(Short Title)

METABOLISM OF NUCLEOTIDES IN RED BLOOD CELLS

by

S.V. Manohar

INCORPORATION OF GLUCOSE CARBON INTO THE ADENINE NUCLEOTIDES OF THE ERYTHROCYTE

by

S. V. Manohar

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of Master of Science.

Department of Biochemistry
McGill University
Montreal.

April 1965

ACKNOWLEDGEMENTS

I wish to express my sincerest appreciation to my research directors Dr. O.F. Denstedt and Dr. D. Rubinstein for their advice and encouragement.

I am most grateful to Dr. J.H. Spencer and Dr. P.G. Scholefield of the Biochemistry Department for their helpful criticisms and discussions.

I am indebted to Mrs. Gertrude Hartmann for the patience and care with which she has typed the thesis, and to Mr. N.S. Randive for the donation of his time for the proofreading.

Special thanks are due to my wife, Malati, who has drawn some of the figures included in this thesis.

This study was supported by a grant from the Defence Research Board of Canada (Project No. 9350-01).

PREFACE

The work reported in this thesis forms a part of the larger programme concerning the preservation of blood. This programme was started at McGill University's Biochemistry Department at the beginning of World War II under the direction of Dr. O.F. Denstedt and has continued, without interruption, to the present time. During these years attention was particularly given to the metabolism of erythrocytes since it was clear that in order to improve the methods of preservation, it is essential to know the nature of the metabolic changes responsible for the loss of cell viability.

With the discovery, by Gabrio and Finch in 1954, of the dramatic action of adenosine and other purine nucleosides on the rejuvination of stored erythrocytes, an impetus was given for the investigation of different aspects of metabolism of these substances in the blood.

Another important discovery in this field was made by Nakao *et al.* in 1959, when they found that in very old specimens of preserved blood (8 weeks) while inosine alone is of very little use in improving the post-transfusion viability of erythrocytes, the addition of adenine together with inosine had a dramatic effect.

The importance of adenine is further enhanced by the discovery of Simon *et al.* in 1962, that adenine alone added to the stored blood also increases the post-transfusion survival of

erythrocytes.

It was of interest, therefore, to study the effect of adenine on the metabolism of erythrocytes.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF ABBREVIATIONS

ACD	Acid-Citrate-Dextrose medium of blood preservation
AMP,ADP,ATP	Adenosine mono-, di-, tri-phosphate
UMP,UDP,UTP	Uridine mono-, di-, tri-phosphate
CMP,CDP,CTP	Cytidine mono-, di-, tri-phosphate
IMP,IDP,ITP	Inosine mono-, di-, tri-phosphate
GMP,GDP,GTP	Guanosine mono-, di-, tri-phosphate
NAD	Nicotinamide Adenine Dinucleotide
NADH	Reduced " " "
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced " " " "
R-5-P	Ribose-5-phosphate
R-1-P	Ribose-1-phosphate
Ru-5-P	Ribulose-5-phosphate
Xu-5-P	Xylulose-5-phosphate
S-7-P	Sedoheptulose-7-phosphate
E-4-P	Erythrose-4-phosphate
G-6-P	Glucose-6-phosphate
Hexose-6-P	Hexose-6-phosphate
F-6-P	Fructose-6-phosphate
F-1,6-P	Fructose-1,6-diphosphate
C3P	Glyceraldehyde-3-phosphate

LIST OF ABBREVIATIONS (continued)

PRPP	Phosphoribosylpyrophosphate
P-P	Pyrophosphate
Pi	Inorganic phosphate
Glucose-U-C^{14}	Glucose labelled uniformly in its carbon atoms with C^{14}
Glucose-1-C^{14}	Glucose labelled in its carbon atom-1 with C^{14}
Glucose-6-C^{14}	" " " " " " -6 " "

INTRODUCTION

Preservation of blood

The erythrocyte is specialized for the transport of oxygen and carbon dioxide between the lungs and other body tissues. It is metabolically a relatively inactive cell. The mature human erythrocyte has no nucleus and is incapable of duplication. It is unique among the somatic cells in that little or no work is done in fulfilling its primary function of transporting oxygen and carbon dioxide. Because of the peculiar properties of hemoglobin, these are passive functions not requiring the expenditure of energy by the cell. What energy is expended by the cell is directed at maintaining the cell in a functional state.

Glycolysis is the most important energy yielding process in the erythrocyte. It is well known, however, that the oxygen carrying capacity of the hemoglobin persists, though perhaps the rate of the exchange of gases may be slower after the erythrocyte has ceased to function as a glycolysing cell.

