW OF LITERATURE ON BLOOD PRESERVATION

Physical Behavior of Blood on Storage in the Cold

Before proceeding with a discussion of glycolysis, it is pertinent to review what is known about the behavior of whole blood and erythrocytes during storage in the cold. This subject has been reviewed by Denstedt, et al. (1)

Sedimentation

The first thing to become apparent during storage of whole blood is the sedimentation of the cellular elements. This process commences almost immediately after blood is drawn. The red cells settle out first, and their sedimentation is fairly complete within 36 to 48 hours. Some of the white cells are carried down by the sedimenting red cells but they tend to settle out more slowly than the latter. The distribution of white cells throughout the red cell mass is greatest at the top and diminishes rapidly as one proceeds downward. The remaining leucocytes and platelets settle out in the course of a few days to form a thin whitish layer on top of the red cells. These elements tend to agglutinate and soon consolidate into a membrane. When this occurs, it is impossible to disperse the white cells individually again by shaking. The mass can be broken only into small fragments. In centrifuged samples, the white cell layer

becomes sufficiently compact to be removable en masse by forceps or by aspiration.

Several factors influence sedimentation. Among these are the density and size of the cells, the specific gravity and the viscosity of the surrounding medium which, in turn, varies with the degree of dilution with the preservative and the temperature. Some of the cells may breakdown before sedimentation is complete.

Autolytic Changes

The formed elements of the blood and the plasma also contain a large number of enzymes. Like other tissues, blood is subject to autolytic enzymic changes when removed from the body. These changes during storage occur more rapidly in the nucleated elements. The polymorphonuclear cells begin to breakdown early and their destruction is almost complete in seven to ten days. Platelets also are easily destroyed, though not as rapidly as the polymorphs. Some of the eosinophils and monocytes may remain intact for as long as two months.

The disintegration of the leucocytes and platelets during storage is indicated by an apparent swelling of the white-cell layer and the developement of a "fuzzy" or "woolly" appearance. At this time the diffusion of free haemoglobin into the plasma is often detectable. Haemolysis actually commences much earlier, but the diffusion of the free haemoglobin liberated is hindered by the white-cell mass.

Other autolytic changes include the slow liberation of ammonia, the accumulation of lactic acid and inorganic phosphate, and possibly the formation in the plasma of lysolecithin. These

products have a tendency to increase haemolysis.

Haemolysis

Haemolysis in preserved blood appears to be due largely to swelling as a result of the increase of osmotic pressure within the red corpuscles. During storage potassium gradually escapes from the red cells and sodium enters from the plasma. In the circulation sodium remains in the plasma and potassium in the cells. The red-cell membrane under these conditions is nearly impermeable to these ions. Maizels (1a) states that the sodium gained by the cell during storage is greater than the potassium lost. Thus the sum of sodium and potassium in the cell is greater after a period of storage than when the cell is in its normal physiological environment. This increase in osmotic pressure is an important cause of swelling. Maizels and Whittaker (2) observed this swelling and found that in the process of swelling the cells become more spherical in shape and the long diameter decreases. This has been noted also by Rapoport (3). The colloidal osmotic pressure of haemoglobin within the cell is greater than the plasma colloidal osmotic pressure. This has an effect in preventing the establishment of an equilibrium. Other factors which tend to prevent the attainment of an equilibrium are the formation of new ions as lactate, phosphate, and ammonium, and certain non-diffusable ones resulting from autolytic changes.

Rous and Turner in 1914 observed that dextrose has a beneficial influence in retarding haemolysis of rabbits' erythrocytes, and Maizels and Whittaker (4) observed that human red-cells when placed in hypotonic saline increase about 60%

in volume before haemolysing but in dextrose solution there is an increase of 83% before haemolysis takes place. The presence of dextrose, therefore, appears to enable the cell membrane to stretch more extensively without breaking down.

Haemolysis is an important factor in the glycolysis of the red cell as it has been pointed out by Solomon, Hald, and Peters (5) that haemolysis of blood by repeated freezing or by saponin retards the rate of glycolysis and Rapoport and Guest (6) have shown that intact cells are necessary for the rephosphorylation of adenylic acid in the regeneration of adenosinetriphosphate.

DeGowin, Harris, and Plass (7) have pointed out the effect of the temperature of storage on haemolysis. They observed that haemolysis is much greater when blood is stored at 20° C than at 5° C.

GLYCOLYSIS

Glycolysis is defined as the biological conversion of glucose to lactic acid. MacLeod (8) observed that glycolysis is an active process in the corpuscles. Maizels (9) says that the process is confined to the cellular elements and does not occur in the plasma. Bird (10) also has shown that the major part of the glycolysis in blood is carried out by the red cells. He states that the relative contribution of red to white cells is 8 : 1. However, since in numbers the red to white cell ratio is about 1,000 : 1, cell for cell, the white cells are more active by far glycolytically than the red cells. This is to be expected since the white cells are nucleated, and

some are motile, and have a relatively high rate of respiration.

Many factors influence the rate of glycolysis. Studies on glycolysis necessarily must be carried out in vitro and have indicated that individual bloods differ greatly in glycolytic ability. Bose and De (11) have shown that the rate of glycolysis in various blood samples differs to such an extent that no estimation of the rate can be predicted. Schmitz and Glover (12), studying ten normal individuals, found that the rate of glycolysis in the blood varied between ten and twenty-three milligrams per hundred ml of blood per hour. MacLeod (8) reported that the rate of red-cell glycolysis varies from individual to individual and may vary even in the individual's own cells at different times.

The second factor in order of importance which influences the glycolytic rate of the preserved red cell is the composition of the so-called "preservative medium". A large number of preservatives has been used in the preservation of blood. Weil in 1915 on this continent reported the successful infusion of citrated human blood which had been stored in the cold for several days. Rous and Turner in 1915 and 1916 observed that the addition of dextrose to citrated rabbit's blood greatly improved the preservation of the corpuscles. They demonstrated also that such blood stored in the cold for two weeks was highly efficacious. Preserved blood was used for human transfusion for the first time by Robertson (13) in 1918 near the end of the first World War. In one of the British casualty clearing stations he collected the blood in the citrate-dextrose mixture of Rous and Turner and after

storing it for as long as three weeks, transfused it into the wounded with good results.

For almost a decade after the close of the first World War little attention was paid to blood preservation until interest was revived by Shamov and his colleagues in Southern Russia who demonstrated that blood taken from dogs killed by violent means could be transfused into severely anaemic animals with completely satisfactory results. Yudin in 1930 in Moscow, perceiving the significance of Shamov's experiments, performed the first transfusion of human cadaver blood. The recipient was a young patient'who had attempted suicide and was at the point of death from loss of blood. Yudin, shortly afterwards, organized in Moscow a very efficient system of collecting blood from the victims of fatal accidents. The cadavers were promptly collected and were exanguinated within six to eight hours after death, provided the corpse was found to be free of the likelihood of infection. No victims of poisoning or diseased persons, or cadavers that had been mutilated so as to infect the blood, were used. No citrated or other anticoagulant was required since, as Skundina found in 1935, blood from cases of sudden death coagulates soon after it is drawn, but reliquefies within an hour or so. Dissolution of the clot is said to occur by a process of "fibrinolysis", which as yet is not fully understood. The blood was stored at about 2^{o} C, and was discarded if after sterility tests was found to be infected.

The Russian work stimulated interest in blood preservation on the continent of Europe, but in American and in Great

'The patient made a good recovery.

Britain little active interest was taken in the use of stored blood until after the war in Spain in which the work of the various transfusions units of Jorda, Bethune and others demonstrated the indispensability of preserved blood for treating the wounded in war.

Little interest in cadaver blood has been evidenced in countries other than Russia; however, placental blood has been used. In 1936 Novikova and Farberova described a method for collecting and preserving placental blood, and later Bagdassarov introduced a preservative mixture containing sodium citrate, magnesium sulfate, potassium and sodium chloride. Workers in America (13 a)also experimented successfully in the clinical use of placental blood but the practice soon was abandoned largely because of the inconvenience of collecting and storing small amounts and the fear of contamination unless sterility tests are performed on every sample. Particularly since the outbreak of the second World War, only blood from healthy donors has been used for preservation and transfusion.

World War II provided a tremendous stimulus for a renewed interest in blood preservation. A number of preservative solutions have been introduced since 1939 varying from simple citrate solutions to quite complex mixtures. DeGowin, Harris, and Plass (7) in 1940 proposed a modification of the citrate-dextrose mixture of Rous and Turner. Ross and Chapin (14), and Belk and Rosenstein (15) used citrate as preservative. Maizels and Whittaker (2) employed a citrate-saline mixture.

Muether and Andrews (16) advocate the use of a citrate-dextrose preservative solution buffered with phosphate.

One of the most important turns in the developement of preservative mixtures was the introduction by Loutit, Mollison, and Young (17) of a mixture of citric acid, sodium citrate and dextrose. The presence of citric acid lowers the pH of the mixture and prevents caramelization of the sugar during autoclaving. Bushby (18) was the first to try this type of mixture, and while he obtained good preservation, he abandoned the use of citric acid because of the severe reactions he encountered in transfusions of the blood. On this continent these acidified preservative mixtures are usually referred to as "ACD" (acid-citrate-dextrose). They vary slightly in the proportions of the three ingredients used. Among the authors advocating this type of preservative solution are Loutit, Mollison, and Young (17); Rapoport (3); Gibson, et al. (19); Ross, et al. (20); Strumia, Blake and Wicks (21); and Strumia, Blake and McGraw (22).

Mollison and Young (23) tried a citrate-saline-dextrose preservative mixture, but found that the presence of sodium chloride resulted in inferior preservation.

Rapoport (3), in 1947, compared glycolysis in blood preserved in three mixtures, namely, citrate, neutral citrate-dextrose, and acid-citrate-dextrose. He found that the glycolytic capacity was lost first in the simple citrate solution and retained longest in the acid-citrate-dextrose mixture.

Temperature has a pronounced influence on the rate of glycolysis. Arthus, quoted by Bose and De (11) observed the

effect of temperature and Tolstoi (24) showed that glycolysis was slower at room temperature than at 37° C. Bose and De also state that the rate of glycolysis varies with the temperature.

The hydrogen ion concentration of the medium also has a very marked effect on the rate of glycolysis in red cells. As has been stated previously, glycolytic ability is retained longest in acid-citrate-dextrose solutions. Rapoport (3) says that erythrocytes in neutral media such as citrate or citrate-dextrose, have a higher initial rate of glycolysis than those in acid-citrate-dextrose. Wurmser, et al. (25) showed that glucose disappears much more slowly from blood at pH 6 than at pH 7.5. In general it is agreed that lowering the pH retards the rate of glycolysis and hence it is assumed that the glycolytic capacity of the cells is better preserved. This is shown in the work of Rapoport (3) and Rapoport and Guest (6) who demonstrated that in acidified defibrinated blood the lower pH causes an immediate liberation of phosphate from diphosphoglycerate, which is one of the intermediates in glycolysis.

The question as to what concentration of glucose is optimal for red cell preservation has received much consideration and still is not entirely settled. De and Bhattacharyya (26) claim that the percentage of sugar glycolysed in the first 24 hours varies inversely with the initial concentration of sugar up to 280 mgm. per 100 ml of blood. Cajori and Crouter (27) on the other hand claim that the rate of glycolysis varies directly with the amount of sugar present.

In this connection the influence of starvation and of diabetes is of interest. Reid and Narayana (28) state that starvation decreases, and food intake increases, the rate of glycolysis in blood. As regards diabetes, there are conflicting claims. MacLeod (8) and Tolstoi (24) claim that there is no difference in the glycolytic rate of diabetic and of normal bloods. Similarly, Cajori and Crouter (27) found no evidence that diabetic blood possesses a diminished glycolytic power. On the other hand, De and Bhattacharyya (26) and Thalhimer and Perry (29) found that the glycolytic rate in diabetic bloods is somewhat reduced.

Schmitz and Glover (12) report that potassium cyanide increased the glycolytic rate in cases of myelogenous leukemia, while Reid and Narayana (28) report that cyanide had no effect on the glycolysing power of the blood.

Bueding and Goldfarb (30) observed that neither iodoacetate nor sodium fluoride alone completely stopped glycolysis but that complete inhibition was observed when a mixture of the two was used.

A study on the comparison of aerobic and anaerobic glycolysis in human blood has been published by Bird (10) who correlated chemical analyses with manometric measurements. Both types of analysis showed that anaerobic glycolysis exceeds aerobic glycolysis. Bird states that the glycolytic rate of blood is small compared to that of other tissues. He confirmed the findings of Thalhimer and Perry (29) in this respect.

Sugar

Claude Bernard in 1876 was the first to demonstrate that sugar disappears from blood on standing at room temperature. According to Kleiner and Halpern (31) some of the early workers, particularly Lepine and Barral, found marked increases in the sugar content of blood incubated at 58° C for short intervals. This they called "sucre virtuel". MacLeod in 1913 (8) found no such rises in blood sugar when the blood was incubated at body temperature for periods up to one hour. Kleiner and Halpern (31) noted these rises not only in dialysed blood, but also in the undialysed controls. Suspecting that the sugar method was at fault, they changed methods twice, and found that the same events occurred in each of the three methods, namely the Folin-Wu, Shaffer-Somogyi, and the Hagedorn-Jensen methods. Lepine and Barral thought that these rises were the result of enzyme action on some substance having a glucosidic linkage. However, Neuwirth (32) could not confirm the work of Kleiner and Halpern.

Andreae (33) in our laboratory in 1946, studying blood preserved in citrate-dextrose, noted characteristic sharp rises in the glucose content, or at least in the reducing power of the blood at certain times usually between the second and tenth and the nineteenth and twenty-sixth days of storage. The abrupt increases always were followed by equally abrupt decreases within a couple of days.

The experimental work to be described later in this thesis partly confirms Andreae's observations. Rises in the

dextrose concentrations were noted in blood containing added dextrose, but they were not as sharp, nor did they always occur at the times reported by Andreae.

Pyruvic Acid

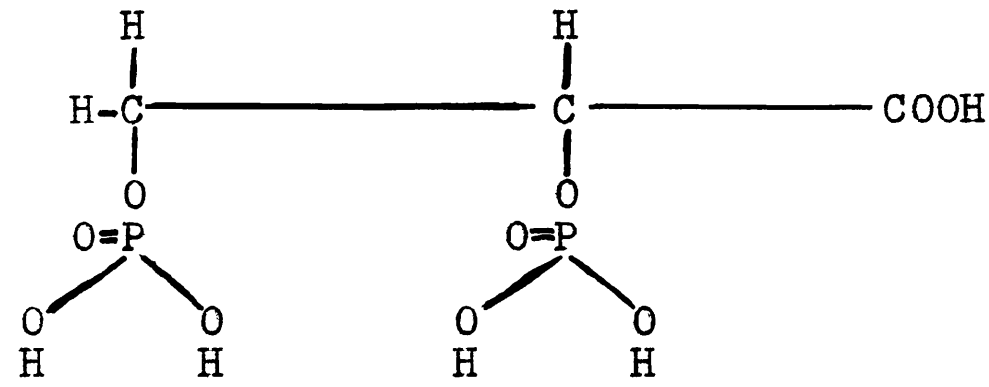
Bueding and Goodhart (34) published a paper in 1941 on the behavior of pyruvic acid in blood. They state that in defibrinated, heparinized, citrated, or oxalated blood, pyruvate disappears from the blood within thirty minutes after blood has been removed from the body. Thereafter in heparinized or defibrinated blood the pyruvate increases, and may rise to above the original level. They observed that oxalated blood did not show the later increase. They found that removal of pyruvate was accelerated by incubation with 0.075 M phosphate and that pyruvate was unaffected by the addition of thiamin, cocarboxylase, or glucose, or by the oxygen tension of the blood. In the presence of 2% fluoride from 83% to 95% of the decrease in pyruvate could be accounted for as lactate. Apparently an enzyme is responsible for the pyruvate removal since it is heat-labile and is present in the red-blood cell. A heat-stable activator also was found which appeared to be a phosphorylated intermediate of carbohydrate metabolism, probably triosephosphate.

Dische (35), and also cited by Bueding and Goodhart (34), found that the removal of hexose phosphate by blood cells was increased by the addition of pyruvate. Under these conditions a phosphate ester difficult to hydrolyse was formed which was thought to be phosphoglycerate. He concluded that the reaction:

pyruvate + triosephosphate $\rightleftharpoons$ lactate + phosphoglycerate, which previously had been described for muscle also occurs in blood.

Phosphate Compounds

Numerous phosphorus compounds are associated with blood glycolysis. Among those which have received the most attention is diphosphoglyceric acid. This compound was first isolated by Greenwald (36) in 1925 who gave the formula as follows:



2.3 diphosphoglyceric acid

Guest and Rapoport (37) state that this compound accounts for about half of the organic acid-soluble phosphate of the blood. According to these authors (38), the phosphate compounds of blood may be divided roughly into three classes, namely, inorganic phosphates, easily hydrolysable phosphates, and non-hydrolysable phosphates. The total minus the inorganic phosphates makes up the organic acid-soluble phosphate. The easily hydrolysable phosphates include the hexose phosphates and adenosinetriphosphate.

The term "non-hydrolysable phosphate" is used for convenience to indicate the difficultly hydrolysable organic phosphates, chiefly diphosphoglycerate. This fraction is not hydro-

lysable with bone phosphatase, but it is split by 1 N HCL on heating in a boiling water bath for 48 hours (39). The normal values for human red-blood cells according to Guest and Rapoport (38) are as follows:

	(as phosphorus)
Inorganic phosphate	1 - 3 mgm %
Adenosinetriphosphate	9 - 12 mgm %, average about 10 mgm %
Hexosephosphates (mono- and di-)	15 mgm %
Diphosphoglycerate	25 - 30 mgm %

As regards the distribution of these compounds in normal blood, Guest and Rapoport (39) state that acid-soluble phosphorus has been found in mature erythrocytes, reticulocytes, and in leucocytes. They point out that there is no significant difference between the bloods of males and females of various species. However, as might be expected, younger animals generally have higher phosphorus levels than adults.

Diphosphoglycerate performs many roles in the general maintenance of bodily functions other than its role as an intermediate in glycolysis in blood. Among the various functions, it aids in the maintenance of electrolyte equilibrium. Guest and Rapoport (39) have demonstrated, for example, that in pyloric obstruction in the dog the most important factor compensating for the decrease in chloride is the increase in diphosphoglyceric acid. They show furthermore, that the distribution of diffusible ions between cells and serum depends mainly on the concentration of anion equivalency of haemoglobin and diphosphoglycerate, which are the two most important non-

diffusible constituents of the cells.

Evidence has been presented by these authors (40) that diphosphoglycerate also may serve as a transporter of phosphorus in the body. They have shown that marked decreases of the diphosphoglycerate content of the cells are seen in rickets, and that before healing occurs, there is a marked increase in the diphosphoglycerate of cells.

Again, these workers have shown (40) that overdosage with irradiated ergosterol in rabbits results in an increase in the organic acid-soluble phosphate. The increase was found to be largely in the diphosphoglycerate fraction.

In both diabetic and ammonium-chloride acidosis (38-39), when there is adequate renal function, there is an increased excretion of phosphate in the urine. This is correlated with a diminished concentration of organic acid-soluble phosphate in the blood, which reduction has been found to occur in the fraction not hydrolysed by bone phosphatase, namely, the diphosphoglycerate. This excretion of phosphate in the urine indicates that the acid-soluble phosphorus of the soft tissues, which are closely related to the phosphorus of the blood, also are drawn upon. Thus the acid-soluble phosphorus of the blood may serve as a guide to the state of the phosphorus in the other tissues.

These points are mentioned, not as having a direct bearing on glycolysis in blood, but to show how the various body processes are integrated, and that conditions directly affecting one of these processes may influence phosphate metabolism of others.

Lactic Acid

The apparent end product of glycolysis in blood is lactic acid. Evans (41) cites Slosse (42) and Kraske, Kondo, and von Noorden (43) as being among the first to show that glycolysis involves the conversion of glucose to lactic acid. Evans (41), in 1922, showed that the fall in carbon-dioxide-carrying power of shed blood was due to the glycolytic production of lactic acid from glucose. In 1947 Bird (10) applied manometric methods to studying the glycolytic process.

Mechanism of Glycolysis

The mass of evidence accumulated so far indicates that the sequence of reactions occurring in the glycolytic cycle of blood and muscle are very closely related. Barron (44) gives a table of these reactions for muscle which is in agreement with that given by Baldwin (45).

TABLE TWO - Barron
COURSE OF GLYCOLYSIS

Stage	Reaction
1.	Glycogen + H_3PO_4 $\rightleftharpoons$ glucose - 1 - phosphate
2.	Glucose - 1 - Phosphate $\rightleftharpoons$ glucose - 6 - phosphate
3.	Glucose - 6 - phosphate $\rightleftharpoons$ fructose - 6 - phosphate
4.	Fructose - 6 - phosphate + ATP $\rightleftharpoons$ fructose -1,6-diphosphate +ADP
5.	Glucose + ATP $\rightleftharpoons$ glucose - 6 - phosphate + ADP
6.	Fructose - 1,6 - diphosphate $\rightleftharpoons$ dioxyacetonephosphate + 3 - glyceraldehyde phosphate
7.	Dioxyacetone phosphate $\rightleftharpoons$ 3 - glyceraldehyde phosphate
8.	3 - Glyceraldehyde phosphate + H_3PO_4 $\rightleftharpoons$ 1, 3 - diphospho-glyceraldehyde

9. 1, 3 - diphosphoglyceraldehyde + DPN $\rightleftharpoons$ 1, 3 diphospho-glycerate + DPN-H

10. 1, 3 - diphosphoglycerate + ADP $\rightleftharpoons$ 3 - phosphoglycerate +

11. 3 - phosphoglycerate $\rightleftharpoons$ 2 - phosphoglycerate

12. 2 - phosphoglycerate + H_2O $\rightleftharpoons$ phosphoenolpyruvate

13. Phosphoenolpyruvate + ADP $\rightleftharpoons$ pyruvate + ATP

14. Pyruvate + DPN-H_2 $\rightleftharpoons$ Lactate + DPN

According to Barron, reactions 8 and 10 are non-enzymatic. The enzymes concerned with the various steps in this sequence are listed below.

TABLE THREE
Enzymes of Glycolysis

Stage	Enzyme	Nature of Enzyme
1.	Phosphorylase	(protein-Mg-adenylic acid)
2.	Phosphoglucomutase	(protein -Mg)
3.	Hexose isomerase	(protein-M')
4.	Phosphohexokinase	(protein-ATP?)
5.	Hexokinase	(protein-ATP?)
6.	Zymohexase	(protein-M')
7.	Triose isomerase	(protein-M')
8.	Non-enzymatic	
9.	Phosphoglyceraldehyde oxidase	(protein-DPN)
10.	Non-enzymatic	
11.	Triose mutase	(protein-M')
12.	Enolase	(protein-Mg)
13.	Phosphopyruvate phosphatase	(protein-ADP-Mg)
14.	Lactate oxidase	(protein-DPN)

'M = Metal

Lactic acid is the end product of anaerobic glycolysis. Where there is a mechanism present for the aerobic destruction of pyruvic acid, the latter rightly should be regarded as the end product of glycolysis. A mechanism for the aerobic destruction of pyruvate, however, has not been demonstrated in blood up to the present time.

According to Greenwald (36) and Rapoport and Guest (46) the diphosphoglycerate of human blood is the stable 2,3-diphosphoglycerate. This isomer occurs in the majority of mammalian bloods; however, in avian bloods with nucleated erythrocytes and in beef blood where the concentration of organic acid-soluble phosphates is low, there appears monophosphoglycerate. Rapoport and Guest (46) state that where this compound occurs it is preceeded by a labile isomer which differs structurally from the stable 2,3-diphosphoglycerate.

Guest and Rapoport state that glucose is phosphorylated by ATP in blood and that the blood cells form diphosphoglycerate and not the monophosphoglycerate in the majority of mammals.

Experiments with radioactive phosphorus have shown that there the turnover is rapid both *in vivo* and *in vitro*. Incubation of human blood cells with NaF and pyruvate causes an increase in diphosphoglycerate.

The same authors (39) have shown that there is a rough correlation between the rate of glycolysis and the amount of ATP present in the red-blood cells.

Inhibitors and Stimulators

Cajori and Crouter (27) observed that in diabetic blood

oxalate increased the rate of glycolysis. Bose and De (11) confirmed this, and found also that if fluoride is used, glycolysis is markedly retarded. Bueding and Goodhart (34), as mentioned previously, found that the presence of 2% fluoride caused the conversion of pyruvic acid to lactic acid.

In 1932 Braunstein (47) studied the effect of arsenate on glycolysis and concluded that when it was present in blood, arsenate impeded the esterification of the hexose with phosphate by "competitive inhibition". It thus prevents the resynthesis of the organic phosphorus compounds. Braunstein also states that the enzymic hydrolysis of the phosphate esters is not affected by arsenate.

Morgulis and Pinto (48), on the other hand, state that arsenate may either stimulate or depress glycolysis in red cells depending on the species of animal. In any case the magnitude of the effect is related to the concentration of the arsenate. They say that regardless of the effect of the arsenate, the inorganic phosphate invariably rises due to the stimulation of the phosphotase activity.

MacFarlane, according to Braunstein (47), claims that arsenate accelerates dephosphorylation, but only when the coenzyme is active. Furthermore, arsenate does not exert a direct effect on hydrolysis, but acts through the coenzyme. Likewise, it does not act directly on the phosphatase.

Bueding and Goldfarb (30) state that glycolysis is not completely arrested by either iodoacetate or sodium fluoride, but that a mixture of the two is necessary for complete inhibition.

Bueding and Wortis (49) describe a more specific effect of iodoacetate in that monoiodoacetate prevents the disappearance of pyruvate from non-precipitated blood while fluoride by itself does not. Furthermore, cyanide increases the rate of disappearance of pyruvate from which they concluded that cocarboxylase is the enzyme responsible for the removal of pyruvate since this enzyme, which is inhibited by iodoacetate, is not affected by sodium fluoride, and is activated by sodium cyanide.

Dische (35) states that when citrated human blood is kept for about 24 hours at 4° C, fructose diphosphate and triosephosphate accumulate in the cells. These esters remain in the cells during washing with saline. When the washed cells, laden with fructose diphosphate, are incubated for thirty minutes at 40° C, the ester falls and lactic acid accumulates. Sodium bromacetate prevents the breakdown of fructose diphosphate.

Schmitz and Glover (12) report that in cases of myelogenous leukemia, potassium cyanide causes a marked increase in the rate of glycolysis. In one case the rate was more than doubled. However, they claim that potassium cyanide has very little effect on the rate of glycolysis in normal blood.

The Action of Dyestuffs

The action of methylene blue on the respiration and glycolysis of the red cell has been studied by Harrop and Barron (50). They showed that the respiratory metabolism of non-nucleated mammalian (human) erythrocytes is greatly

accelerated by methylene blue, approaching the magnitude of the metabolism of the nucleated bird erythrocytes. This effect was obtained also, though in smaller degree, with Bindschedler's green, indigo disulfonate, o-chlorophenol indophenol, phenol indophenol, and toluylene blue. Toluylene blue was the only one of these which was as effective in stimulating respiration as methylene blue. They found that in the presence of methylene blue the respiration was accelerated even in the presence of KCN. The respiration of avian erythrocytes also was increased but not as much as with human cells.

The same authors (51) showed that the addition of methylene blue, or other dyes of a similar oxidation-reduction potential, would increase the breakdown of sugar and decrease the formation of lactic acid. They point out that the action of methylene blue does not take place until glycolysis already has been initiated. Moreover, the action is not on glucose, but on some intermediate. According to Barron (52), and also according to Quastel and Wheatley (53), the methylene blue accepts hydrogen from the intermediate and then the hydrogen reacts with molecular oxygen. In other words, methylene blue enables non-nucleated red cells to respire as do nucleated bird's cells. In the light of later work summarized by Barron and illustrated in Table II, page 17 and 18, this is easily explained in that methylene blue accepts hydrogen from the reduced DPN. This prevents the reduction of pyruvic acid to lactic acid. The final step of the glycolytic cycle involving the reduction of DPN takes place relatively long after the initiation of the cycle with the entrance of glucose.

Barron (52) states that narcotics inhibit the action of methylene blue. He postulates that narcotics adhere to the surface of the cell thus preventing the dye from entering the cell. Furthermore, he states that the increase of oxygen consumption produced by methylene blue is proportional to the level of anaerobic metabolism in the cell. The increase is marked in cells with a high "fermenting power", as in mammalian erythrocytes; and is much less, or even nil, in cells with low anaerobic metabolism such as avian red-blood cells.

Quastel and Wheatley (53) studied the action of 29 dyestuffs on the oxidation of glucose, lactate, succinate, and formate by B. coli. They found that all of the dyestuffs which have a vigorous toxic action are basic in character, the acid dyestuffs having little or no action. The molecular structure of the dyestuff is important in determining toxicity. They also found that the dyes exhibited a specificity in their behavior toward dehydrogenases. This also confirms the observations of Barron. The nature of the buffer used also was found to influence the toxicity of the dye thus, the toxicity of methylene blue was greatly diminished in the presence of phosphate when compared to the effect in veronal buffer. In the oxidation of succinate and methylene blue, the oxidation was inhibited to the extent of 90% with veronal buffer but only 30% with phosphate buffer.

Quastel (54) prepared cell-free preparations of fumarase and found that both acid and basic dyestuffs were toxic to this enzyme. He postulated that the acid or basic groups of the dye combine with oppositely charged groups in the

active center of the enzyme and thus inhibit its action. He showed that not only are the acid or basic groups important in determining the activity of the dye, but that the spatial configuration of the molecule also was a determining factor in its toxicity.

Quastel found that the addition of an excess amount of the substrate would protect the enzyme from the inhibiting influence of the dye. Similarly proteins exert a protective action by taking up some of the dye. Once the enzyme is inhibited, however, the addition of protein or a dye with oppositely charged groups does not reverse the inhibition. By means of dyes and protective substances, it is possible, therefore, to gain information about the active groups of the enzyme.

Relationship of Sugar and Phosphorus during Glycolysis

Guest (55) in 1932 published a study on the relationship between sugar and phosphorus during glycolysis in normal human blood. He observed that in defibrinated blood, incubated at 37° C, glycolysis occurs at a fairly constant rate, the fall in glucose usually amounting to 13 - 16 mgm. % per hour. The process ceased when the reducing substance had fallen to about 20 mgm. per cent. During the first few hours, the inorganic phosphorus either remained at a constant level or diminished slightly. At the time the free sugar was exhausted, usually within 6 to 8 hours, the inorganic phosphorus rose sharply and steadily to reach a concentration of about 20 to 25 mgm. per cent at about the fifteenth hour. This rise

in inorganic phosphorus took place at the expense of the organic acid-soluble phosphorus, generally designated "ester phosphorus". The increase occurred very rapidly in hypoglycemic blood from insulin-treated animals. It was delayed in normal or hypoglycemic blood for several hours by the addition of an excess of either levulose or dextrose.

Guest, in his paper, reviews the literature up to 1932 on this subject. The following facts were brought out:

1. When freshly drawn, defibrinated blood is incubated, the sugar falls. The inorganic phosphorus is low for the first few hours, remaining either stationary or falling slightly. Then, as glycolysis slows down, or stops from lack of sugar, inorganic phosphorus is liberated rapidly from the organic phosphorus compounds of the cells.

2. During glycolysis in blood, two main reactions take place:
   a) Synthesis of hexose phosphoric-acid esters, ar
   b) The hydrolysis of these esters.

3. According to the speed of the two reactions, any of the following three possibilities may occur, namely:
   a) Excess of synthesis, with the removal of inorganic phosphate;
   b) The two reactions may proceed at the same rate;
   c) Excess of hydrolysis, with liberation of inorganic phosphate.

4. Among the various factors which may influence glycolysis, and the synthesis and hydrolysis of esters are:
   a) The number of cells in a given sample (glycolysis occurs only in the cells).
   b) Haemolysis, since this arrests glycolysis but not the hydrolysis of esters.
   c) Dilution of the blood with isotonic salt solution merely slows glycolysis. Dilution of the serum with a phosphate mixture accelerates glycolysis more than does an equal dilution with NaCL or Ringer's solution.
   d) The addition of glucose and an appropriate phosphate buffer encourages synthesis of esters and consequently speeds up glycolysis.

e) The optimum pH for glycolysis, and approximately the optimum for ester synthesis, is about pH 7.8. Ester synthesis and glycolysis proceed only at an alkaline reaction, and are impeded even by lowering the pH to 7.3. Ester-hydrolysis on the other hand occurs over a pH range from 6.0 to 9.0. In the absence of sugar, it is most rapid at pH 8.0.

The claim that glycolysis can proceed only in an alkaline medium would seem to require qualification in view of the work of many investigators (3, 6, 25) who have shown that glycolysis does occur in slightly acid solutions such as ACD though at a greatly retarded rate. Lowering the pH definitely retards the rate of glycolysis.

Potassium in Glycolysis

The role of potassium in glycolysis is obscure. Harris (56) believes that the passage of potassium across the red-cell membrane during storage possibly is an effect of glycolysis, though glycolysis *per se* does not appear to be the only factor controlling its passage across the cell membrane. He and others have shown that some of the potassium lost from the cells of blood stored in citrate at refrigerator temperatures is regained when the temperature is raised to 25° C or to 37° C. Addition of glucose will increase the amount of potassium re-entering as long as the cell is glycolysing. Thus temperature and glycolysis both have an effect on the exchange of potassium.

EXPERIMENTAL

METHODS

Collection of the Blood

Blood was collected aseptically and in the usual manner from an antecubital vein and without aspiration from students and from patients at The Verdun Protestant Hospital (Mental). The collection flasks containing the preservative were fitted with rubber stoppers with a half inch hole in the center to admit the bleeding tube. The entire system was kept sterile and finally the hole in the large stopper was closed with a number "0" rubber stopper. For storage the flask was protected on top with a sterile covering of gauze and cellophane. Samples were removed from the flask periodically, by agitating the contents lightly to suspend the cells, and then removing a few ml. of blood with a sterile pipette. The small hole in the large stopper then was closed with a fresh sterile number "0" rubber stopper and after adding a drop or two of dilute formaldehyde to the gauze, the flask was covered as usual and replaced in the refrigerator which was kept at 5° C.

Preservative Solutions

Details of the preservative mixtures and the proportions of blood and preservative used in the various storage experiments are given below:

Experiment A

1. Isotonic (3.2%) trisodium citrate solution
   Isotonic (5.0%) C. P. dextrose solution
   Blood : Citrate : dextrose - 10 : 4 : 1
2. Same as above

3. Same as above

4. Same as above

Experiment B

1. Isotonic (3.2%) trisodium citrate $\cdot 2H_2O$
   7.7% C. P. dextrose solution
   Blood : citrate : dextrose - 5 : 1 : 2/3

2. Same as above

3. Baxter's ACD solution F - 9X
   Brilliant cresyl blue, 0.0032 M

Experiment C

1. Isotonic (3.2%) sodium citrate
   Isotonic (5.4% dextrose
   Blood : citrate : dextrose - 5 : 1 : 2/3

2. Isotonic (3.2%) citrate
   Isotonic (5.4%) dextrose
   Brilliant cresyl blue, 0.0032 M
   Ratio 5 : 1 : 2/3, as above

3. Baxter's ACD (F-9X)
   Ratio of blood to preservative - 5 : 1 2/3

Experiment D

1. Baxter's ACD
   Ratio of blood to preservative, 10 : 3

2. Isotonic (3.2%) sodium citrate
   Isotonic (5.0%) dextrose
   Brilliant cresyl blue, 0.0032 M
   Ratio blood : citrate : dextrose - 10 : 2 : 1

3. Isotonic (3.2%) trisodium citrate
   Isotonic (5.0%) dextrose
   Ratio of blood : citrate : dextrose - 10 : 2 : 1

4. Isotonic (3.2%) trisodium citrate
   Ratio of blood to citrate, 10 : 3

Experiment E

1. Isotonic (3.2%) trisodium citrate
   Isotonic (5.0%) dextrose
   Ratio of blood : citrate : dextrose - 10 : 2 : 1

2. Same as above

"ACD" designates citric acid-sodium citrate-dextrose preservative mixtures. Baxter's F-9x contains the following: 2.48 Gm USP Dextrose, 2.24 Gm USP Sodium citrate, 0.80 Gm USP citric acid per 100 ml of solution.

Experiment F

1. Isotonic (3.2%) trisodium citrate
   Isotonic (5.0%) dextrose
   Ratio of blood : citrate : dextrose - 10 : 1 : 2

Experiment G

1. Isotonic (3.2%) trisodium citrate
   Isotonic (5.0%) dextrose
   Ratio of blood : citrate : dextrose - 10 : 2 : 1

In the additional preservative solutions of this experiment the same amounts of citrate and dextrose were used and the ratio was kept the same but the following dyes were added:

2. Nile blue, 0.0032 M
3. Methylene blue, 0.0032 M
4. Evans' blue, 0.0032 M
5. Vital Red, 0.0032 M

Storage of the Blood Samples

Immediately after collection, the flasks containing the blood were stoppered with a sterile stopper and placed in the refrigerator at 4° C. The blood was swirled before sampling in order to ensure thorough mixing.

Chemical Methods

1. Sugar
   Sugar was determined by Somogyi's (57) modification of the Shaffer-Hartman method except for a slight modification which consisted of using 0.2 ml. of blood mixed with 7 ml. of water. To this was added 2.0 ml. of 0.7% copper sulfate and 0.8 ml. of 2.5% sodium tungstate

for precipitation of the proteins. Because of the dextrose contained in the preservative the above reduction in the amount of blood taken for analysis was necessary.

2. Pyruvic Acid
This compound was determined by the method of Friedemann and Haugen (58).

3. Lactic Acid
The determination of lactic acid at first gave considerable difficulty. In the beginning we used the method of Barker and Summerson as described by LePage (59). The procedure gave extremely irregular results and we were forced to investigate it and other methods at a very substantial cost of time and labor.

Our first attempt was to modify the procedure of Miller and Muntz. We employed fuming sulfuric acid to develop the color and concentrated sulfuric acid for dilution of the sample so that the color could be read using the 10-ml. aperture of the Evelyn photoelectric colorimeter. This procedure also gave erratic results. Continued experimentation showed that the use of fuming sulfuric acid was unnecessary but it became evident that the amount of water present in the sample was an important factor in determining the development of color intensity. The following experiment therefore was carried out to determine the amount of water necessary for maximum color development.

0.2 ml., containing 20 mcg., of a standard zinc lactate solution was used for all determinations. Water was added in varying amounts up to 2.8 ml. Concentrated sulfuric acid then

was added to the sample in the cold to make a final volume of 10.2 ml. The development of color was timed. By this means it was found that the maximum color developed by adding 1.3 ml. of water to the 0.2 ml. sample and by using 8.7 ml. of concentrated sulfuric acid. The color took between one and two hours to reach a maximum. This procedure gave consistently improved results, but occasionally for reasons unknown the method seemed to become erratic.

Kwiecinska (60), working at the same time, modified this method so as to overcome the difficulties, hence her modification was employed in the latter experiments. The procedure is as follows:

> The blood is precipitated by washing 0.2 ml. of blood into 7.1 ml. of water. To this is added 2.0 ml. 0.7% copper sulfate and 0.8 ml. of sodium tungstate. The mixture is then centrifuged and aliquots of the supernatant are removed for analysis.
>
> Ten ml. of concentrated sulfuric acid are added to 1.5 ml. aliquots of the sample protein-free filtrate. The sulfuric acid is added drop by drop to the sample in a colorimeter tube while it is being vigorously shaken in an ice water-bath. After addition of the acid is complete, the tube is stoppered and placed in a boiling water-bath for exactly 5 minutes. It is then removed and cooled. One drop of 4% copper sulfate is added and mixed and then two drops of p-hydroxydiphenyl reagent (prepared according to LePage (59)) are added and mixed. Color is developed by allowing the tube to stand at room temperature for one hour. At the end of this time the tube is again placed in a boiling water-bath for ninety seconds after which it was cooled in a cold water-bath. The color then is read in an Evelyn photoelectric colorimeter using the 565 mu. filter.

4. Phosphate

The method of Fiske and Subbarow (61) was used for the determination of inorganic phosphate both before and after

liberation from the esters. Color was developed by adding ammonium molybdate and amino naphthal sulfonic acid to the samples. The color was then read in an Evelyn photoelectric colorimeter using a 660 mu. filter.

Phosphate was partitioned in the following manner:

(a) Inorganic phosphate was determined on an unhydrolysed aliquot of the blood filtrate after the proteins had been precipitated with trichloroacetic acid.

(b) Easily hydrolysable phosphate is represented by the additional phosphate liberated on heating in a boiling water-bath with 2 N HCl for 100 minutes. The value for this fraction is obtained by subtracting the inorganic value from the combined inorganic and easily hydrolysable value obtained after hydrolysis. Compounds found in this section are adenosinetriphosphate, phosphocreatin, and hexosephosphates.

(c) Total organic acid-soluble phosphate. This is determined by digesting an aliquot of the blood filtrate with 10 N sulfuric acid on an electric wire-furnace. The value obtained from the 100 minute hydrolysis subtracted from the total organic acid-soluble phosphate leaves the value representing diphosphoglycerate. Andreae ( ) cites evidence to show that this is true.

5. Haemoglobin

In order to ensure that the red cells in the samples removed for analysis had been suspended uniformly, a haemoglobin determination was carried out on each specimen taken for analysis. 0.02 ml. of blood was added to 10 ml. of distilled water in a colorimeter tube. One drop of 10% ammonium hydroxide was added to haemolyze the cells in the solution. The color intensity was then read in an Evelyn colorimeter using a 540 mu. filter.

Calculation of Results

In order to make the analytical results readily comparable they were calculated and expressed in millimoles per

liter. The values were first determined in mgm. % of blood and conversion was accomplished by use of the following formulae:

Sugar, lactic acid, pyruvate :

$$\frac{\text{Mgm. \% x 10}}{\text{Mol. Wt.}} = \text{Millimoles per liter}$$

Diphosphoglycerate :

Total acid-soluble phosphate - 100 minutes hydrolysis value = mgm. % diphosphoglycerate phosphate

$$\frac{\text{mgm. \% diphosphoglycerate phosphate x 10}}{2 \times 95} = \text{millimoles per liter of diphosphoglycerate}$$

EXPERIMENTAL RESULTS

The experimental data are presented in the following tables and certain of the corresponding graphical presentations will be found beginning with page 37. All values are expressed as millimoles per liter. The experiments lasted for varying periods of time up to 72 days, and analyses were done at regular intervals during the period. The preservatives used in the various experiments are listed in the preceding section and are indicated in the tables.