Blood transfusion is performed mainly to provide the patient with red cells while his hemopoietic system produces new cells. The ability of preserved red cells to remain in the recipient's circulation after transfusion compared with that of fresh red cells affords an index of the efficiency of the method of preservation. On transfusion of fresh blood slightly less than

1 per cent (0.8%) of the donor's cells are removed from the recipient's circulation each day. This is the normal rate of cell turnover in the healthy individual. Thus, if a blood specimen is preserved under favourable conditions at 5°C for 10 days, about 10 per cent of the cells have become nonviable. If the specimen is transfused, the nonviable cells will be removed from the circulation within a few (3 or 4) hours. Those cells which survive the initial rapid destruction thereafter are eliminated from the circulation at the normal rate of about 1 per cent per day.

Mollison and Young (1, 2) have demonstrated that the red cells may become nonviable during preservation without undergoing hemolysis. Furthermore the cells which would undergo hemolysis if placed in slightly hypotonic saline do not necessarily undergo hemolysis when placed in the circulation; they may survive for a long time. Since the rapid phase of destruction of preserved red cells after transfusion is virtually complete within 3-4 hours after transfusion, estimation of the percentage of the transfused cells remaining in the circulation 24 hours after transfusion affords a satisfactory index of the post-transfusion survival.

Donohue and Finch (3) found that the red cells of the rabbit and the human behave much the same during preservation under the comparable conditions. Similarly the survival after transfusion into the respective species has been found to be comparable. Thus,

preservation and survival studies with rabbit blood have proved to be convenient and reliable for evaluating the merits of blood preservatives and methods of preservation for human blood specimens.

Blood usually is stored at 4°C. At this temperature the rate of metabolic reaction is greatly reduced as compared with that at body temperature. Preservation of blood in isotonic trisodium citrate alone at 4°C is unsatisfactory because the red cells deteriorate rather rapidly after the endogenous glucose becomes used up within 5 to 10 days. As early as 1916, Rous and Turner (4) demonstrated that addition of glucose to the preservative medium greatly prolonged the period of preservation of red cells. The energy required for sustaining the cells is solely provided by the utilization of glucose. The membrane of the human red cells is freely permeable to glucose.

Aylward et al. (5) and Maizels (6) reported that the addition of glucose reduces the hydrolysis of ester phosphorus in the red cells. Acidification of blood with citric acid as a means of retarding the metabolic activity of the red cells during storage was first shown by Bushby et al. (7), in 1940, with animal blood. But on reinfusion it was found to have toxic effects. Later, in 1943-44, Loutit et al. (8) found the method satisfactory with human blood. The effect of acidification is mainly to shift the pH

away from the optimum for glycolytic enzymes and thus retard the metabolic activity of cells during storage (9).

In blood specimens stored with the medium containing citric acid, sodium citrate and dextrose (i.e. 'ACD blood'), the rate of utilization of glucose by the red cells is decreased from the beginning of storage. The preserved specimens remain free from hemolysis for 4 weeks or longer. During preservation the pH continues to fall because of the production of lactic acid. As shown by Kashket et al. (9), at the lower than normal pH values certain glycolytic enzymes, notably hexokinase, undergo a progressive and apparently irreversible inactivation.

The metabolic deterioration of red cells during storage is a process different from that of aging in vivo. During storage the red cells undergo characteristic and critical physical changes: sphering with an increase in volume, a decrease in diameter and in surface area. The osmotic and mechanical fragility of cells is increased during storage. With the accumulation of lactic acid the hydrogen ion concentration in the red cells and in the blood is slowly increased and the ability of red cells to utilize glucose by glycolysis is progressively diminished. Rapoport (10) has reported that the inorganic phosphorus concentration increases sharply in the red cells with corresponding decrease in the other acid soluble phosphorus compounds. Gabrio and Finch (11) have re-

ported that the adenosine triphosphate (ATP) concentration of the cells was progressively diminished during the storage of blood.

In the circulation, on the other hand, the ATP concentration in the red cells appears to undergo little change with aging of the cells (11). Gabrio and Finch (11) observed also in the dog that there was no significant change in osmotic or mechanical fragility of the circulating red cells over a period of 10 to 150 days. Furthermore, there was no appreciable diminution in the ability of cells to utilize glucose. In the same study (11) it was observed that the organic and the inorganic phosphate concentration remained constant during the aging of the circulating erythrocyte mass and the easily hydrolyzable phosphate fraction showed only random fluctuation. Lowy et al. (12), however, suggest that the ATP may fall slightly during the aging of the cells, in vivo.