SERIES A

The object of this series of experiments was to follow the destruction of sugar and the formation of lactic acid. The work of Andreae (33) showed that there are characteristic rises in the reducing power of stored blood followed by equally abrupt falls. This series of experiments was designed to seek confirmation of this. It was also desired to find out if the lactic acid formed could be accounted for by the glucose destroyed. A comparison of the four experiments will be made at the end of the series.

Experiment 1.

In this experiment there are rises in reducing power occurring on the ninth, 16th and 37th days, and a plateau on the 29th day. Glycolysis proceded during the whole experiment.

SUGAR AND LACTIC ACID IN BLOOD PRESERVED IN CITRATE-DEXTROSE SOLUTION
(See A-1, pg.27)
Millimoles per liter

Day	Sugar	Lactic Acid
0	26.67	3.00
3	25.28	3.33

Experiment 1 continued -

Day	Sugar	Lactic Acid
5	21.00	6.33
7	19.61	4.33
9	20.00	1.67
11	14.44	--
14	10.56	5.67
16	14.17	15.89
18	13.50	19.67
21	11.11	13.33
23	9.44	12.00
25	7.94	9.33
27	7.78	8.89
29	5.50	8.87
32	5.50	13.33
35	3.67	35.00
37	5.61	10.33
42	4.61	38.00
44	3.61	33.67
72	1.56	32.00

Millimoles glucose destroyed: 25.11
Millimoles lactate formed : 29.00

Experiment 2.

Rises occurred in reducing power on the 9th, 16th and 29th days. Plateaux occurred on days 23 and 37 in this blood.

Sugar was destroyed during the whole period.

SUGAR AND LACTIC ACID IN BLOOD PRESERVED IN CITRATE-DEXTROSE SOLUTIOI

(See A-2, pg.27)

Millimoles per liter

Day	Sugar	Lactic Acid
0	26.50	3.33
3	26.11	4.33
5	19.61	5.67
7	19.44	4.33
9	21.61	1.33
11	20.78	--
14	16.83	4.00
16	17.50	12.56
18	16.94	10.00
21	14.44	9.00
23	14.61	7.33
25	12.50	10.67
27	12.22	7.33
29	13.06	10.67
32	12.22	12.00
35	11.11	26.67
37	11.28	7.33
42	9.67	33.33
44	9.44	25.00
72	5.89	28.67

Millimoles glucose destroyed : 20.61
Millimoles lactate formed : 25.34

Experiment 3.
(See fig. 1 , pg.37)

This blood behaved in a different manner from the others in the series. Glycolysis appeared to proceed at a much faster rate, and the minimum reducing value was approached about the 26th day. There were slight rises in sugar on the 10th and 15th days, and a plateau occurred beginning with the 4th day.

SUGAR AND LACTIC ACID IN BLOOD PRESERVED IN CITRATE-DEXTROSE SOLUTION
(See A-3, pg.28)
Millimoles per liter

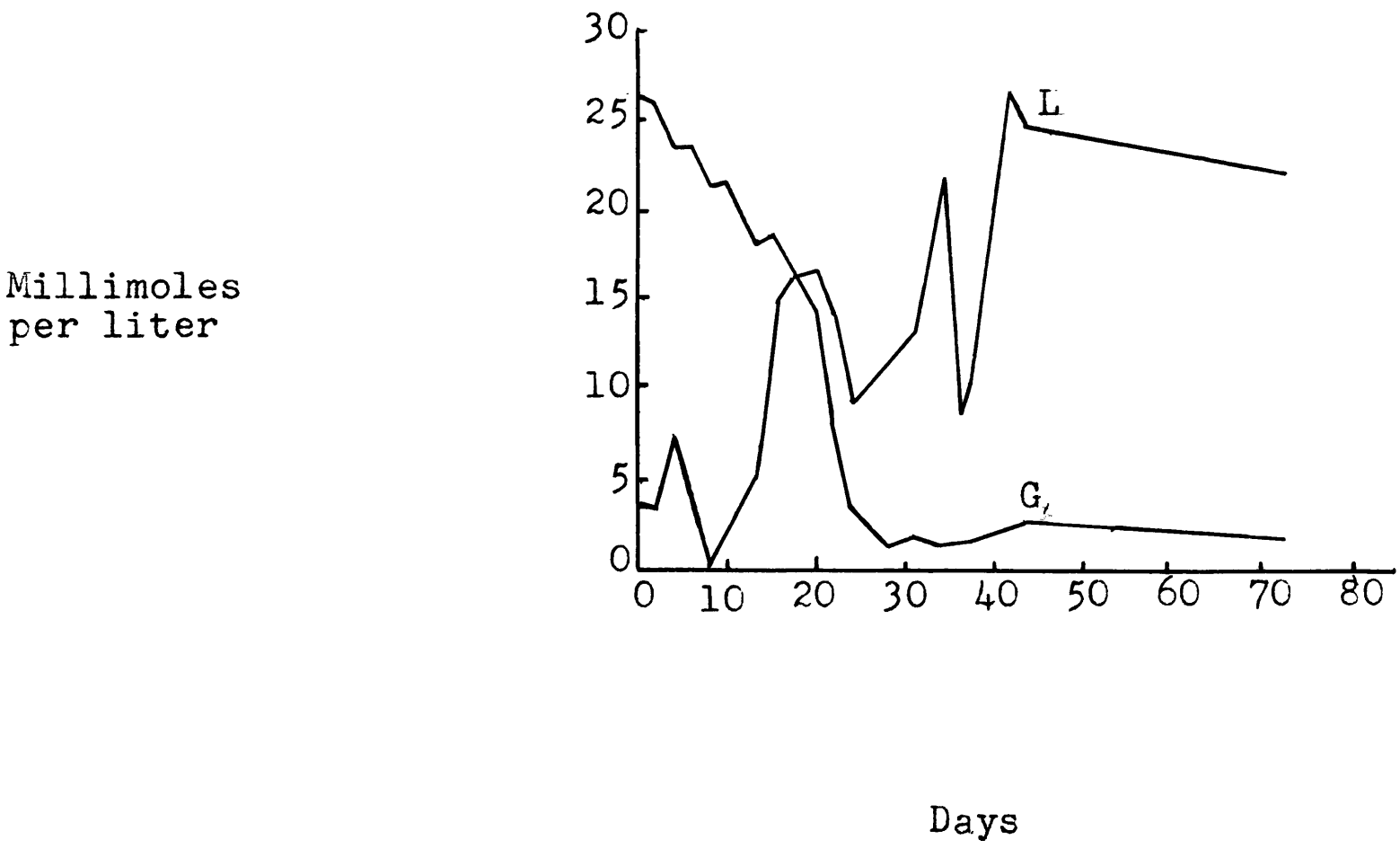
Day	Sugar	Lactic Acid
0	26.94	3.33
2	26.00	3.33
4	23.61	7.33
6	23.61	4.00
8	21.39	0.00
10	21.56	--
13	18.50	5.00
15	18.78	14.33
17	17.39	16.00
20	14.61	16.67
22	7.11	13.67
24	3.39	9.00
26	1.94	10.33
28	1.29	11.33
31	2.11	13.33
34	1.56	21.67
36	1.67	8.33
41	1.94	26.00
43	2.39	24.67
71	1.56	22.00

Millimoles glucose destroyed : 25.38
Millimoles lactate formed : 18.67

Experiment 4.

Rises in sugar in this experiment will be noted on days 15, 22-26, and 43. The last rise was only a slight one. Plateaux occurred beginning on days 4 and 31. Sugar fell throughout the entire experiment.

Fig. 1
Sugar And Lactic Acid In Blood Preserved In
Citrate-Dextrose Solution (A-3)



Legend;
L : Lactic Acid
G : Glucose
P : Pyruvic Acid
D : Diphosphoglycerate

SUGAR AND LACTIC ACID IN BLOOD PRESERVED IN CITRATE-DEXTROSE SOLUTION
(See A-4, pg.28)
Millimoles per liter

Day	Sugar	Lactic Acid
0	24.89	3.67
2	24.72	5.00
4	21.83	9.00
6	21.83	5.33
8	21.83	--
10	21.56	--
13	17.67	5.67
15	18.33	13.33
17	18.06	17.33
20	16.11	15.33
22	11.67	13.00
24	11.83	10.00
26	12.22	11.67
28	11.28	15.67
31	11.39	10.00
34	11.39	24.67
36	10.00	44.00
41	6.33	47.00
43	6.56	50.33
71	3.39	35.33

Millimoles glucose destroyed : 21.50
Millimoles lactate formed : 31.66

Comparison of Bloods of Series A

The bloods of Series A were donated by four healthy students and collected into citrate-dextrose. It will be seen from the above tables that disturbances in the disappearance of sugar took place at fairly regular intervals, namely on or about days 4, 9, 16, 22, 29, and 37, and once on day 43. These disturbances showed apparent inhibition of sugar removal, and were manifest either as actual rises in reducing substance or as plateaux.

The overall increase in the lactic acid content of the bloods was less than expected, but this may be attributable to difficulties encountered in the method used for the determination of lactic acid at this time.

SERIES B

The experiments of this series were designed to compare the glycolytic process in three different types of preservative solutions. They were to be citrate-dextrose, citrate-dextrose with added Brilliant Cresyl Blue, and Baxter's ACD. Inadvertently in the preparation of the preservatives, the dye was placed in the Baxter's solution instead of the second citrate-dextrose. It was decided to continue the experiment for about a week or so to determine what the effect of the dye would be. It was anticipated that pyruvate formation would be affected since the dye can act as a hydrogen acceptor. The increased pyruvate and the decreased lactic acid bear out the expectation. The blood used in this series was given by a single donor.

Experiment 1.- Blood in Citrate-Dextrose

It will be noted in the following table that there was an initial rise in reducing substances which continued throughout the experimental period. For this reason no comparison between the amount of sugar destroyed and the amount of lactate formed can be made. The diphosphoglyceric acid, pyruvate, and lactic acid all rose.

GLYCOLYTIC BEHAVIOR OF BLOOD PRESERVED IN CITRATE-DEXTROSE SOLUTION
(See B-1, pg.28)
Millimoles per liter

Day	Sugar	Lactic Acid	Diphosphoglycerate	PyruvicAcid
0	64.4	3.19	1.43	0.12
3	89.6	5.10	2.14	0.20
6	91.1	9.53	2.41	0.22

Experiment 2. - Blood in Citrate-Dextrose

The same general behavior is exhibited in this experiment as in experiment 1 above, except for pyruvic acid. Pyruvate rose on the third day, but fell on the 6th day to a level between those found initially and on the 3rd day.

GLYCOLYTIC BEHAVIOR OF BLOOD PRESERVED IN CITRATE-DEXTROSE SOLUTION
(See B-2, pg.28)
Millimoles per liter

Day	Sugar	Lactic Acid	Diphosphoglycerate	Pyruvic Acid
0	44.4	2.53	1.62	0.13
3	65.1	4.58	2.16	0.19
6	76.7	8.10	2.42	0.17

Experiment 3. - Blood in Baxter's ACD with Added Dye

The most striking feature of this experiment is the large increase in pyruvic acid. This compound increased steadily and by the 6th day had reached a concentration of 2.72 millimoles per liter. The sugar content rose on the 3rd day, but fell again on the 6th day to a value between those of days 0 and 3. The diphosphoglycerate fell on the 3rd day and rose again on the 6th. Lactic acid increased throughout the experiment.

GLYCOLYTIC BEHAVIOR OF BLOOD PRESERVED IN BAXTER'S ACD SOLUTION CONTAINING BRILLIANT CRESYL BLUE
(See B-3, pg.28)
Millimoles per liter

Day	Sugar	Lactic Acid	Diphosphoglycerate	Pyruvic Acid
0	31.7	1.76	1.26	0.69
3	56.7	2.68	0.38	1.73
6	53.3	5.50	2.03	2.72

Comparison

It will be noted that in all three cases the sugar content rose during the first three days of storage. In the third experiment alone, there was a decrease on the sixth day. As this was not anticipated, the sugar method was suspected.

However, subsequent experiments showed that the increase in reducing substances early during storage is a fairly constant occurrence. The source of the reducing substance as yet has not been established.

In the first two experiments of this series, the diphosphoglycerate showed a marked rise on the third day. In the blood preserved in ACD with the dye, there is a fall to a very low level at this time. At the end of the experimental period diphosphoglycerate was still approximately 0.40 millimoles lower in experiment 3 than in 1 and 2.

In the bloods of Experiments 1 and 3, pyruvic acid increased throughout the experiment; in blood 2, there was a fall on the sixth day. However, in Experiments 1 and 2, the pyruvic acid content at no time exceeded 0.22 millimoles; but in Experiment 3, there were 1.72 millimoles of pyruvate on the third day and 2.72 millimoles on the sixth, over 12 times as much as in either of the first two experiments.

The lactic acid values are worthy of attention. In the first two bloods lactic acid was 50%, at least half, again as high as in Experiment 3. This situation is discussed in greater detail on page 83 .

As the experiment was terminated while the sugar values were higher than the initial values, no ratio of the glucose destruction to lactate formation can be presented.

SERIES C.

The experiments comprising this series were essentially a repetition of Series B, however, in this case the dye was added to citrate-dextrose. There are three types of preser-

vative solution: citrate-dextrose, citrate-dextrose with added dye, and Baxter's ACD.

In this series of experiments, and those to follow, ratios of the substances disappearing to those formed will be worked out as follows:

The amount of diphosphoglycerate in millimoles per liter disappearing will be added to twice the number of millimoles of sugar disappearing. Doubling the amount of sugar is done in order to make a theoretical ratio with the sum of pyruvic acid and lactate of 1 : 1 possible. The ratio at the end of each experiment will thus be a comparison of the sum of those substances destroyed to the sum of those formed.

Experiment 1. Blood in Citrate-Dextrose

The sugar showed a rise on the 4th day and one commencing on the 22nd day. Lactic acid showed a steady rise throughout the experimental period except for a high value of 30.80 millimoles on the 22nd day. This abnormal value is probably the result of a fluctuation in the analytical method. Diphosphoglycerate was steady for the first few days and then showed a marked rise on the 7th day. Following this there was a rapid fall until the 19th day when a value of 0.82 millimoles was reached. From this point onwards the fall in diphosphoglycerate was very slow. Pyruvate rose slightly on the third day only to fall again by the 7th. There was a moderate rise on the 11th day followed by a marked increase on the 15th. An almost constant value was attained by the 19th day.

GLYCOLYTIC BEHAVIOR OF BLOOD PRESERVED IN CITRATE-DEXTROSE SOLUTION
(See C-1, pg.28)

	Millimoles per liter		
Day	Sugar	Lactic Acid	Diphosphoglycerate	Pyruvic Acid
0	34.5	2.27	2.72	0.17
3	47.8	--	2.71	0.20
7	40.0	8.47	4.25	0.16
11	36.7	10.57	3.79	0.96
15	31.1	13.12	2.32	1.93
19	24.4	16.50	0.82	2.18
22	31.9	30.80	0.74	2.14
26	32.9	18.41	0.57	2.12
30	31.8	19.21	0.53	2.12

Summary.

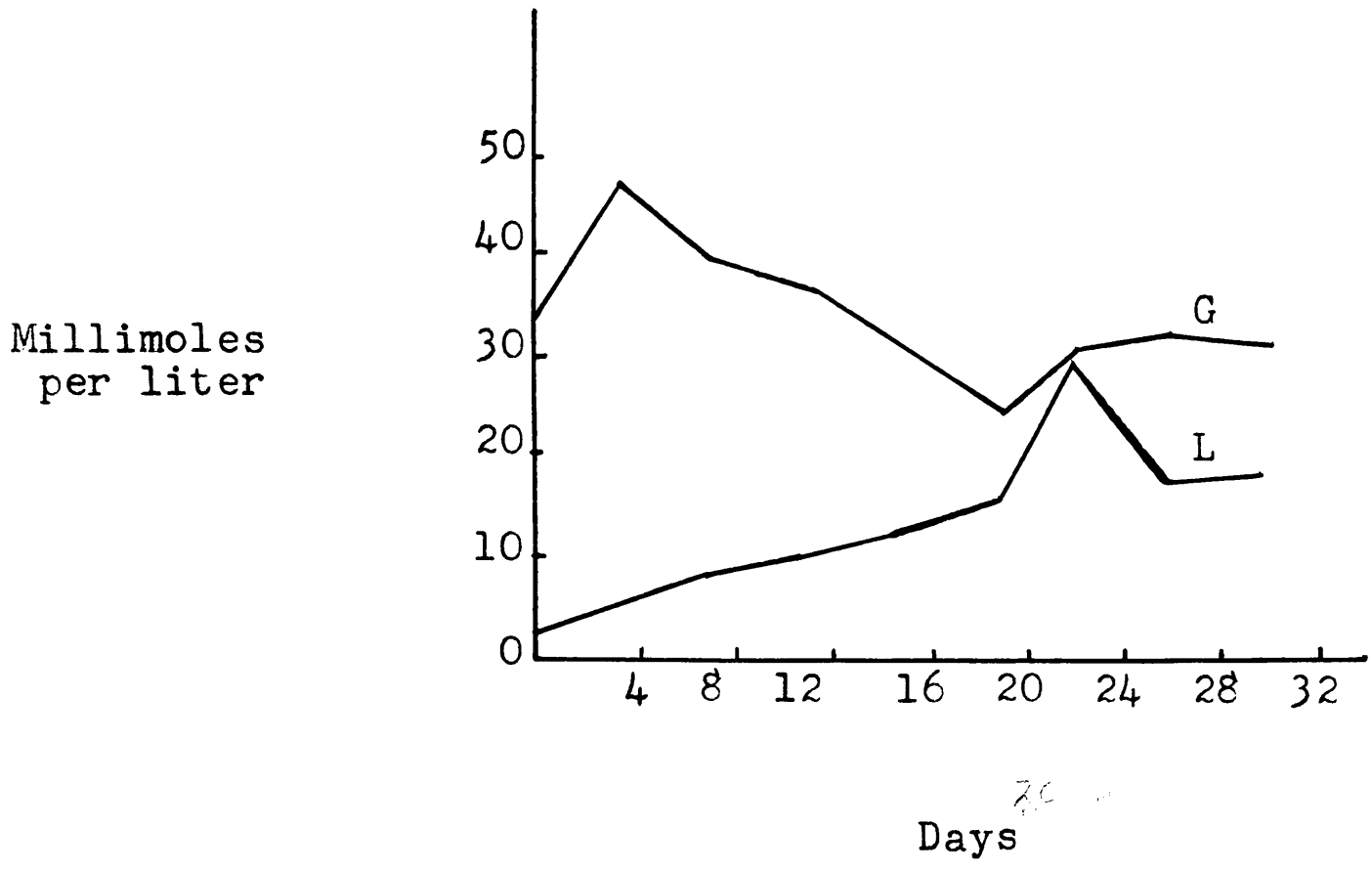
	Destroyed	Formed
Glucose	2.7	
	x 2	
	5.4	
DPG	2.19	
Lactate		16.94
Pyruvate		1.95
Total	7.59	18.89

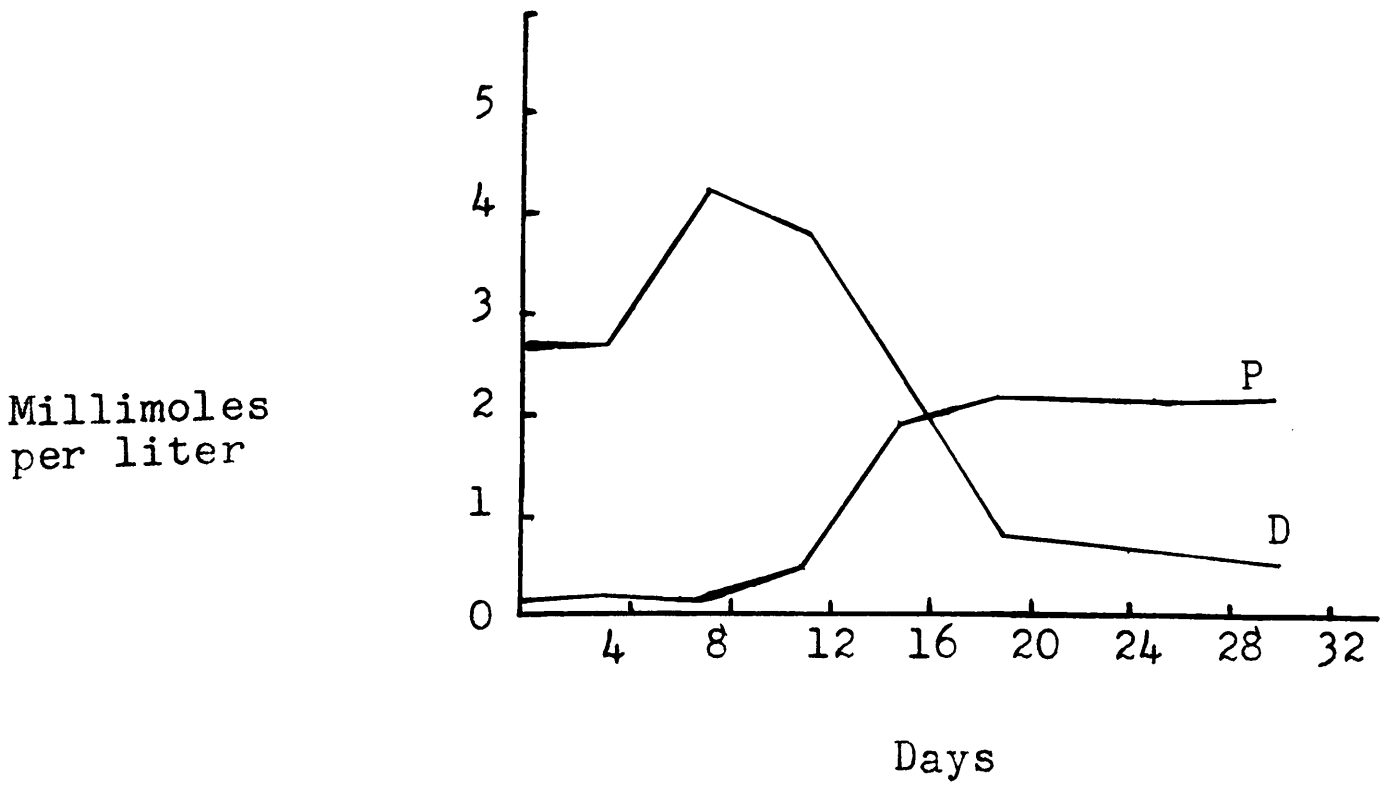
Ratio: approximately 1 : 2

Experiment 2. Blood in Citrate-Dextrose with Added Brilliant Cresyl Blue.

It will be noted in the following table that a great deal more sugar was destroyed in this experiment than in the preceding one. The sugar content of the blood showed increases on days 11 and 19. Lactic acid showed a steady increase until the 26th day except for a marked rise on day 22, which is believed to have no significance. Diphosphoglycerate increased slightly on the 3rd day and markedly on the 7th. It then fell until the 19th day when the values remained fairly constant at considerably more than 1 millimole. This is a high terminal concentration. The pyruvic acid, as might be expected from Series B above, exhibited the most interesting behavior.

Fig. 2
Glycolytic Behavior Of Blood Preserved In
Citrate-Dextrose Solution (C-1)





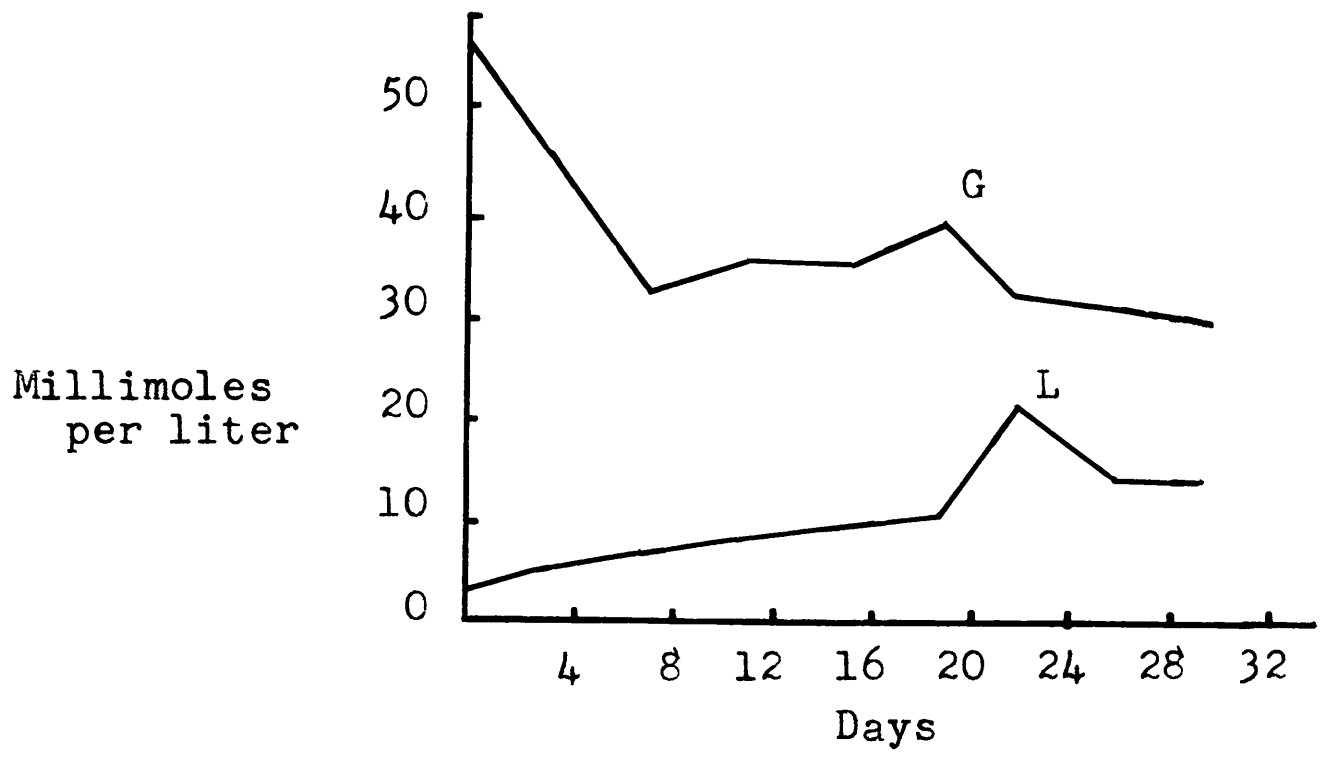
The initial concentration of this substance was 1.83 millimoles. This is well above the normal range. It should be explained at this point that in all of these experiments a time interval of about three hours existed between the time blood was drawn and the first sample taken. This was done, not only as a matter of convenience, but also to allow time for intimate mixing of the blood with the preservative solution and for cooling to refrigerator temperature in order that sampling at all times would be uniform. Thus there was a period in which the action of the dye would take place before the first sample was removed. The disadvantage of this is offset because the blood in all of the experiments of this series came from the same donor and the initial concentration of pyruvate of the citrate-dextrose sample could be assumed to be the approximate concentration in the dye preservative at the time of collection.

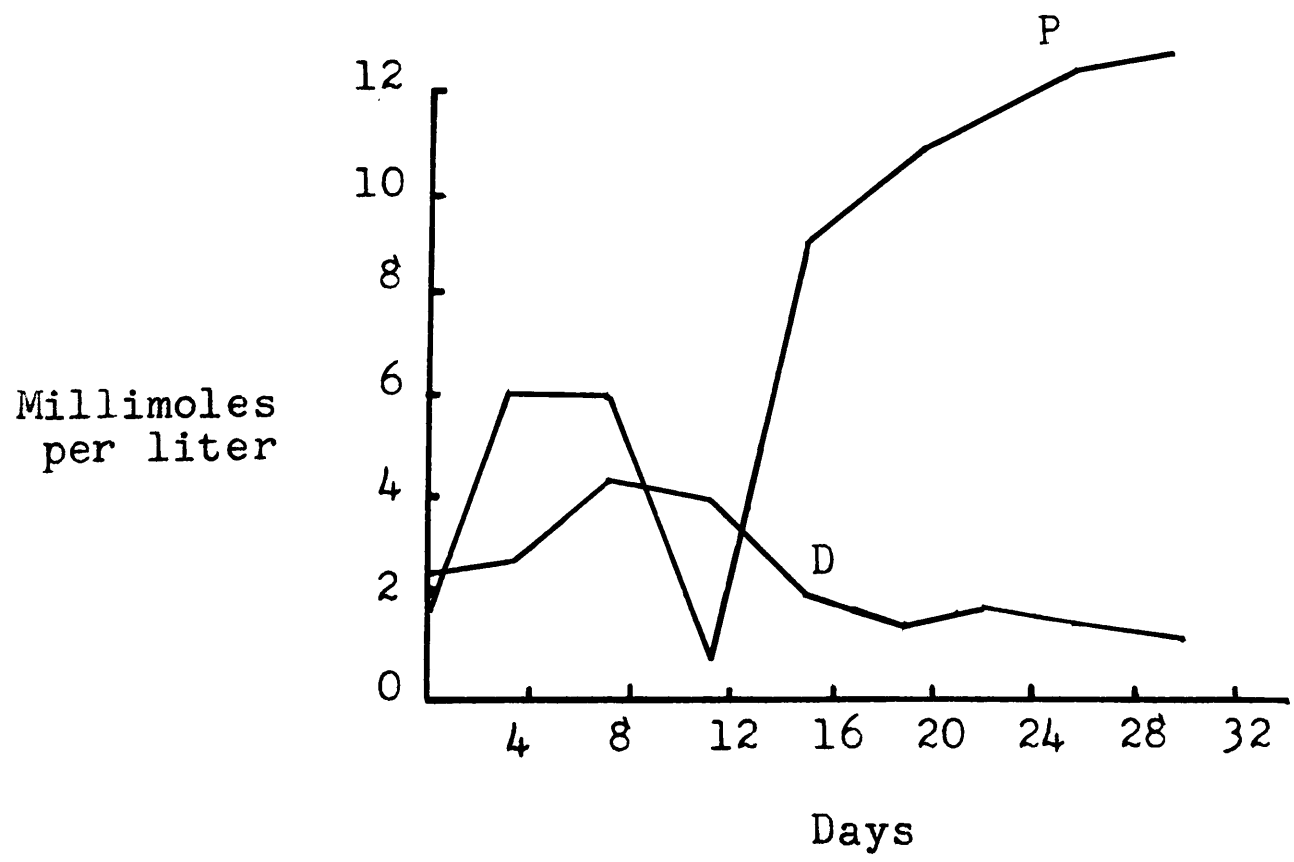
The pyruvate showed an increase in the first three days. After this there was a slight fall on the 7th day and a greatly diminished concentration of 0.76 millimoles on the 11th day. From this time on the pyruvic acid content increased at an extremely rapid rate until the 26th day when it started, apparently, to level off.

GLYCOLYTIC BEHAVIOR OF BLOOD PRESERVED IN CITRATE-DEXTROSE SOLUTION CONTAINING ADDED BRILLIANT CRESYL BLUE
(See C-2, pg.28)

		Millimoles per liter		
Day	Sugar	Lactic Acid	Diphosphoglycerate	Pyruvic Acid
0	55.6	2.35	2.51	1.83
3	46.7	4.77	2.68	6.08
7	32.9	6.39	4.25	5.91
11	36.7	8.12	3.89	0.76
15	36.2	9.17	2.12	8.90
19	40.0	10.63	1.37	10.58
22	33.33	21.44	1.78	11.29
26	31.8	14.67	1.35	12.40
30	30.00	14.13	1.24	12.75

Fig. 3
Glycolytic Behavior Of Blood Preserved In Citrate-Dextrose Solution Containing Added Brilliant Cresyl Blue (C-2)





Summary:

	Destroyed	Formed
Glucose	25.6	
	x 2	
	51.2	
DPG	1.27	
Lactate		11.78
Pyruvate		10.92
Total	52.47	22.70

Ratio: approximately 2 : 1

Experiment 3. Blood in Baxter's ACD

Sugar in the blood of this experiment commenced a rise on the 11th day which continued until the 19th. After this time there was a steady fall until the end of the experimental period. Lactic acid showed a constant rise until the 26th day at which time the content of this substance remained constant, except for an exceptional rise on the 22nd day, which again may be disregarded. Diphosphoglycerate fell slightly on the third day but rose a considerable amount on the 7th. After this there was a rapid fall until the 19th day when a moderately steady value was reached. There was, however, a slight rise on the 22nd day. Pyruvic acid increased on the 3rd day, fell again until the 11th day, and then remained approximately constant for a few days. On the 19th day a steady rise in pyruvate commenced which lasted throughout the remainder of the experimental period.

GLYCOLYTIC BEHAVIOR OF BLOOD PRESERVED IN BAXTER'S ACD SOLUTION
(See C-3, pg.28)

	Millimoles per liter			
Day	Sugar	Lactic Acid	Diphosphoglycerate	Pyruvic Acid
0	58.9	1.47	2.29	0.49
3	53.3	3.34	2.01	0.93
7	44.4	4.99	3.26	0.57
11	55.1	5.54	3.05	0.35
15	58.4	6.90	1.92	0.38
19	60.0	8.58	0.51	0.45
22	54.00	17.31	0.97	0.55
26	51.11	10.93	0.57	0.70
30	45.55	10.93	0.30	0.90

Summary:

	Destroyed	Formed
Glucose	13.35	
	x 2	
	26.70	
DPG	1.99	
Lactate		9.46
Pyruvate		0.41
Total	28.69	9.87
Ratio:	approximately	3 : 1

Comparison.

In Experiment 1 where the blood was preserved in citrate-dextrose solution, the sugar content demonstrated the smallest fall and the overall increase in lactic acid was greatest. The small decrease in sugar was contrary to expectation. The greatest fall in sugar occurred in the blood preserved with the dye. The final lactic acid level here was lower than in Experiment 1, and this is not surprising because since the dye is a hydrogen acceptor, it would be expected to compete with the pyruvic acid for the hydrogen carried by the reduced DPN in the glycolytic cycle shown on page 17. This would result in diminished lactic acid formation and increased pyruvate, and was actually found. More sugar was apparently removed from the blood stored in ACD than from the one in citrate - dextrose solution. This is not what one would expect from the reports of various workers (3) who say that glycolysis is retarded in blood stored in ACD. A possible explanation of this is offered later on page 77. The terminal lactic acid concentration was lower, however, than that found in citrate-dextrose.

The diphosphoglycerate content of the blood in ACD was lower than that of the blood in citrate-dextrose. This is in

accordance with Rapoport (3) who says that in ACD there is an immediate rise in inorganic phosphorus at the expense of the organic acid-soluble phosphorus compounds.

There is an interesting fact to be noted in the relationship between diphosphoglycerate and pyruvic acid. In the second experiment (containing the dye) the pyruvate level was very much higher than in the other two media, and the diphosphoglycerate was a great deal higher also. In the third experiment (in ACD), the concentration of both of these compounds was the lowest.

SERIES D.

All of the bloods reported in this series were given by a single donor. The object of this set of experiments was to confirm the findings of the previous one. One other preservative, citrate (see D-3, pg.28), was added to this set. Thus there are four experiments to be reported here. Blood is stored in citrate-dextrose, citrate-dextrose with added Brilliant Cresyl Blue, citrate, and Baxter's ACD.

Experiment 1. - Blood in Baxter's Solution

There are three rises in the sugar content of this blood occurring on the 4th, 10th, and 18th days. After the 18th day, the rate of sugar destruction is slow. Lactic acid showed a steady increase until fluctuations became apparent after the 22nd day. Diphosphoglycerate commences falling immediately and reaches a fairly constant low level about the 10th day. Pyruvic acid rose on the 4th day and then fell until the 14th. Afterwards it commenced to rise and continued rising until the end of the experiment.

GLYCOLYTIC BEHAVIOR OF BLOOD PRESERVED IN BAXTER'S ACD SOLUTION
(See D-1, pg.28)

		Millimoles per liter		
Day	Sugar	Lactic Acid	Diphosphoglycerate	Pyruvic Acid
0	25.4	1.98	2.1	0.38
4	26.1	5.46	1.13	0.97
7	22.7	8.69	--	0.83
10	23.3	8.89	0.31	0.44
14	20.6	12.56	0.71	0.36
18	22.5	18.33	0.61	0.45
22	21.4	17.22	0.76	0.56
26	21.3	24.44	0.50	0.72
30	20.8	22.44	0.29	0.91

Summary:

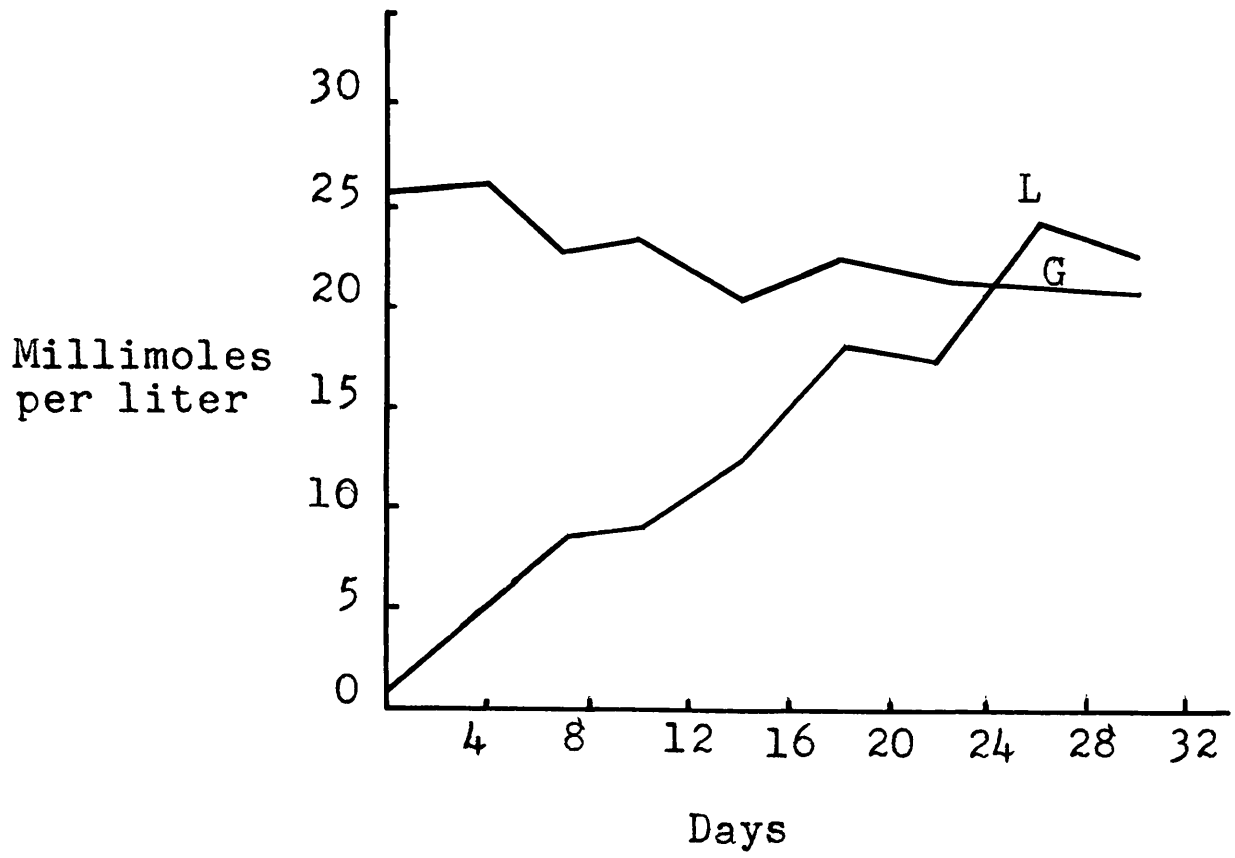
	Destroyed	Formed
Glucose	4.6	
	x 2	
	9.2	
DPG	1.81	
Lactate		20.46
Pyruvate		0.53
Total	11.01	20.99

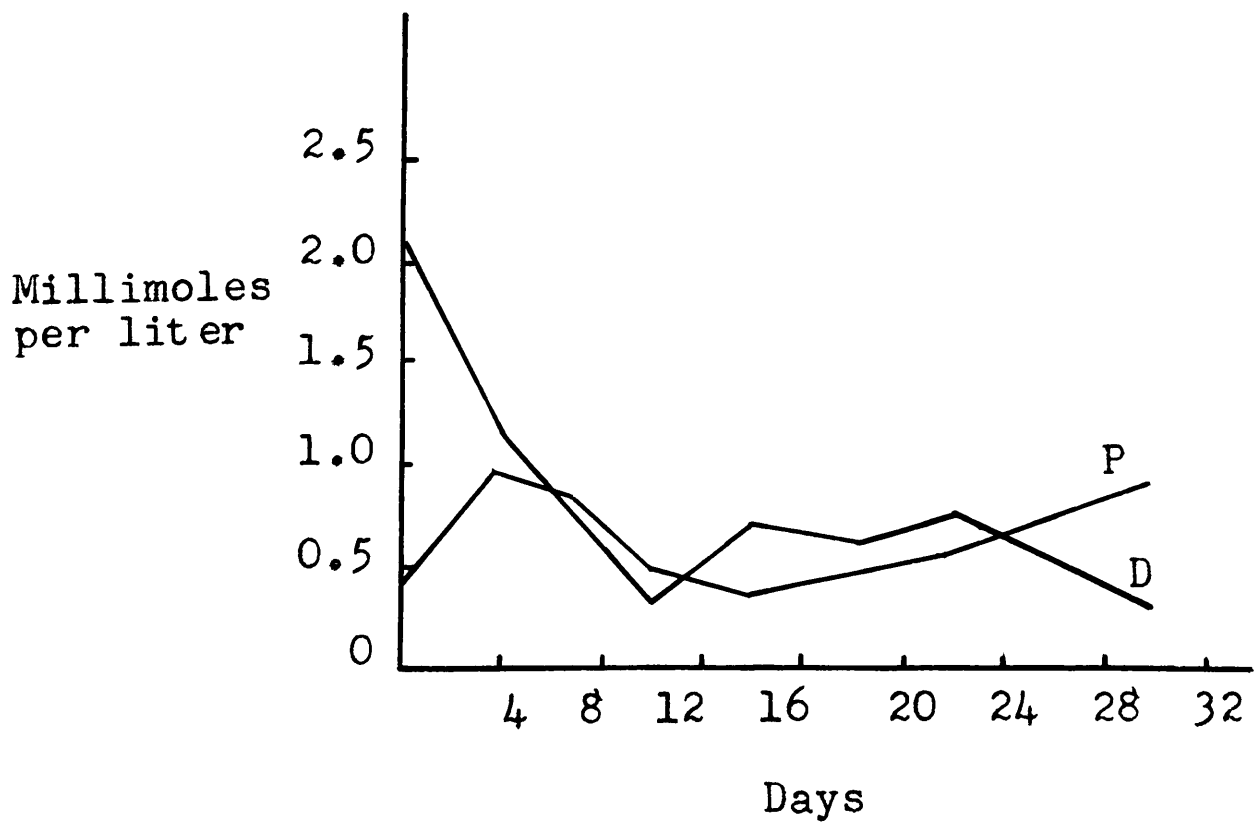
Ratio: approximately 1 : 2

Experiment 2. Blood in citrate-dextrose with Added Brilliant Cresyl Blue.