The capacity of the preserved cells to survive in the circulation after transfusion decreases with the duration of storage of the blood and the ATP content similarly undergoes progressive diminution (10, 11). It would appear, therefore, that the post-transfusion survival of the red cells is related to their ATP content. However, as observed by Mollison and Robinson (13), after 60-80 days of storage the ATP content appears to be less significant in its influence on the post-transfusion survival of the red cells.

When the relationship of ATP concentration to post-transfusion survival of preserved red cells first became apparent, efforts were made to increase the ATP content of the preserved cells by the addition of various substances to the blood specimens. Judged by the behavior of added adenosine monophosphate (AMP), Albaum et al.(14) concluded that the nucleotides cannot pass through the red cell membrane. Under appropriate conditions, however, it was found possible to alter the composition and concentration of nucleotides in the red cells. Addition of riboflavin to the blood, for example, produces an increase in the rate of flavin nucleotide synthesis in the cells, both in vivo and in vitro (15). It was apparent, therefore, that certain molecular groups of nucleotides can enter the red cell.

Gabrio and Finch (16), in 1954, reported that the addition of carbohydrate intermediates, amino acid, liver particulates to the preserved blood was ineffective in reconstituting the organic phosphate compounds of the red cells. Similarly, the addition of succinate, α-ketoglutarate, fumarate and nicotinamide had no effect on resynthesis of organic phosphate compounds (17). Nor was the addition of adenine, ribose, inorganic phosphate, ribose-5-phosphate (R-5-P) individually, or in combinations, found to increase the concentration of labile phosphate in the preserved red cells. Adenosine diphosphate (ADP) and ATP likewise gave

negative results, while AMP was only slightly effective.

Gabrio and Finch, in the same study (16), found that the addition of adenosine stimulated a rapid and marked regeneration of labile organic phosphates in the preserved cells on incubation of the specimens for one hour at 37°C. The regeneration of labile organic phosphate gave rise to improvement in the posttransfusion survival of the red cells compared to that of specimens not incubated with adenosine before transfusion. Adenosine was shown to be effective also when added to the blood specimens at the beginning of storage at 4°C. The addition of adenosine to the preserved blood samples enhances the glucose utilization by the cells (17). The optimal concentration of adenosine was found to be 2,000-2,500 μmoles per 100 ml of red cells (17, 18).

In 1955, Gabrio and Huennekens (19) observed that other purine nucleosides such as inosine, deoxyadenosine, guanosine and xanthosine similarly stimulate the resynthesis of organic phosphates when added to the preserved red cells. The beneficial effect of guanosine on ATP maintenance was shown also by Prankerd (20).

Donohue et al. (21) found deoxyadenosine to be as effective as adenosine in causing the regeneration of organic phosphates and maintenance of posttransfusion viability of the red cells. At the same time they pointed out that the hypotensive action of small amounts of adenosine (1-10 μmoles), administered to the rab-

bit and the dog, indicates the potential hazard of using this nucleoside in the preservation of blood for clinical use. Related purine nucleosides such as guanosine, xanthosine and inosine in dosage 10 times the minimal hypotensive dosage of adenosine caused no observable effect. The hypotensive activity of deoxyadenosine is less than one-twentieth of that of adenosine.

Gabrio et al. (22), in 1956, compared the effectiveness of various purine nucleosides as a preservative for the red cells. They found that when added individually in equimolar amounts (1300 μmoles per 100 ml of cells) to the stored blood specimens, inosine proved to be most effective in promoting the resynthesis of high energy organic phosphates. Next to inosine in the descending order of effectiveness were adenosine, guanosine and xanthosine.

The weight of evidence now indicates that adenosine is superior to inosine in preserving the red cell viability during preservation (13). Lange et al. (23) studying the efficasy of inosine in the preservation of red cells obtained posttransfusion survival results comparable to that reported by Gabrio et al. (22) only with about 19 per cent of specimens. As a rule the posttransfusion survival was inferior with specimens stored for more than 28 days with inosine. Also, Overgaard-Hansen et al.(24) reported that the effectiveness of adenosine in the resynthesis of ATP from ADP and AMP in the cell is about 25 per cent greater than with

inosine. They suggested, therefore, the probability that adenosine, like inosine, provides ribose phosphate but serves also as a substrate for the direct synthesis of adenine nucleotides. Jones et al.(25) found that adenosine, after incubation for one hour at 37°C, increased the total nucleotide content of red cells which had been preserved for 1-2 years at -20°C. This observation also suggests that the purine moiety of adenosine is used as a substrate for ATP synthesis.