The sugar in this blood showed a plateau beginning with day 4 and a rise on day 18. Lactic acid increased until the 26th day when a slight fall occurred. There was a fall on the 22nd day, but this is believed to be due to a fluctuation in the method. The concentration of diphosphoglycerate remained fairly steady for the first few days. A rise occurred on the 10th day which was followed by a steady fall until a concentration of 0.84 millimoles was reached on the 30th day. The pyruvate content increased on the 4th day, fell on the 7th, and then rapidly increased until the 18th day. On the 22nd day there was again a moderate fall which was followed by increased values until the end of the experiment.

Fig. 4
Glycolytic Behavior Of Blood Preserved In
Baxter's ACD Solution (D-1)





GLYCOLYTIC BEHAVIOR OF BLOOD PRESERVED IN CITRATE-DEXTROSE SOLUTION WITH ADDED BRILLIANT CRESYL BLUE
(See D-2, pg. 28)

		Millimoles per liter		
Day	Sugar	Lactic Acid	Diphosphoglycerate	Pyruvic Acid
0	21.0	1.98	2.3	1.11
4	15.0	6.45	2.25	4.13
7	15.0	8.43	--	3.62
10	11.4	8.11	2.53	4.95
14	11.1	11.44	1.86	6.71
18	16.9	18.00	1.76	10.39
22	7.4	16.44	1.37	9.43
26	6.6	26.67	1.11	10.91
30	4.2	26.11	0.84	11.63

Summary:

	Destroyed	Formed
Glucose	16.8	
	x 2	
	33.6	
DPG	1.46	
Lactate		24.13
Pyruvate		10.52
Total	35.06	34.65

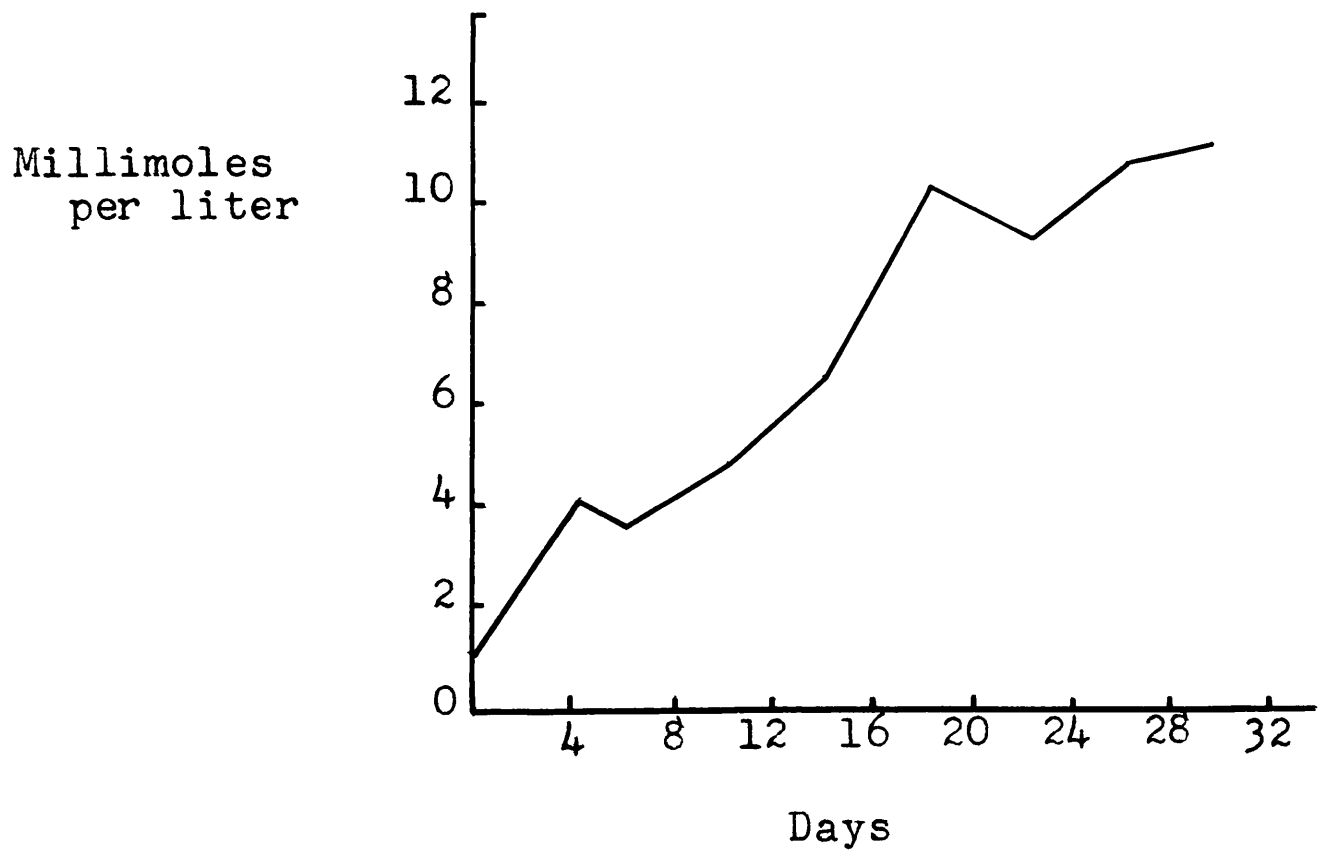
Ratio: approximately 1 : 1

Experiment 3. Blood in Citrate.

The sugar concentration fell immediately and reached its minimum level about the 7th day. No rises were noted except for fluctuations in the non-sugar reducing substances after the sugar was exhausted. Lactic acid rose until the 7th day, fell until the 14th, and rose again on the 18th day. This was followed by a fall on the 22nd day, and then there was a steady rise until the end of the experiment. The values for the 18th and 30th days are very close, so the lactic acid may have reached its greatest concentration about the 18th day and fluctuated around this level from this time onwards.

The diphosphoglycerate content fell slightly on the 4th day and reached its minimal concentration about the 10th.

Fig. 5
Behavior Of Pyruvate In Blood Preserved In Citrate-Dextrose Solution Containing Added Brilliant Cresyl Blue (D-2)



Pyruvic acid showed a marked increase on the 7th day and again on the 10th. After this there was a gradual rise until the 18th day which was followed by a fall on the 22nd. From this time onwards the pyruvate content of the blood showed a small continuous rise.

GLYCOLYTIC BEHAVIOR OF BLOOD PRESERVED IN CITRATE
(See D-3, pg. 28)

		Millimoles per liter		
Day	Sugar	Lactic Acid	Diphosphoglycerate	Pyruvic Acid
0	4.3	2.38	2.2	0.17
4	2.1	8.03	2.04	0.21
7	0.7	9.13	--	1.72
10	1.0	8.33	0.17	4.40
14	0.8	8.11	0.39	5.06
18	1.6	15.00	0.47	5.29
22	0.8	12.44	0.50	4.40
26	0.8	13.33	0.08	4.67
30	0.8	15.56	0.32	4.74

Summary:

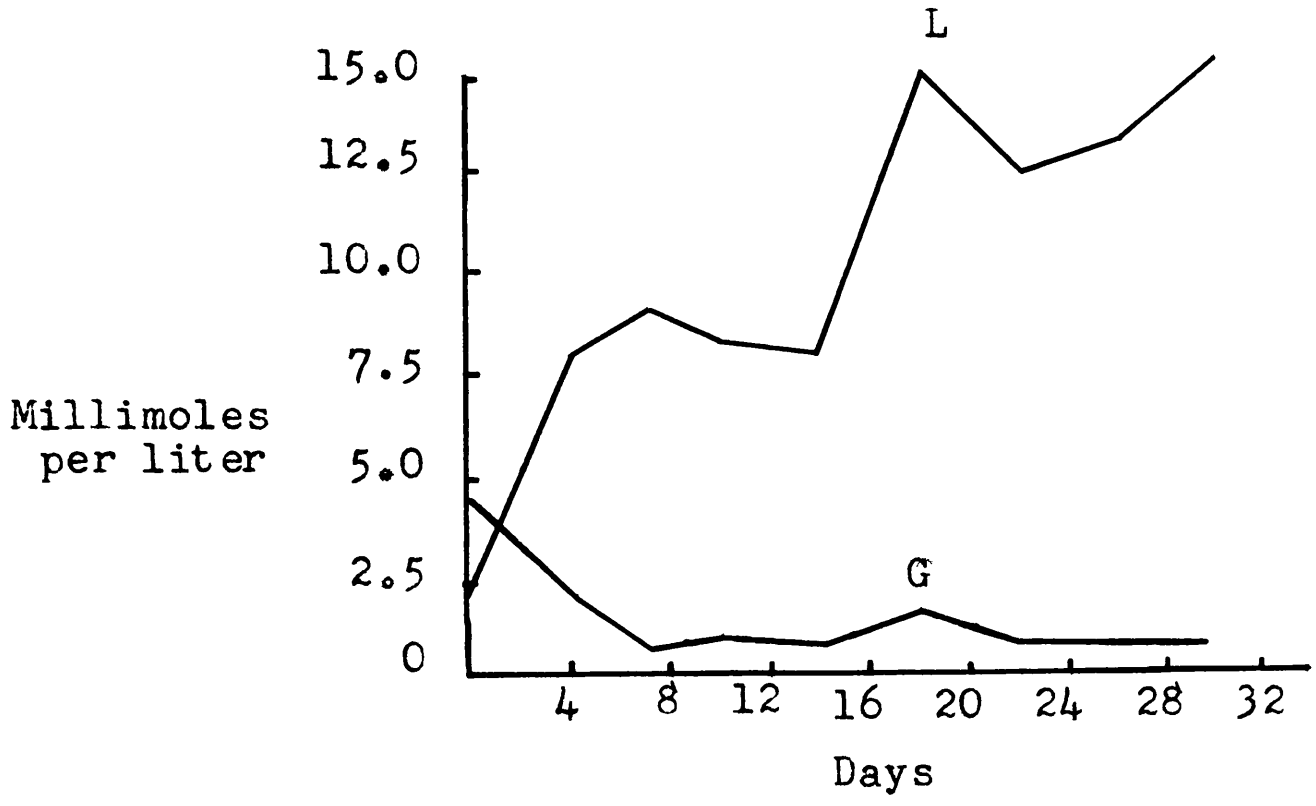
	Destroyed	Formed
Glucose	3.5	
	x 2	
	7.0	
DPG	1.88	
Lactate		13.18
Pyruvate		4.57
Total	8.88	17.75

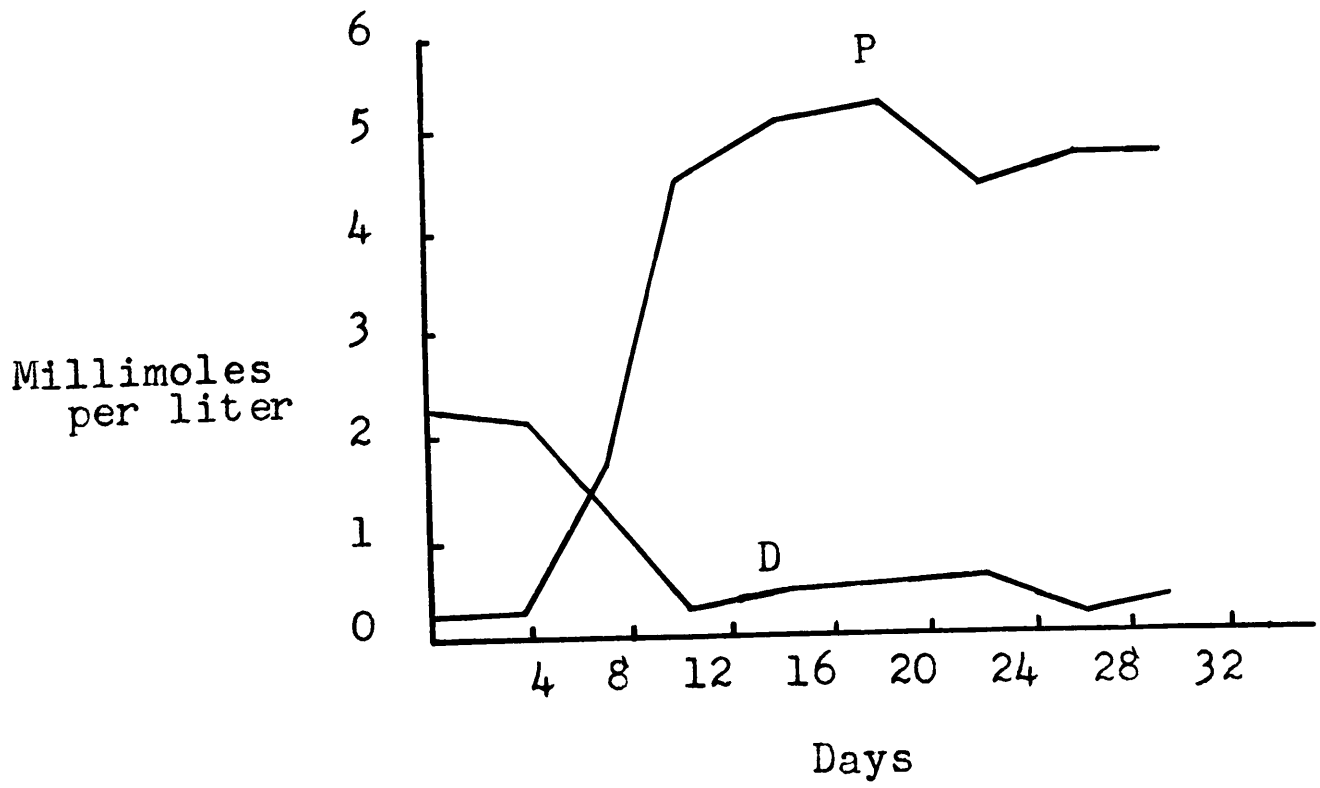
Ratio: approximately 1 : 2

Experiment 4. Blood in Citrate-Dextrose.

There is a slight rise in the concentration of sugar on the 10th day and a moderate rise on the 18th. The lactic acid content increased until the 18th day, fell on the 22nd, and subsequently rose again to about the level of the 18th day. Diphosphoglycerate fell slightly on the 4th day and then markedly until about the 14th day. The approximate minimal concentration was reached at this time. Pyruvic acid

Fig. 6
Glycolytic Behavior Of Blood Preserved
In Citrate Solution (D-3)





showed a slight rise on the 4th day and an even smaller decrease on the 7th. From this time until the 18th day there were marked increases in the content of pyruvate. On the 22nd day there was a fall again, followed by a steady rise until the end of the experiment.

GLYCOLYTIC BEHAVIOR OF BLOOD PRESERVED IN CITRATE-DEXTROSE
(See D-4, pg. 28)

	Millimoles per liter			
Day	Sugar	Lactic Acid	Diphosphoglycerate	Pyruvic Acid
0	21.4	3.26	2.3	0.15
4	17.4	8.25	2.05	0.20
7	14.2	10.93	--	0.18
10	14.6	14.44	1.37	1.17
14	10.6	20.89	0.74	1.85
18	11.1	31.33	0.71	2.05
22	9.6	25.56	0.63	1.50
26	8.6	31.67	0.45	1.64
30	6.9	29.89	0.58	1.74

Summary.

	Destroyed	Formed
Glucose	14.5	
	x 2	
	29.0	
DPG	1.72	
Lactate		26.63
Pyruvate		1.59
Total	30.72	28.22

Ratio: approximately 1 : 1

Comparison.

There were apparent disturbances in glucose removal in Experiments 1, 2, and 4 on days 4, 10, and 18. In Experiment 2, the day 4 disturbance was merely a plateau, in the other instances rises occurred on these days. Sugar was exhausted first in the blood stored in citrate alone, as was expected; in the other experiments, glucose removal continued throughout the 30 day experimental period. The greatest destruction of sugar took place in the blood preserved with

citrate-dextrose and Brilliant Cresyl Blue where 16.8 millimoles of sugar were destroyed. The blood showing the next greatest amount of sugar destruction was the one stored in citrate-dextrose. Sugar removal amounted to 14.5 millimoles here. The blood in Baxter's solution destroyed only 4.6 millimoles, approximately 25% of that destroyed by the dye solution. The citrated blood, with no added dextrose, showed the least amount of sugar destruction. However, practically all of the sugar was destroyed in this blood while a considerable amount remained in the others.

Two bloods, those in Experiments 3 and 4, showed the approximate maximum concentration of lactic acid in about 18 days. The maximum concentration in the other two bloods did not appear for about 26 days. The greatest amount of lactic acid was produced by the blood in citrate-dextrose, with 26.63 millimoles formed. The one in citrate-dextrose-Brilliant Cresyl Blue came next with 24.13 millimoles. It will be remembered that this blood showed the greatest amount of sugar loss. The blood in ACD produced only 20.46 millimoles. The citrated blood, as anticipated, produced the smallest amount of lactate.

Experiments 1 (ACD) and 3 (citrate) showed the lowest terminal concentration of diphosphoglycerate; slightly more of this substance was destroyed in the blood preserved in citrate than in the one preserved in ACD. The blood stored with the dye showed the largest terminal content and the smallest amount of destruction. This is in accordance with the results found in the preceding series.

Pyruvate increased most rapidly and to the greatest extent in the blood preserved in citrate-dextrose and dye. Two and a half times as much was formed here as was formed in citrate, which showed the next largest concentration. Here again the highest concentration of pyruvate was found with the highest concentration of diphosphoglycerate, and the lowest concentrations of these two substances were found together. However, no correlation was found between the two intermediate concentrations.

SERIES E.

This series is the first in a study of the enzymology of red cells in diseased states. It was considered of exploratory interest and value to follow the progress of glycolysis in two different types of mental derangement, namely in a schizophrenic and a depressive patient. Through the kind co-operation of Dr. H. Lehmann of the Verdun Protestant Hospital (Mental), blood was obtained from two patients who were definitely representative of the above mentioned types of mental disturbance. The bloods were stored in citrate-dextrose solution and the course of glycolysis investigated.

Experiment 1. Blood of a Schizophrenic Patient Preserved in Citrate-Dextrose.

Only one rise in the sugar concentration was noted in the blood of this patient; it occurred on the 3rd day. Following this rise there was a steady fall until the 36th day when minimal concentration was reached. Lactic acid increased until the 36th day, but on the 63rd day the value was considerably diminished. Diphosphoglycerate remained steady for

three days, fell on the 6th day, rose on the 9th, and then fell until about the 36th day when approximately the minimal concentration was reached. Pyruvic acid showed a slightly increased content on the 3rd day, but fell again until the 9th. On the 13th the pyruvate concentration commenced rising at a rapid rate until the 21st day, when the values remained moderately constant for a time. There was another rise on the 36th day and by the 63rd day the concentration had nearly doubled.

GLYCOLYTIC BEHAVIOR OF A SCHIZOPHRENIC'S BLOOD PRESERVED IN CITRATE-DEXTROSE
(See E-1, pg.28)

	Millimoles per liter			
Day	Sugar	Lactic Acid	Diphosphoglycerate	Pyruvic Acid
0	14.61	3.44	2.04	0.30
3	15.72	8.22	2.05	0.38
6	13.50	11.44	1.95	0.30
9	11.28	13.44	2.28	0.23
13	9.06	18.00	1.78	1.11
17	7.77	20.22	1.25	1.73
21	7.50	22.22	0.95	1.93
25	6.56	28.22	0.74	1.96
29	6.28	30.78	0.74	1.82
36	4.33	31.22	0.39	2.16
63	4.06	20.44	0.46	4.18

Summary:

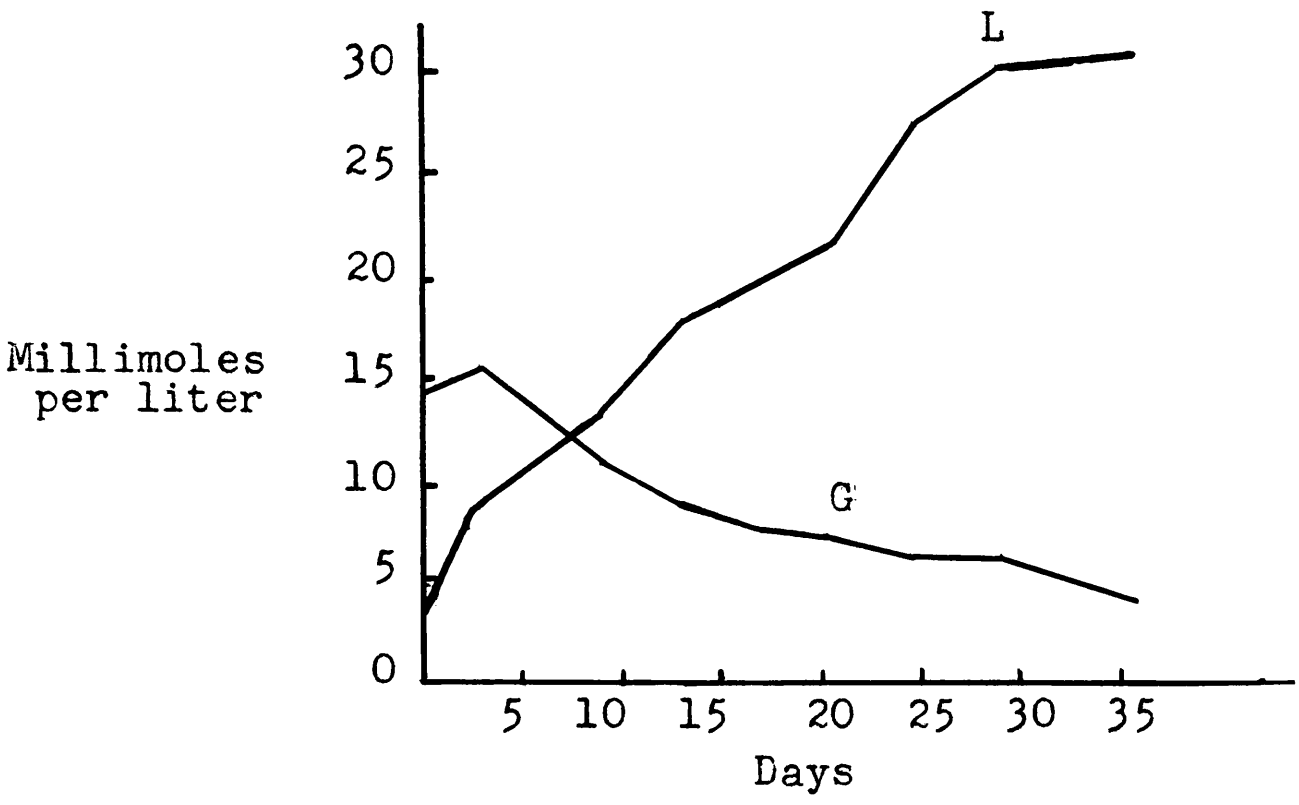
	Destroyed	Formed
Glucose	10.55	
	x 2	
	21.10	
DPG	1.58	
Lactate		17.00
Pyruvate		3.88
Total	22.68	20.88

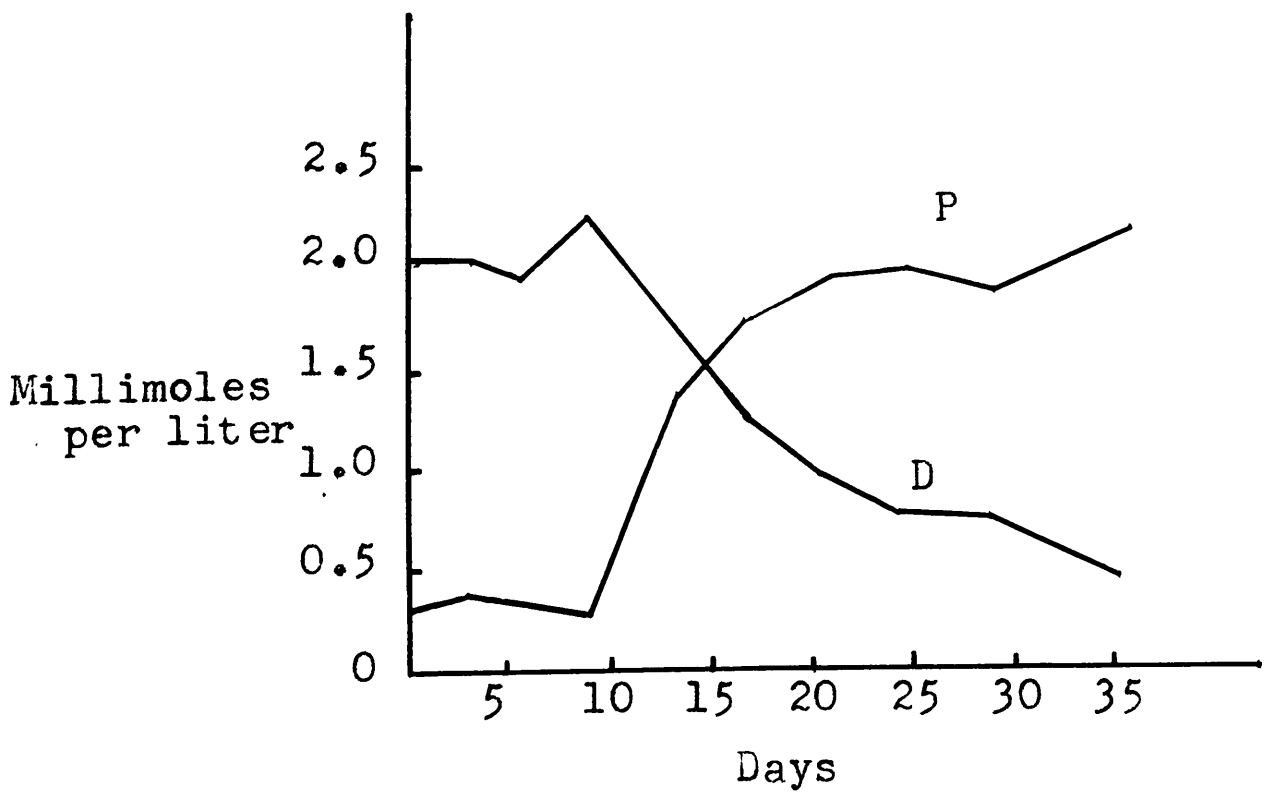
Ratio: approximately 1 : 1

Experiment 2. Blood of a Depressive Preserved in Citrate-Dextrose.

One rise in sugar concentration occurred, and as in the experiment above, it took place on the 3rd day. After this there was a steady destruction of glucose until the end of the

Fig. 7
Glycolytic Behavior Of A Schizophrenic's Blood Preserved In Citrate-Dextrose Solution (E-1)





experiment. The lactic acid content rose rapidly until the 6th day, after which the rate of formation diminished somewhat. On the 29th day, however, there was a fall in lactic acid, followed by a rise again on the 36th. By the 63rd day the concentration had again fallen. Diphosphoglycerate commenced falling immediately and fell until the 9th day, on which day there was a marked rise. This continued until the 13th. From then until the end of the experimental period diphosphoglycerate fell constantly. It should be noted that the rate of fall was very much slower in this blood than the one in Experiment 1 of this series. The concentration on the 36th day in this blood (Experiment 2) was slightly higher than on the 21st day in the Schizophrenic blood.

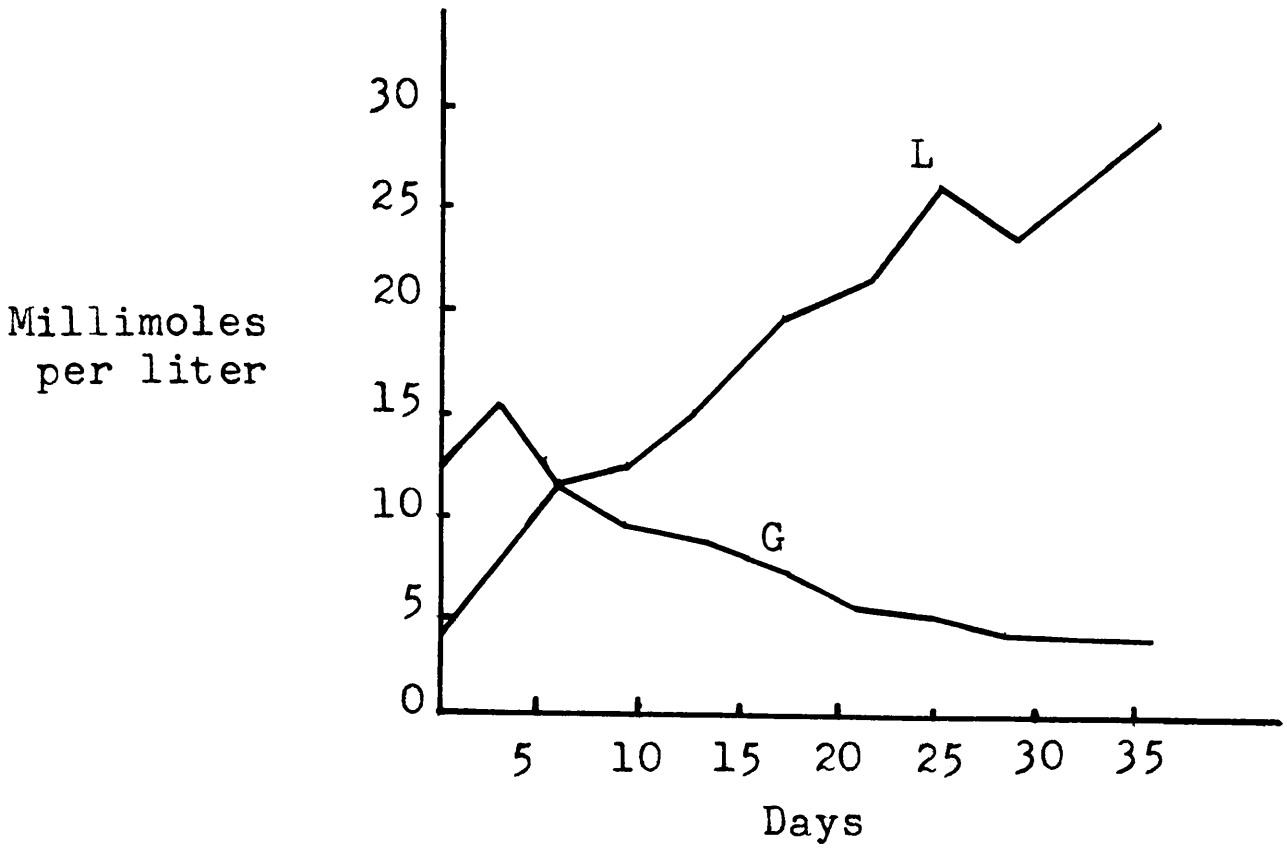
Pyruvic acid formation also was delayed in the Depressive blood. There was an increase on the 3rd day but the concentration fell until the 13th day. After this there was a rise until the end of the experiment. The concentration on the 63rd day was almost twice as high as on the 36th.

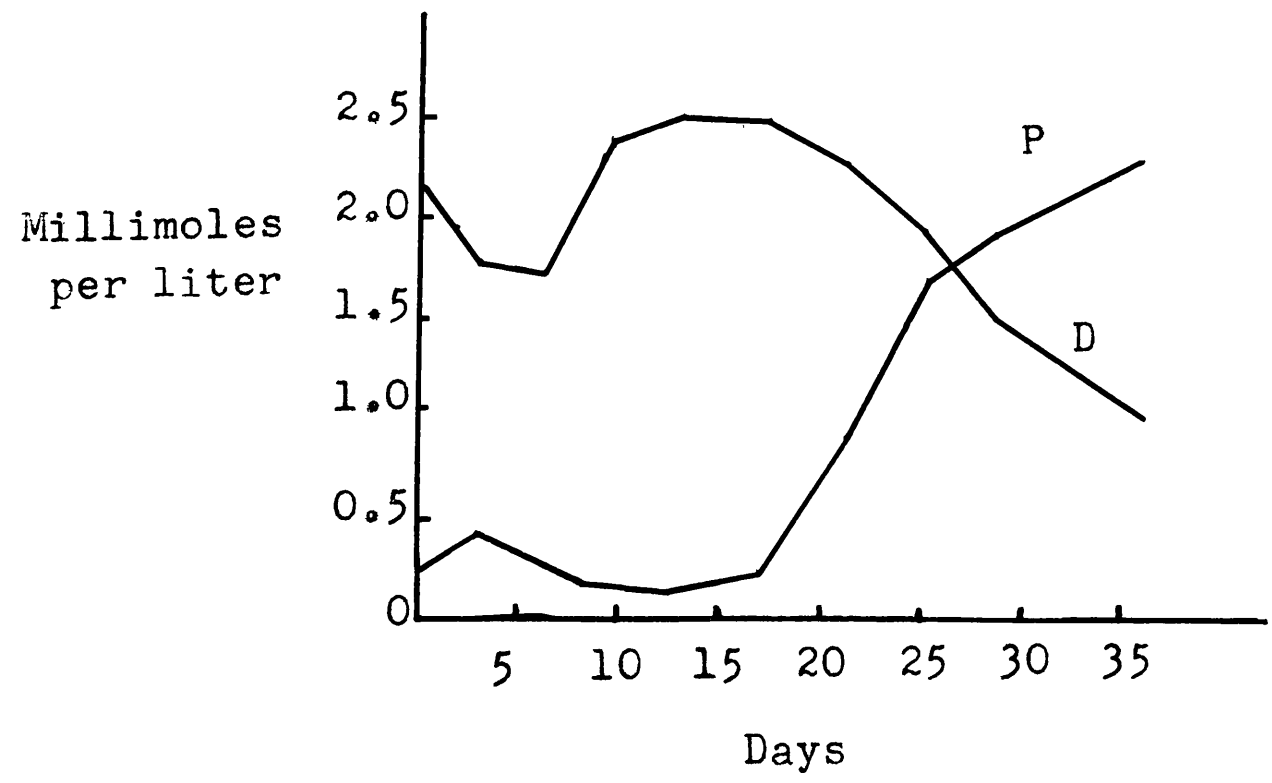
GLYCOLYTIC BEHAVIOR OF A DEPRESSIVE'S BLOOD PRESERVED IN CITRATE-DEXTROSE

(See E-2, pg.28)

		Millimoles per liter		
Day	Sugar	Lactic Acid	Diphosphoglycerate	Pyruvic Acid
0	12.22	3.89	2.16	0.26
3	15.44	7.67	1.76	0.45
6	11.39	11.22	1.71	0.33
9	9.72	12.22	2.38	0.14
13	9.06	15.67	2.51	0.14
17	7.50	19.89	2.47	0.22
21	5.72	21.89	2.27	0.84
25	5.28	26.67	1.92	1.65
29	4.44	23.78	1.39	1.93
36	4.06	30.00	1.00	2.27
63	2.11	20.44	0.49	4.23

Fig. 8
Glycolytic Behavior Of A Depressive's Blood Preserved In Citrate-Dextrose Solution (E-2)





Summary:

	Destroyed	Formed
Glucose	10.11	
	x 2	
	20.22	
DPG	1.67	
Lactate		16.55
Pyruvate		3.97
Total	21.89	20.52

Ratio: Approximately 1 : 1

Comparison.

Sugar behaved in nearly the same manner in both bloods, showing a rise on the 3rd day, and then progressively falling. However, the Depressive's blood showed a slower subsequent rate of fall.

In the blood of Experiment 2 there was a fall in the lactic acid concentration on the 29th day, when on this day there was an increase in Experiment 1. Both bloods showed approximately the same low terminal value.

The metabolism of diphosphoglycerate was slower in the Depressive's blood than in the Schizophrenic's. The concentration on the 36th day in Experiment 2 was about three times as high as in Experiment 1. However, the values for the terminal concentrations were very close.

The rate of formation of pyruvic acid was slower in the Depressive's blood than in the Schizophrenic's, but the values for the 36th day were nearly the same in the two bloods, as were the terminal ones.

SERIES F

There is only one blood in this series. The Schizophrenic who donated the blood for Experiment 1, Series E also

donated this blood. As the patient had been showing a progressive deterioration in his condition, it was considered of value to repeat the previous experiment after a lapse of time.

Experiment 1. The Blood of a Schizophrenic Patient Preserved in Citrate-Dextrose.

Three rises in the sugar content of this blood were noted. They occurred on the 4th, 11th, and 20th days. Lactic acid increased until the 20th day, and then fell on the 25th, which was the end of the experiment. The pyruvic acid content of this blood rose slightly on the 4th day and fell just a little on the 7th. After this time there was a continuous increase in the pyruvate concentration. The diphosphoglycerate content fell on the 4th day and rose again on the 7th. After this the concentration showed a continual decline.

GLYCOLYTIC BEHAVIOR OF A SCHIZOPHRENIC'S BLOOD IN CITRATE-DEXTROSE
(See F-1, pg. 29)

	Millimoles per liter			
Day	Sugar	Lactic Acid	Diphosphoglycerate	Pyruvic Acid
0	31.67	1.78	1.50	0.07
4	33.89	4.22	1.03	0.13
7	27.64	6.44	1.80	0.11
11	32.22	7.00	1.72	0.27
15	28.89	10.11	1.28	1.01
20	29.72	16.78	0.68	1.44
25	23.78	9.67	--	1.51

Summary:

	Destroyed	Formed
Glucose	7.89	
	x 2	
	15.78	
DPG	0.82	
Lactate		7.59
Pyruvate		1.44
Total	16.60	9.03

Ratio: approximately 2 : 1

SERIES G. Normal Blood Preserved in Citrate-Dextrose Solution With Various Dyes.

This experiment was designed to determine the effect of various dyes, in addition to the Brilliant Cresyl Blue reported earlier, on the glycolytic cycle. The results of the following experiments show that all of the dyes except one, which have an effect on the glycolytic cycle, exert their effect on pyruvate removal. The single exception is found in Vital Red in Experiment 5 where there is no effect on diphosphoglycerate and pyruvic acid, but glucose utilization is apparently inhibited and the lactic acid increases to slightly above the control level.

In those experiments where the dyes have inhibited the removal of pyruvate, the lactic acid is found to be diminished. An explanation of this will be offered in the general discussion on page 83.

Experiment 1. Normal Blood in Citrate-Dextrose

There was one rise in the concentration of glucose in this blood. It occurred on the 8th day. The lactic acid remained constant for the first three days and then commenced rising at an increasingly rapid rate. The diphosphoglycerate content fluctuated to some extent, but remained essentially unchanged. The pyruvic acid increased in amount during the first eight days of the experiment and decreased during the second eight day period. Here again, the changes in concentration were very small.

In all of the five bloods of this series the concentration of diphosphoglycerate showed an overall rise during the experimental period. Therefore an adjustment had to be made

in computing the ratio. The increase in diphosphoglycerate was added to that of the lactate and pyruvate and this total was compared with twice the number of millimoles of glucose destroyed.

GLYCOLYTIC BEHAVIOR OF BLOOD PRESERVED IN CITRATE-DEXTROSE SOLUTION
(See G-1, pg.29)

		Millimoles per liter		
Day	Sugar	Lactic Acid	Diphosphoglycerate	Pyruvic Acid
0	25.00	1.22	2.00	0.09
3	22.22	1.22	2.03	0.16
8	23.61	3.11	2.08	0.19
12	23.50	8.78	1.85	0.15
16	22.50	17.89	2.17	0.13

Summary:

	Destroyed	Formed
Glucose	2.50	
	x 2	
	5.00	
DPG		0.17
Lactate		16.67
Pyruvate		0.04
Total	5.00	16.88

Ratio: approximately 1 : 3

Experiment 2. Blood Preserved in Citrate-Dextrose with Added Nile Blue.

In this experiment sugar rose on the 12th day, but by the 16th day had started falling again. Lactic Acid showed a moderately steady rise throughout the whole experiment. Diphosphoglycerate increased to a small extent, but pyruvate showed a tremendous increase over the control level. This compound did not show the usual slight rise and fall at the beginning of the experiment before beginning the relatively much greater rise that is common to most citrate-dextrose preservatives, but started at a much greater level and continued rising without interruption.

GLYCOLYTIC BEHAVIOR OF BLOOD PRESERVED IN CITRATE-DEXTROSE SOLUTION WITH ADDED NILE BLUE (0.0032 M)
(See G-2, pg.29)

Day	Sugar	Millimoles per liter Lactic Acid	Diphosphoglycerate	Pyruvic Acid
0	23.33	1.67	2.22	0.58
3	18.06	2.78	2.38	2.72
8	15.86	6.33	2.82	4.19
12	16.94	9.11	2.62	3.90
16	16.11	11.44	2.74	4.60

Summary:

	Destroyed	Formed
Glucose	7.22	
	x 2	
	14.44	
DPG		0.52
Lactate		9.77
Pyruvate		4.02
Total	14.44	14.31

Ratio: approximately 1 : 1

Experiment 3. Blood Preserved in Citrate-Dextrose with Added Methylene Blue.

The sugar content rose on the 12th day, but only by a small amount. On the 16th day there was a slight drop. Lactic acid increased steadily throughout the experiment. Diphosphoglycerate fluctuated to some extent and the terminal concentration was somewhat higher than the initial one. Pyruvic acid increased by leaps and bounds, and, as in Experiment 2, did not show the slight rise and fall at the beginning of the experimental period.