Red cells have been shown to be highly permeable to adenosine and inosine (26, 17). Also, as shown by Conway and Cooke (27) and by Rubinstein et al.(28) there is a powerful enzyme in red cells which deaminates adenosine to inosine. There is also evidence that free adenine is not formed from adenosine in blood (28).

Gabrio and Huennekens (19) partially purified the nucleoside phosphorylase of the red cells. Sandberg et al. (29) studied the activity of purine riboside phosphorylase in red cells of various mammalian species and reported that in the red cells this enzyme has no effect on xanthosine. Gabrio (30) found that this enzyme has no effect on adenosine. It has been obtained in highly purified form from human red cells by Tsuboi and Hudson (31). While investigating the properties of their purified enzyme preparation they found that inosine and guanosine are very susceptible to the action of the enzyme but that xanthosine is less susceptible.

Adenosine and pyridine nucleosides are not attacked by the enzyme (32).

Contradictory to these findings Prankerd (33) held that in human red cells adenine is formed from adenosine. However, as pointed out by Rubinstein and Denstedt (34), the RF values of adenine and hypoxanthine with the solvent used by Prankerd (33) for the separation of purines and their ribosides, are almost identical. It is more likely, as other workers (28) have shown, that adenine was not present in the system.

There is evidence also that the purine moiety of adenosine is not used for ATP synthesis in the human red cell. Lowy et al. (35) incubated fresh human red cells with adenosine-8-C^{14} and found little, if any, C^{14} in the isolated ATP.

Rubinstein and Denstedt (34) found little or no adenosine deaminase activity in human plasma. On the other hand, the adenosine, added to blood, can enter the red cells where it rapidly undergoes deamination to form inosine. The inosine is then split, presumably by phosphorolysis, yielding hypoxanthine and ribose-1-phosphate (R-1-P). Dische (36) has shown that the adenosine, added to hemolysates, gives rise to hexose-6-phosphate (hexose-6-P), fructose-1,6-diphosphate (F-1,6-P) and glyceraldehyde-3-phosphate (C3P). Added R-5-P likewise gives rise to these products.

It appears, therefore, that adenosine, added to the blood,

stimulates the synthesis of ATP by supplying ribose phosphate as a substrate for energy metabolism and not by providing a receptor for the phosphate groups. Inosine stimulates the synthesis of ATP by the same process. Rubinstein et al. (37) explain the superiority of adenosine over inosine on the ground that adenosine, by liberating ammonia on deamination, increases the pH of the stored blood thus favouring the utilization of glucose. The ribose phosphate formed in the metabolism of adenosine is first converted to hexose phosphates and triose phosphate which, in turn, may be converted to lactic acid by way of the glycolytic pathway. ATP is generated incidentally in the glycolysis.

In 1959 Nakao and coworkers (38, 39) observed that when the blood is preserved in ACD medium for several weeks, the regeneration of ATP in the cells no longer can be brought about by incubation at 37°C with inosine alone. They suggested that this may be accounted for by the depletion of adenine moiety which is required for the resynthesis of ATP. In the same study Nakao et al.(39) observed further that while added inosine induces practically no regeneration of ATP in 8-week old specimens of blood, the addition of adenine together with inosine effects a marked increase in the ATP concent of the red cells. In experiments with adenine-8-C^{14} they showed that adenine itself is

utilized for synthesis of adenine nucleotides and that inosine, or adenine and inosine together, did not increase the ATP concentration in fresh blood specimens; but only in the older preserved specimens was the effect, and particularly the superior effect of inosine and adenine together, pronounced in causing regeneration of ATP.