GLYCOLYTIC BEHAVIOR OF BLOOD PRESERVED IN CITRATE-DEXTROSE SOLUTION WITH ADDED METHYLENE BLUE (0.0032 M)
(See G-3, pg.29)

Day	Sugar	Millimoles per liter Lactic Acid	Diphosphoglycerate	Pyruvic Acid
0	16.94	1.11	2.42	1.02
3	14.61	3.67	2.33	3.89
8	13.06	8.33	2.87	5.86
12	13.89	12.44	2.84	6.34
16	13.33	14.67	2.83	8.27

Summary:

	Destroyed	Formed
Glucose	3.61	
	x 2	
	7.22	
DPG		0.41
Lactate		13.56
Pyruvate		7.25
Total	7.22	21.22

Ratio: approximately 1 : 3

Experiment 4. Blood Preserved in Citrate-Dextrose with Added Evans' Blue.

The concentration of glucose in this blood showed a small increase on the 3rd day and a larger one on the 12th. Lactic acid increased steadily throughout the experiment. Diphosphoglycerate remained nearly constant for the first 8 days and then commenced rising slowly. Pyruvic acid increased on the 3rd day and then fell until the 12th. On the 16th day there was a rise again. The behavior of this blood is almost the same as the control in Experiment 1 of this series.

GLYCOLYTIC BEHAVIOR OF BLOOD PRESERVED IN CITRATE-DEXTROSE SOLUTION WITH ADDED EVANS' BLUE (0.0032 M)
(See G-4, pg.29)

		Millimoles per liter		
Day	Sugar	Lactic Acid	Diphosphoglycerate	Pyruvic Acid
0	17.39	1.11	2.54	0.14
3	17.78	4.56	2.55	0.23
8	14.17	9.56	2.55	0.10
12	15.83	15.56	2.63	0.09
16	14.44	18.89	2.92	0.27

Summary:

	Destroyed	Formed
Glucose	2.95	
	x 2	
	5.90	
DPG		0.38
Lactate		17.78
Pyruvate		0.13
Total	5.90	18.29

Ratio: approximately 1 : 3

Experiment 5. Blood Preserved in Citrate-Dextrose With Added Vital Red.

Sugar rose in this blood on the 8th and 16th days. The falls in between were small and the terminal rise was to a concentration slightly higher than the initial one; so the sugar content apparently remained stationary. This is surprising because the lactic acid rose to a concentration a little higher than the control. The diphosphoglycerate fell slightly on the 3rd day and then rose until by the 16th day it was higher than the initial level. The pyruvic acid concentration increased very slightly on the 3rd day and then fell just a little until the 12th. After this there was another minute rise. The concentrations, therefore, of the sugar, diphosphoglycerate, and the pyruvate can be assumed to have remained almost constant.

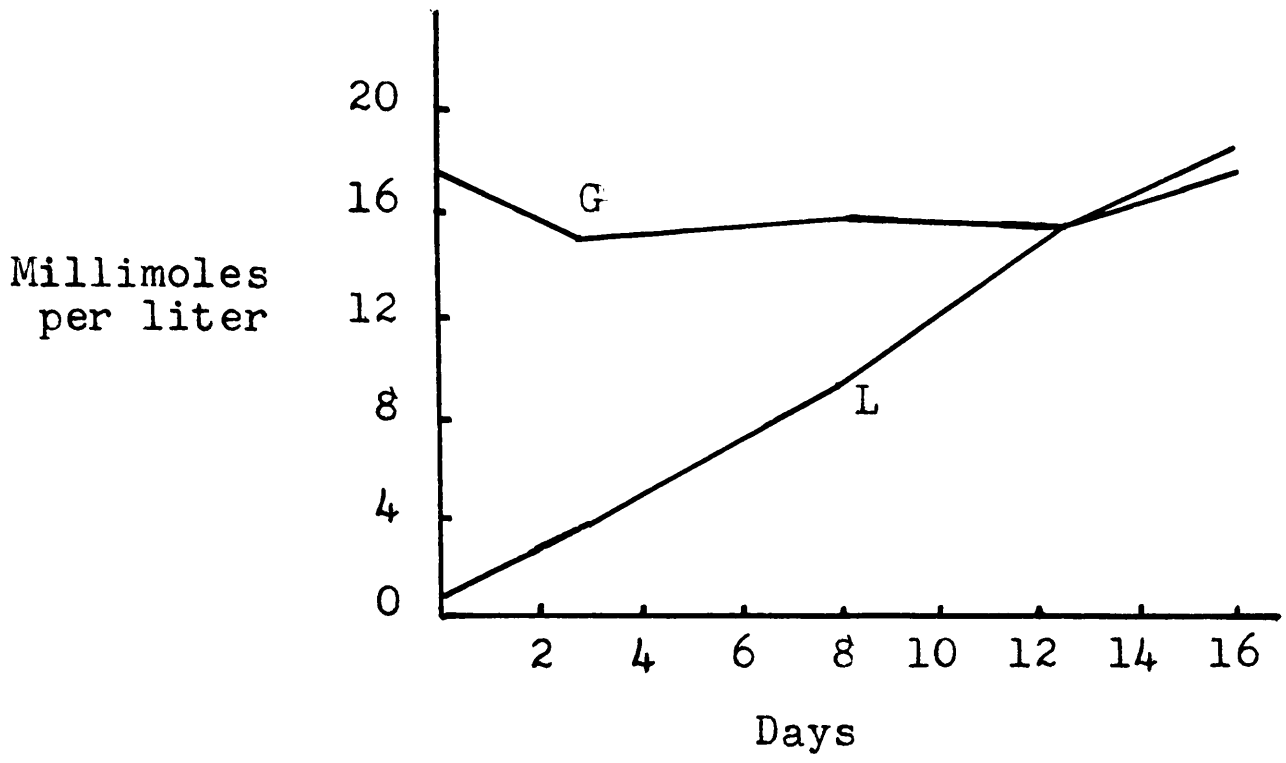
GLYCOLYTIC BEHAVIOR OF BLOOD PRESERVED IN CITRATE-DEXTROSE SOLUTION WITH ADDED VITAL RED (0.0032 M)
(See G-5, pg. 29)

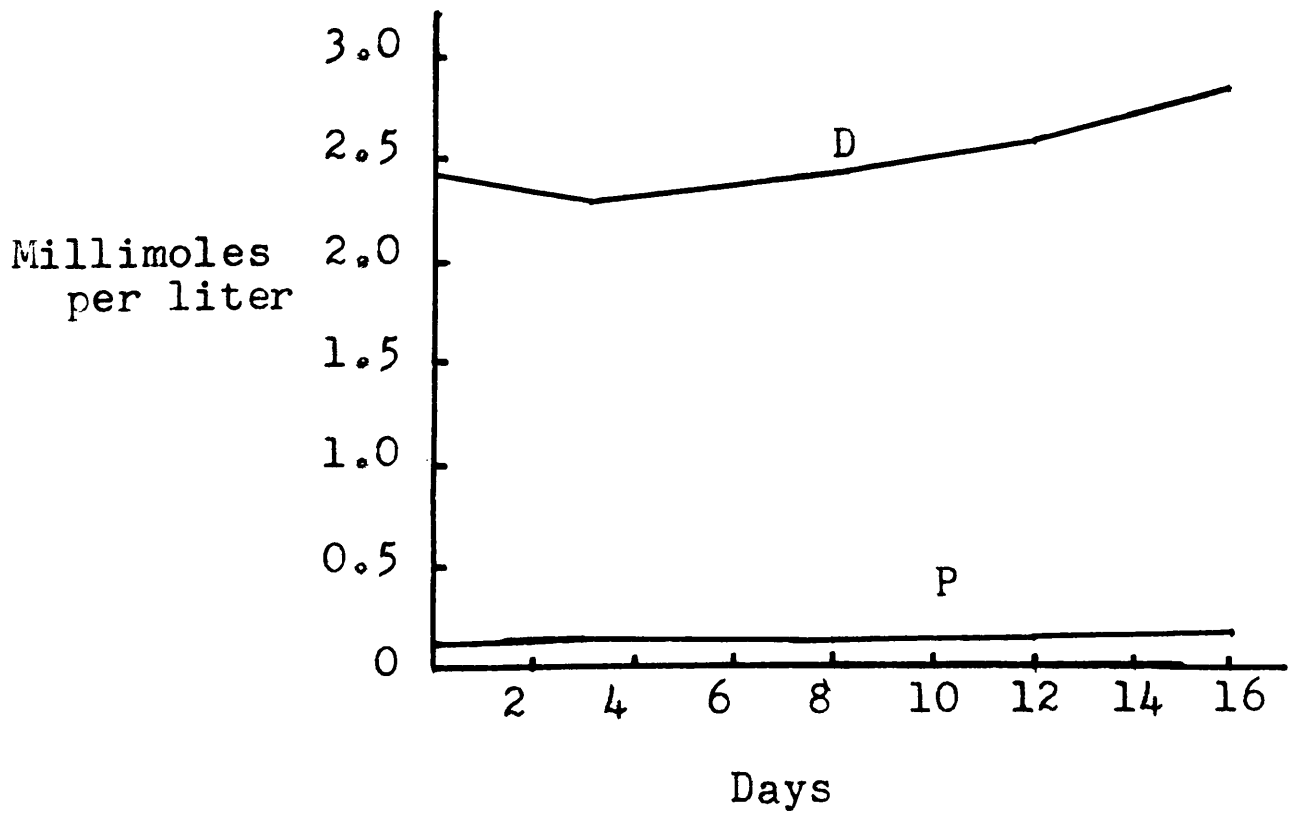
	Millimoles per liter			
Day	Sugar	Lactic Acid	Diphosphoglycerate	Pyruvic Acid
0	17.50	0.89	2.38	0.09
3	15.00	3.78	2.30	0.13
8	16.00	9.22	2.43	0.12
12	15.83	15.56	2.59	0.11
16	17.78	18.89	2.86	0.14

Summary:

	Destroyed	Formed
Glucose		0.28
DPG		0.48
Lactate		18.00
Pyruvate		0.05
Total		18.81

Fig. 9
Glycolytic Behavior Of Blood Preserved
In Citrate-Dextrose Solution Containing
Added Vital Red (G-5)





Comparison.

It is apparent from a survey of the preceding experiments that the actions of Nile Blue and Methylene Blue are quite similar. Both dyes accept hydrogen from the reduced DPN which results in increased pyruvate formation and decreased lactate production. Also, where these two dyes are used, there is a greater destruction of glucose. Barron and Harrop (51) noted that with Methylene Blue and other dyes of a similar oxidation-reduction potential there was an increased sugar degradation and a diminished lactic acid formation. Pyruvate estimations were not reported in this paper.

Evans' Blue appeared to be quite inert. The glycolytic behavior of the blood stored with this dye was almost exactly the same as the control in citrate-dextrose alone.

The most amazing results were found with Vital Red. This dye seemed to inhibit the breakdown of both glucose and diphosphoglycerate and the formation of pyruvate; yet the formation of lactic acid was quite normal. The source of the lactate under these conditions has not yet been established. A possible explanation of these results is offered in the general discussion on page 77.

Additional Phosphate Determinations.

In Series E, F, and G, a fractional phosphate analysis was carried out. This gave information regarding the labile (easily hydrolysable) phosphate. The partition may be described as follows:

A. Inorganic Phosphate. This is the inorganic phosphate found in the filtrate from blood after removal of the proteins with trichloroacetic acid.

B. Organic Labile Phosphate. This is the increase in inorganic phosphate after hydrolysis of the trichloroacetic acid filtrate for 100 minutes at 100° C with 2 N HCL. Thus the

Labile Phosphate = B - A.

Compounds included in this fraction are adenosinetriphosphate, phosphocreatin, and hexose phosphates.

C. Total Acid-Soluble Phosphate. This is the phosphate found after complete digestion of the organic compounds in the TCA blood filtrate with 10 N sulfuric acid.

C - B = Diphosphoglycerate.

The following tables are given in terms of milligrams of phosphate per 100 ml. of whole blood. Two tables are presented for each blood of each series, the first represents the Labile Phosphate and the second is a table of Inorganic Phosphate values.

Series E. (See page 58)

Experiment 1.

Day	Labile Phosphate	Inorganic Phosphate
0	10.50	13.75
3	12.25	11.75
6	12.00	12.00
9	8.25	16.50
13	4.75	31.00
17	0.00	50.50
21	0.00	59.00
25	3.00	51.00
29	0.00	55.50
36	2.00	54.50
63	0.00	61.50

Experiment 2.

Day	Labile Phosphate	Inorganic Phosphate
0	12.50	12.50
3	14.75	10.25
6	17.75	9.25
9	12.70	12.50
13	9.75	15.50
17	4.50	22.00
21	0.50	31.50
25	0.50	33.50
29	0.00	40.50
36	1.50	48.00
63	0.00	59.50

Series F. (See page 63)

Experiment 1.

Day	Labile Phosphate	Inorganic Phosphate
0	13.50	10.00
4	15.00	7.50
7	8.25	14.00
11	5.25	19.00
15	4.25	30.50
20	5.50	39.00

Series G. (See page 65)

Experiment 1.

Day	Labile Phosphate	Inorganic Phosphate
0	9.75	12.25
3	12.00	12.50
8	6.00	14.50
12	8.75	12.00
16	6.50	12.00

Experiment 2.

Day	Labile Phosphate	Inorganic Phosphate
0	7.00	18.75
3	12.25	13.00
8	3.00	14.00
12	6.75	11.50
16	5.50	12.00

Experiment 3.

Day	Labile Phosphate	Inorganic Phosphate
0	7.00	20.00
3	13.75	15.00
8	2.00	15.50
12	4.50	12.00
16	1.75	16.50

Experiment 4.

Day	Labile Phosphate	Inorganic Phosphate
0	7.75	20.00
3	12.50	14.00
8	6.00	16.50
12	7.00	13.50
16	2.00	16.50

Experiment 5.

Day	Labile Phosphate	Inorganic Phosphate
0	6.75	21.00
3	6.75	14.00
8	8.75	16.00
12	9.75	12.00
16	7.25	12.00

Haemoglobin.

Whenever samples were removed for analysis, haemoglobin determinations were done to ensure that thorough mixing of the contents of the preservative flasks had taken place. The results of these determinations were quite steady in each blood, which showed that representative samples were removed in each case.

DISCUSSION

I. The Glycolytic Behavior of Normal Blood Stored in Citrate-Dextrose Preservative Solutions.

1. Sugar

The fall in glucose in blood preserved in citrate-dextrose proceeds at an irregular rate. With the added dextrose, in the usual citrate-dextrose preservatives, the sugar level rarely falls to zero during storage. In the four bloods reported in Series A, the minimum amount of reducing substances present at the end of the experimental period was equivalent to 1.56 millimoles of glucose, and the most remaining was 5.89. This amounts to between 28 and 106 mgm. per 100 ml. of whole blood. Part of this represents non-sugar reducing substances, probably about 20 mgm. per cent, which is the figure reported in the literature (55), and the remainder is glucose which has not been changed. Glycolysis is most rapid during the first 26 to 40 days. After this time further disappearance of glucose occurs very slowly. Blood in Experiment A-3 showed the most rapid rate of glycolysis of the four bloods in the series. By the 26th day the sugar had fallen from 26.94 millimoles per liter to 1.94 millimoles. In the other bloods the fall was not as rapid, and only in A-1 of the other bloods was such a low level reached.

The removal of sugar from blood containing added dextrose does not follow a smooth curve. There are periods where the sugar, or at least the reducing substances, rises. Andreae in our laboratory, reported these fluctuations occurring at fairly regular intervals, usually between the second

and tenth days and between the nineteenth and twenty-sixth days. In all of the bloods that were stored in citrate-dextrose either transitory increases were observed or plateaux occurred. In some cases increases occurred during the first few days and additional increases took place at about the ninth day, and again about the sixteenth day. Further increases sometimes occurred around the 22nd, 29th, and 37th days. There was one on day 43. The bloods in Experiment B showed an initial transitory rise which either continued or, as in B-1, reached a plateau. In blood C-1, there was an initial rise, and another about the 22nd day. The sugar continued at the increased level until the experiment was terminated. In blood D-4 there were temporary plateaux beginning at the 7th and 14th days respectively. Also in G-1 a rise occurred on the 8th day. To sum up, either rises or plateaux may occur initially, about the 9th day, 16th, 22nd, 29th, and about the 37th days. These observations, then, are in general agreement with those of Andreae.

2. Lactic Acid

Concurrently with the fall in sugar, there was a rise in lactic acid. In none of the bloods of Series A was the lactic acid that was formed enough to account for the fall in sugar. This may be due in part to the fact that the lactic acid method used in these earlier experiments was unsatisfactory. In all bloods in Series B, the sugar was higher at the end of the experiment than it was initially, hence the ratio of sugar removed to lactic acid formed could not be determined. In C-1 and G-1, there was again more

lactic acid than could be accounted for on the basis of sugar disappearance. Only in D-4 was there the expected ratio of 1 : 2 between sugar and lactic acid.

As has been stated in the previous section on sugar, Andreae obtained transitory rises in the glucose concentration. She suggested that the formation of new reducing intermediates might account for these sudden and periodic increases in apparent sugar concentration. The presence of these compounds would mask the disappearance of glucose and lactic acid formation could not be correlated with glucose utilization. One may offer another possibility, namely, that pyruvate and lactate may be formed by other than the glycolytic mechanism. Citrate of the preservative solution was suspected as being a possible precursor, but Kwiecinska (60) in this laboratory disproved this by showing that the same unaccountable lactic acid production occurs in defibrinated blood, namely, in the absence of added citrate.

3. Behavior of Phosphorus Compounds

The behavior of phosphorus compounds was not followed in Experiment A. Experiment B was terminated after six days so the information gleaned here was not great, however, in the two specimens, B-1 and B-2, stored in citrate-dextrose there was a rise in diphosphoglycerate during the first week. In blood C-1, for the first three days the diphosphoglycerate remained unchanged. On the seventh day there was a marked, but brief, increase and then a rapid fall. By the 19th day, the diphosphoglycerate level was quite low and continued to fall though more gradually to the end of the experiment.

In blood D-4 the diphosphoglycerate commenced falling immediately, until a level of approximately 0.74 millimoles per liter was reached at about the 14th day. Thereafter the rate of fall was considerably diminished. In blood G-1, on the other hand, the diphosphoglycerate remained fairly steady through the experimental period of 16 days. Diphosphoglycerate as a rule tends to remain moderately constant during the first few days of storage but in some cases it increases temporarily during this period. A definite fall in diphosphoglycerate content usually occurs after seven to ten days of storage. (See pages 37 to 74 for graphs.)

The amount of easily hydrolysable phosphate esters, ATP, hexose phosphates and phosphocreatin was low at the beginning of Experiment G-1. The phosphate hydrolysed from these compounds in the first determination amounted to 4.75 mgm. per 100 ml. of blood. By the third day this had risen to 12 mgm. per cent. At the eighth day there was a fall to about half of this level which continued to the 16th day.

As the labile phosphorus compounds are determined by subtracting the initial inorganic phosphate from the total inorganic phosphate after 100 minutes hydrolysis with 2 N HCL, it is of interest to examine the behavior of the inorganic phosphate. Guest (55) in 1932 said that inorganic phosphate in normal defibrinated blood remained either at a constant level, or diminished slightly during the first few hours when the blood was incubated at 37° C. Glycolysis is greatly retarded in the cold, and hence continues for several days. Examination of the table for preserved Blood G-1 will reveal that the inorganic phosphate level fell from 17.25 mgm. per cent to

12.5 mgm. per cent by the third day. Thereafter, the level remained fairly constant for the remainder of the 16 day period.

4. Pyruvic Acid

In bloods B-1 and B-2, preserved in citrate-dextrose the amount of pyruvic acid remained approximately constant during the experimental period of six days. The pyruvate content of these two bloods was low, being less than 0.25 millimoles per liter. There were extremely slight rises on the third day in both bloods and in blood B-1 this slightly increased level continued until the sixth day.

In bloods C-1 (graph on page 44) and D-4 preserved in citrate-dextrose, the initial level of pyruvate was approximately the same as in the two bloods in the B Series. A small increase was observed on the third day, but on the seventh day in both cases the pyruvate underwent an abrupt increase. The rise continued until the 18th day in blood C-1 and until the 19th day in D-4, when the level reached approximately two millimoles per liter. After this time the amount of pyruvate concentration remained moderately constant.

In blood G-1, preserved in citrate-dextrose, the pyruvic acid level remained low during the entire experimental period of 16 days. The content of the blood at the beginning of the period was 0.09 millimoles per liter. By the 8th day this had increased to 0.19 millimoles, and by the 16th day had fallen to 0.13 millimoles. In this blood considerable difficulty was experienced during the veni puncture which resulted in obtaining somewhat less than the desired

amount and hence an overdilution with the preservative solution. This explains the relatively low initial concentration of pyruvate.

5. Interrelationships Between Sugar, Phosphate, Pyruvate, and Lactic Acid

As glucose disappears from the blood, lactic acid is formed. In the one experiment D-4, a good correlation was obtained between the amount of sugar removed and the amount of lactate formed. In most cases, however, with the exception of Series A, the amount of glucose removed was not sufficient to account for the lactic acid formed. The writer feels that the discrepancy may be due to the simultaneous formation of some reducing intermediate which is estimated along with the glucose and tends to make the sugar level appear higher than it actually is. The discrepancy is being investigated further.

After glycolysis has been under way for from seven to eleven days a marked fall in diphosphoglycerate usually occurs. During this time the fall in sugar may amount to as much as 125 mgm. per cent. The rather sudden fall in diphosphoglycerate is reflected by an equally abrupt rise in pyruvic acid. In Experiment D-4 slightly more pyruvate was formed than could be accounted for by the fall in diphosphoglycerate. One would not necessarily expect an exactequivalence in the amount of diphosphoglycerate lost and the amount of pyruvate formed because the former of these is being produced continuously from the breakdown of glucose. So, as long as there is some diphosphoglycerate in the system, pyruvate can still be formed, as pyruvate represents a later stage in the glycolytic process.