More recently in 1961, Nakao et al.(40) reported that when the hemolyzate from fresh human blood is passed through Dowex-1 column to remove the acid soluble phosphorus compounds, the addition of adenine-8-C^{14} together with R-5-P, or inosine and inorganic phosphate, magnesium and nicotinamide, gave no incorporation of the adenine-8-C^{14} into the adenine nucleotides. However, on addition of a trace of ATP to the mixture, the labelled adenine was incorporated into AMP but not into ATP. When larger amounts of ATP were added the labelled adenine was incorporated into ATP also. Furthermore, there was a net increase in the concentration of ATP. They explained the regeneration of ATP as indicated in the following reactions:

$$\text{Inosine} \longrightarrow \text{R-1-P} \rightleftharpoons \text{R-5-P}$$

(1) R-5-P + ATP $\rightleftharpoons$ AMP + PRPP

(2) PRPP + Adenine $\rightleftharpoons$ AMP + P-P

(3) 2AMP + 2ATP $\rightleftharpoons$ 4ADP

(4) 4ADP + n R-5-P $\longrightarrow$ 4ATP + other products

Overall: (n+1) R-5-P + Adenine + 3ATP $\longrightarrow$ 4ATP + P-P + other products

Price and Handler (41) have shown that acetone powder extract of human red cells can synthesize phosphoribosylpyrophosphate (PRPP) from R-5-P and ATP, and AMP from adenine and PRPP. The red cell membrane is very permeable to dissolved adenine (26). The enzyme, adenylate kinase, which catalyzes the reaction AMP + ATP $\rightleftharpoons$ 2ADP, was first found in muscle by Glowick and Kalckar (42,43). They called the enzyme myokinase, and observed its action in the phosphorylation of hexose in the presence of ADP and hexokinase. An enzyme with similar activity, but less stable, was purified from liver (44). Other workers (45, 46, 47) have demonstrated the occurrence of similar enzymic activity in many other tissues.

The presence of adenylate kinase in the human red cell has been reported by Kashket and Denstedt (48) and by Tatibana et al. (49). The enzyme is present in the red cell membrane as well as in the cytoplasm (48). This has been confirmed by Askari and Fratantoni (50) but Cerletti and Bucci (51) found the activity only in hemolyzate and not in the stroma of human red cells. Sventsitskaya (52) observed that ATP, added to the stored blood, increased the ATP content of red cells and maintained their metabolic viability. It is possible that in his experiments the added ATP was hydrolyzed to ADP by phosphatases in plasma (53) and that the ADP so formed then gave rise to intracellular ATP by the action

of adenylate kinase in the cell membrane.

The scheme proposed by Nakao et al.(40) for the regeneration of ATP in the red cells, on addition of inosine and adenosine to the blood, can be explained as follows: Inosine enters the red cell and is broken down to R-1-P and hypoxanthine by the action of purine nucleoside phosphorylase. The R-1-P is converted to R-5-P by phosphoribose mutase, and the R-5-P, in turn, is converted to PRPP by the action of ATP. The PRPP reacts with the added adenine in the cells to form AMP. One molecule of AMP and ATP in the presence of adenylate kinase give rise to 2 molecules of ADP. The latter may then be converted to 2 molecules of ATP during the catabolism of the remainder of the R-5-P.

Nakao et al.(54) and Wada et al. (55) reported that not only did the adenine and inosine together maintain the ATP concentration of the eight week old blood at a high level but the posttransfusion survival was also considerably better than that of control ACD-preserved cells. The hexokinase activity, osmotic resistance and morphological integrity also were better maintained (56).

In 1962, Simon et al.(57, 58) reported the beneficial effect of the addition of small quantities of adenine on the posttransfusion survival of preserved red cells. In one experiment, they found that after 42 to 46 days of storage at $4^{o}C$ only 35-39 per

cent of the control cells, preserved in ACD, remained viable, compared to 62-67 per cent of cells preserved in the same medium with addition of 0.5 to 1.0 μmole of adenine per ml. of blood. They observed further that any additional beneficial effect of inosine added along with adenine was relatively slight compared to that with adenine alone.

It is of interest that with the addition of adenine to the preserved blood, the glucose consumption and lactate formation remained decreased throughout the storage period of six weeks, as compared with that of the control specimen. This occurred notwithstanding the maintenance of ATP concentration slightly higher in the presence of adenine throughout the storage period. The ability of preserved red cells to utilize glucose is better maintained also in cells stored with adenine.

De Verdier *et al.*(59), in 1964, also reported the effectiveness of the addition of adenine alone on the viability of red cells. They found that the viability of the red cells in blood stored for 35 days with adenine was as good as that of the ACD-preserved specimens stored for only 21 days.

In the study of Simon *et al.* (57) the influence of adenine on cell viability was found to be more marked than the effect on ATP maintenance, thus suggesting that the ATP alone may not be the sole factor determining posttransfusion viability. Whether the beneficial action of adenine is more related to the maintenance

of the total adenine nucleotides remains to be determined. As reported by de Verdier (59), the presence of adenine appears to favour the maintenance of these nucleotides.