The accumulation of pyruvate and the fall in diphosphoglycerate would be expected to have an effect on the amount of lactic acid produced while these changes are going on, but in citrate-dextrose the concentration of pyruvate and diphosphoglycerate is so small that their reflection in lactic acid formation is masked by the much greater concentration of the latter. In the case of the dye experiments where there is a comparatively tremendous amount of pyruvate accumulation, lactic acid formation is reduced.

In Experiment C-1, (see page 43), there is a closer equivalence between the amount of diphosphoglycerate disappearing and the amount of pyruvate formed.

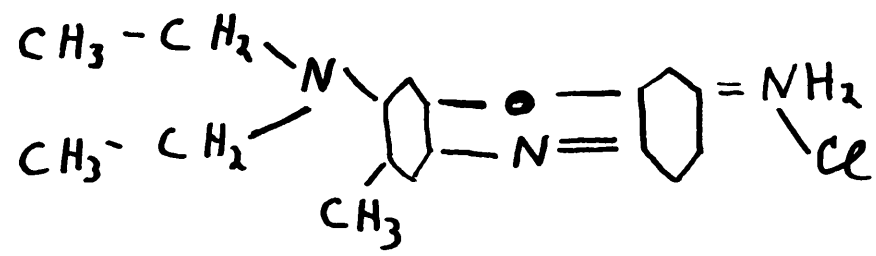
In the only experiment of this set where the inorganic phosphate was measured, namely in G-1, this remained fairly constant after the initial fall referred to above. The rise in inorganic phosphate such as was observed in other experiments (see page 72) did not occur in G-1.

II. The Glycolytic Behavior of Normal Blood Stored in Citrate-Dextrose with Various Added Vital Dyes.

In this investigation the effect of certain vital dyes on glycolysis was studied. The dyes included Brilliant Cresyl Blue, Nile Blue, Methylene Blue, Evans' Blue, and Vital Red. The structural formulae of these dyes are given below:

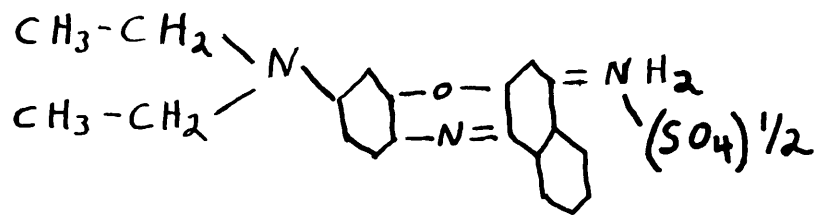
a)

Brilliant Cresyl Blue



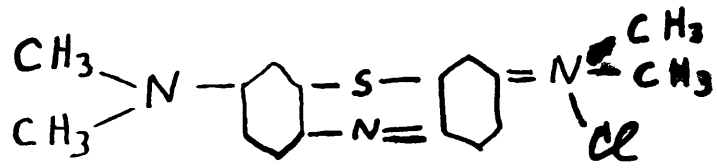
b)

Nile Blue



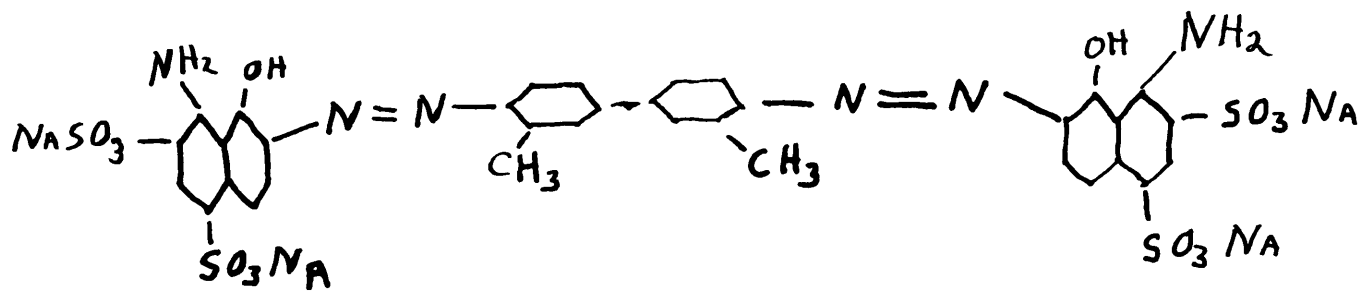
c)

Methylene Blue



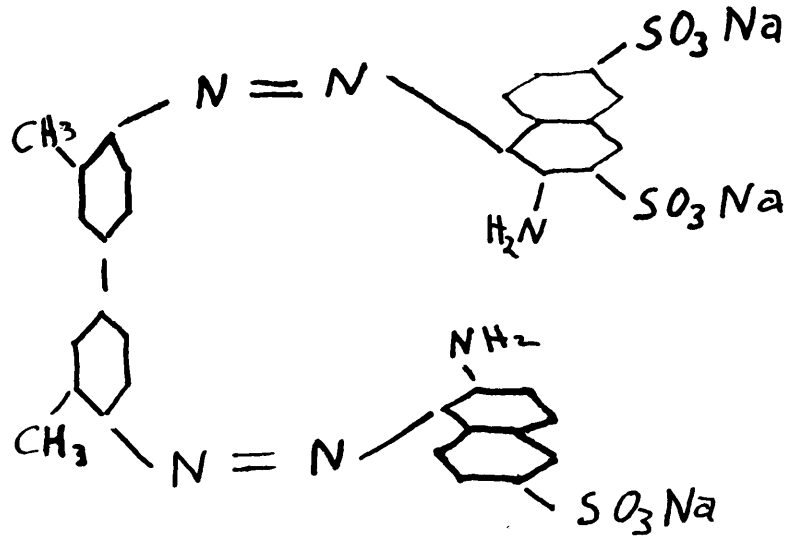
d)

Evans' Blue



e)

Vital Red



It will be seen that three of the dyes are basic, namely, Brilliant Cresyl Blue, Nile Blue, and Methylene Blue. The other two, Evans' Blue and Vital Red are acidic. The three basic dyes had a marked inhibitory action on the removal of pyruvate, and consequently on the formation of lactic acid. The acid dyes were inert in this respect. A table giving the molecular weights of the dyes employed follows:

MOLECULAR WEIGHTS OF DYES

	Dye	Molecular Weight
1.	Brilliant Cresyl Blue	317.811
2.	Nile Blue	732.828
3.	Methylene Blue	319.845
4.	Evans' Blue	960.808
5.	Vital Red	826.759

The Effect of Dyes on Glycolysis.

The three basic dyes referred to inhibit the removal of pyruvic acid, increase the destruction of glucose, and impede the formation of lactic acid. Barron and Harrop (51), as cited previously, state that Methylene Blue and other dyes of a similar oxidation-reduction potential accelerate the utilization of sugar and cause a decreased amount of lactic acid to be produced. These dyes have long been known to be hydrogen acceptors. No pyruvate determinations are reported in the above mentioned paper and it is probable that had they been done, the amount of sugar destroyed and not accounted for by lactic acid would have been accounted for as pyruvate. In only one instance in the present investigation, namely Experiment C-2, page 43 and 46, was there not enough lactate and pyruvate to account for all the glucose removed. In Experiments

D-2 and G-2, where Brilliant Cresyl Blue and Nile Blue, respectively, were used the sugar removed was accounted for in the combined pyruvate and lactate fractions. In Experiment G-3, where Methylene Blue was the dye employed, more lactate and pyruvate were found than would be expected from the amount of sugar destroyed. If one were to postulate the formation of a reducing substance to explain the situations where the fall in sugar does not account for all the lactic acid formed then the formation of this unknown substance also must be inhibited by Nile Blue and Brilliant Cresyl Blue, since in Experiment G-2 with Nile Blue, there was excellent agreement between sugar removal and lactate and pyruvate formation. In Series C where the ratio of 1 : 2 in the control sample might indicate the presence of a reducing substance, more sugar disappears in the Brilliant Cresyl Blue experiment (C-2) than can be accounted for by the formation of lactate and pyruvate.

The dye, Evans' Blue (see Experiment 4, Series G) had no effect whatsoever on the course of glycolysis. The amount of sugar destroyed in the presence of this dye was approximately the same as that destroyed in the control sample.

Vital Red, in Experiment G-5, gave rise to a most interesting phenomenon. In this experiment the sugar, diphosphoglycerate, and pyruvate remained practically unaltered, yet the concentration of lactic acid rose rapidly throughout the experiment. If one were again to postulate a precursor other than glucose one would have to assume that the conversion is stimulated by the dye.

The effect of the various dyes on the diphosphoglycerate was to increase the concentration in Series G more than it was increased in the control experiment of this series; and in Series C and D, Experiments C-2 and D-2 respectively, showed not only a delayed fall in diphosphoglycerate, but a slower rate of fall.

The Glycolytic Behavior of Blood Preserved in Acid-Citrate-Dextrose.

Rapoport (3) describes the glycolytic behavior of blood stored in ACD mixtures. He preserved blood in citrate, citrate-dextrose, and ACD, and, after a period of storage, tested the glycolytic capacity in specimens kept at 37° C for 90 minutes, by determining the fall in sugar and the rise in lactic acid. Blood stored in ACD was found to have a slower initial rate of glycolysis, but glycolytic capacity was retained longest in this medium. In ACD the inorganic phosphate was found to increase sharply in the cells at the expense of the organic acid-soluble phosphate which, he says, is composed mainly of ATP and diphosphoglycerate. He found also that ATP remains longest in ACD. Hence it must have been largely diphosphoglycerate that was hydrolysed. Our findings are in accordance with these.

Experiment 1, Series D, (graphs on page 51/2) is a good illustration of the above behavior. ATP was not measured, but diphosphoglycerate was found to fall rapidly when compared with the control experiment, D-4. The minimal concentration of diphosphoglycerate was reached by the 10th day in Experiment D-1 but not until the 14th in D-4.

The rate of glucose removal also was slower in the ACD medium than in the control in citrate-dextrose. Experiment D-1 showed a loss of sugar of only 4.6 millimoles per liter, whereas in D-4 the loss was 14.5 millimoles during 30 days.

Lactic acid production is lower in ACD than in citrate-dextrose, the concentrations being 20.46 and 26.63 millimoles respectively during the 30 day experimental period of Series C and D.

Production of pyruvate, too, was found to be delayed in ACD. The increase of this substance during 30 days in ACD was 0.53 millimoles and in citrate-dextrose was 1.59.

The Glycolytic Behavior of Blood Preserved in ACD Containing Brilliant Cresyl Blue.

In Experiment B-3 blood was preserved in ACD containing Brilliant Cresyl Blue. There is no control for this experiment. However, since the glycolytic behavior of blood stored in ACD and in citrate-dextrose containing this dye has already been described, it is of interest to use them as a basis for comparison.

Since the concentration of reducing substances rose, no comparison of the rate of sugar removal can be made. Preservation in ACD tends to retard the rate of glucose utilization, but the dye tends to increase the rate. The production of lactic acid, as would be expected, was less than in the two bloods of Series B preserved in citrate-dextrose. This undoubtedly results from two causes: first, a diminished rate of glycolysis in ACD, and secondly, inhibition of the conversion

of pyruvate to lactic acid.

The behavior of pyruvate is as anticipated. Considerably more of this compound was formed than in ACD alone or in citrate-dextrose alone, but it was less than was found in citrate-dextrose with added Brilliant Cresyl Blue, Nile Blue, or Methylene Blue when one compares the values for about the 6th day.

The behavior of diphosphoglycerate also is interesting. The concentration of this substance was expected to fall immediately due to the influence of the ACD, but actually increased concentrations were found in those experiments employing dyes which inhibit the removal of pyruvate. The initial concentration was lower in B-3 than in B-1 and B-2, and it fell to a very low level on the 3rd day. However, on the 6th day the concentration had increased to a value less than 0.40 millimoles lower than in the two citrate-dextrose mixtures. Thus, diphosphoglycerate behavior appears to follow that expected from observations on blood in ACD during the first 3 days. After this time, when the pyruvate rises, it behaves according to observations in the dye experiments.

Glycolytic Behavior of Blood Preserved in Citrate Alone.

Only one experiment of this type was carried out, Experiment 3, Series D, (graphs on page 55). Sugar was destroyed very quickly, reaching a minimal value on the 7th day which probably represents the non-sugar reducing substances. Lactic acid, however, continued to rise until about the 18th day. The source for the additional lactic acid which

was formed in excess of the equivalent of glucose removed is not known. About twice as much lactate and pyruvate was formed as could be accounted for by the removal of sugar and diphosphoglycerate.

Diphosphoglycerate commenced falling at about the time the glucose was exhausted, but pyruvate, like lactate, continued to rise until the 18th day.

Comparison of the Glycolysis of Blood From Mental Patients With That of Normal Blood.

In both the bloods of Series E (graphs on pages 60, 62) glucose was removed at the normal rate. In both bloods there were rises in the sugar concentration on the third day followed by a steady fall until the end of the experiment. Approximately 10 millimoles per liter were destroyed in each blood. These bloods were preserved in citrate-dextrose solution. In both good agreement was found between the sum of sugar and diphosphoglycerate destroyed and the formation of lactate and pyruvate. The terminal concentrations of glucose, lactate, diphosphoglycerate, and pyruvate were much the same in the two bloods. This experiment was carried on for 63 days at the end of which time glycolysis had virtually ceased; despite the numerous similarities in behavior, however, there were certain marked differences. During the experiment the rate of fall in sugar concentration was about the same, in both cases, but the fall in diphosphoglycerate was delayed in the depressive's blood, and in this blood the accumulation of pyruvate and lactate also was delayed.

The fall in diphosphoglycerate concentration occurred beginning with the 13th day. This is within the range of 10 and 15 days found in Experiments C-1 and D-4, for two normal bloods stored in citrate-dextrose. The fall was delayed in Experiment E-2, namely in the depressive's blood, until the 25th day. The rise in pyruvate also was delayed about 10 days.

The initial concentrations of pyruvate were slightly higher in the bloods of the two mental patients than in normal subjects, the schizophrenic (Experiment E-1) being slightly the higher of the two.

The schizophrenic patient had been showing a steady deterioration in his condition, so it was decided to repeat the experiment a couple of months later. This was Experiment F-1. The glycolytic picture was not as clear-cut as in the previous experiment (E-1). Almost twice as much sugar disappeared as could be accounted for in the lactic acid and pyruvate production. The initial concentration of diphosphoglycerate was low, 1.50 millimoles, and the fall was delayed until the 15th day. The pyruvic acid rise also was delayed for the same time. The initial concentration of pyruvate was at a quite low level, 0.07 millimoles, and the concentration of lactate at the beginning of this experiment was about half of what it was initially in Experiment E-1. These three experiments represent the first of a series on bloods of mental patients that will be carried out. The main differences found so far in these bloods are that in both conditions there is an elevated pyruvate level and that in the depressive's blood the fall in diphosphoglycerate and the rise in pyruvate

may be delayed. In the second experiment employing the schizophrenic's blood, there were marked disturbances in the glycolytic mechanism in that the glucose and diphosphoglycerate fall did not balance the rise in lactate and pyruvate.

Conclusions.

In five of the ten experiments comprising Series C, D, E, and F, the day on which the first marked fall in diphosphoglycerate occurred, the pyruvic acid commenced to rise. These were Experiments C-3, D-3, D-4, E-1, and F-1. In two of the Experiments, C-2 and D-2, (graphs on pages 46 and 53) in which Brilliant Cresyl Blue was added to the preservative, the pyruvate commenced to rise immediately in both cases. The diphosphoglycerate fell on the 15th and 14th days respectively. In two citrate-dextrose experiments there was no correlation. In the experiment, D-3, where citrate alone was the preservative, the beginning of the rise in pyruvate coincided with the time of exhaustion of glucose, and, on the same day, the results of hydrolysis indicated that diphosphoglycerate had fallen markedly. This was the only instance when there was a definite correlation between sugar disappearance and the fall in diphosphoglycerate or the rise in pyruvate. In all of the experiments with added dextrose there was no correlation between the fall in sugar and the rise in lactic acid on the one hand, and the fall in diphosphoglycerate and the rise in pyruvate on the other hand.

The behavior in acid-citrate-dextrose of pyruvate and diphosphoglycerate is somewhat different. In this preservative

medium diphosphoglycerate commences an immediate fall, but the rise in pyruvate is slow and gradual, and does not commence until later than is usual in citrate-dextrose, and here again, there is no obvious correspondence on the sugar disappearance curve or on the lactic acid accumulation curve to the point when the diphosphoglycerate reaches a minimum level. Thus, in these two preservative media, two pairs of compounds, sugar and lactic acid, and diphosphoglycerate and pyruvate, are found side by side with apparently no relationship between them.

In the blood preserved in citrate alone, three events occurred simultaneously. On the seventh day glucose fell to its minimum concentration, diphosphoglycerate commenced a marked fall, and pyruvic acid commenced a marked rise. Thus, good correlation in the behavior of these three compounds was observed.

In those experiments where the dye employed caused an increase in pyruvate, there was a decrease in the amount of lactic acid formed, and the concentration of diphosphoglycerate was somewhat increased, and the fall was delayed.

Thus, in citrate alone and in citrate-dextrose with certain added dyes, the connection between these four compounds is established. So it is seen that the results of the experiments reported in this study are explainable on the basis of the modern glycolytic scheme proposed by Barron (see page 17).

Three events need explaining by this scheme:

1. The connection in citrate alone between the attainment of the minimum concentration of glucose with the fall in diphosphoglycerate;

2. The correlation in citrate alone between the fall in diphosphoglycerate and the rise in pyruvic acid; and

3. The accumulation, in the dye preservatives, of pyruvate at the expense of the lactic acid formation.

Exhaustion of the glucose would result in a failure to form diphosphoglyceraldehyde. When the latter is used up there would be no hydrogen available for the reduction of DPN, nor could the diphosphoglyceric acid level be maintained, hence the fall in this substance. Lack of reduced DPN eliminates the source of hydrogen for the reduction of pyruvic acid to lactate, thus the rise in pyruvate.

Brilliant Cresyl Blue, Nile Blue, and Methylene Blue are hydrogen acceptors, and thus compete with the pyruvate for the hydrogen of the reduced DPN. Therefore, there is an increase in the concentration of pyruvate, and lactic acid formation is retarded.

The answer as to why the relationships are not as clear as when blood is preserved in citrate-dextrose solution, is that the added sugar naturally requires a longer time to be removed. During this added time haemolysis takes place, which prevents the rephosphorylation of ATP (6). There is for this reason a breakdown in the glycolytic cycle before the glucose is exhausted, which obscures the various relationships noted in the citrate preservative.

SUMMARY

1. Various factors influencing the glycolytic process in stored blood were investigated.

2. In citrate-dextrose preservative mixtures sugar fell at an irregular rate and reached minimal concentrations between the 29th and 72nd days. Unaccounted for rises in the sugar concentration occurred initially and around days 9, 16, 22, 29, and 37. The diphosphoglycerate content remained fairly constant for about 10 to 15 days and then fell to a minimal level about the 14th to 19th day. Pyruvic acid usually increased very slightly on the 3rd or 4th day and then decreased for about 3 to 6 days. After this time there were rapid increases in the concentration of pyruvic acid until about the 19th day when the content remained nearly constant for the remainder of the experimental period. Lactic acid accumulated throughout the experimental period, except when this was longer than about 36 days.

3. Baxter's ACD Solution (F-9x) was found to slow the overall glycolysis when compared with the citrate-dextrose controls. This may be accounted for by an immediate liberation of some of the phosphate from the diphosphoglycerate fraction. There was a slower rate of fall in the glucose concentration, a delayed rise in pyruvate, and a diminished accumulation of lactic acid.

4. Three basic dyes, Brilliant Cresyl Blue, Nile Blue, and Methylene Blue, were found to exert a strong inhibition on the removal of pyruvate. This is explained by the fact

that these dyes accept hydrogen from the reduced DPN present, thus inhibiting reduction of pyruvate to lactate. In these instances, the formation of lactic acid was diminished and the fall in diphosphoglycerate was delayed. There was also an increased removal of glucose.

5. One acid dye, Evans' Blue, was found to exert no influence on glycolysis in blood stored in citrate-dextrose. Another acid dye, Vital Red, was found apparently to inhibit the utilization of sugar, the fall in diphosphoglycerate, and the rise in pyruvate, yet lactic acid accumulated in the normal manner.

6. The results of the experiments on blood from the mental patients show little difference from the normal in the case of the schizophrenic. The depressive patient's blood, on the other hand, showed a delay in the fall of diphosphoglycerate and in the rise in pyruvate, also the rate of formation of lactic acid was somewhat slower.

7. In seven experiments more lactate and pyruvate were formed than could be accounted for by sugar and diphosphoglycerate removed. Three possible explanations are offered:

a) The remote possibility that citrate or some other precursor may be a source of additional pyruvate and lactate, thus complicating the glycolytic balance sheet,

b) That a copper-reducing intermediate may be found thus tending to maintain the reducing level despite the removal of glucose,

c) That the failure to balance the glycolytic balance sheet is due to failure to take into consideration other glycolytic intermediates such as hexose phosphates, etc.

This is most likely the correct explanation.

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