It is significant also that small amounts of adenine were consistently found to be more effective than large amounts (57). It is of incidental interest that although adenine is readily incorporated into mammalian cells in tissue culture, it inhibits cell multiplication at concentrations about 10^{-3} M (60,61). This concentration also has a deleterious effect on the viability and the glycolytic mechanism of preserved red cells.

The striking feature of the beneficial action of adenine on red cell preservation is its pronounced effectiveness in relatively minute concentrations. Thus, the amount of adenine for maximum effectiveness is about 150 µmoles per 100 ml. of red cells compared to an equivalent of about 2500 µmoles per 100 ml. of red cells with adenosine.

Hexose monophosphate shunt

The Embden-Meyerhof metabolic pathway in the tissue cells is the major route by which glucose is converted to pyruvic acid for further oxidation in the Krebs cycle. There are, however, other routes by which glucose can be oxidized. It is well known that even after addition of inhibitor substances such as arsenate, iodoacetate and fluoride which block one or other of the reactions in the glycolytic system, glucose can still be utilized by various animal and plant tissues.

In one of the alternative pathways known variously as the "hexose monophosphate shunt", "Warburg Dickens Pathway" or the "pentose phosphate pathway", glucose is converted to pentose phosphate, through glucose-6-phosphate (G-6-P) and 6-phosphogluconic acid.

In 1935, Warburg et al. (62, 63) showed that 6-phosphogluconic acid is formed by oxidation of G-6-P in red blood cells, and that the 6-phosphogluconic acid underwent further oxidation, in the presence of NADP, with the production of CO_2. Cori and Lipmann (64) showed that G-6-P is oxidized first to 6-phosphogluconо-δ-lactone which readily undergoes hydrolysis to 6-phosphogluconic acid. Brodie and Lipmann (65) showed that the hydrolysis of the lactone also is enzymatic. Glock and McLean (66) demonstrated the presence of G-6-P dehydrogenase and 6-phosphoglucon-

ate dehydrogenase in many tissues of rat, rabbit, ox and mouse. The high activity of these dehydrogenases in adrenal cortex (66, 67) and in lactating mammary glands (66) also was demonstrated.

Horecker and Smyrniotis (68) purified 6-phosphogluconate dehydrogenase from yeast and many animal tissues and showed that it catalyzes the following reaction:

$$\text{6-Phosphogluconic acid} + NADP^{+} \xrightleftharpoons{\text{Enzyme}} \text{Pentose-5-phosphate} + CO_2 + NADPH + H^{+}$$

The enzyme requires Mg^{++} ions probably for the decarboxylation step. Both reactions appear to be catalyzed by the same enzyme as they cannot be segregated.

Horecker _at al_. (69) identified the pentose phosphate formed in the above mentioned reaction as ribulose-5-phosphate (Ru-5-P) and showed that it is converted to R-5-P by the action of an enzyme R-5-P isomerase, which they purified from yeast. Axelrod and Jang (70) also purified the isomerase from alfalfa and various other plant sources.

Another enzyme, phosphoketopentose epimerase, catalyzes the interconversion of Ru-5-P and xylulose-5-phosphate (Xu-5-P). The presence of the epimerase in yeast and rabbit muscle was demonstrated by Svere _et al_. (71), and by other workers, in L.pentosus (72), horse erythrocytes (73) and calf spleen (74).

Thus, G-6-P is converted to Ru-5-P which, in turn, is con-

verted to R-5-P and Xu-5-P. Though all the reactions from G-6-P to pentose phosphates in the pathway are reversible, energy is required for conversion of pentose phosphate to hexose phosphate in the reverse direction (75). However, there is an alternative pathway by which pentose phosphates can be converted to hexose phosphates. In this pathway the formation of sedoheptulose-7-phosphate (S-7-P) and erythrose-4-phosphate (E-4-P) occurs as intermediates.

The first reaction of the alternative pathway is catalyzed by the enzyme, transketolase, which transfers a ketol group ($-CO-CH_2OH$) from a ketose donor to a suitable aldose acceptor.

Horecker et al. (76) purified the transketolase from rat liver and spinach, and from yeast, by Haba et al. (77). Horecker et al. (76) were the first to identify S-7-P as the product of the transketolase reaction in pentose metabolism. Thiamine pyrophosphate and Mg^{++} ions were shown to be essential cofactors in the reaction (76, 77). At first it was thought that Ru-5-P is the donor of the ketol group (78, 77), but later, the donor was shown to be Xu-5-P (71, 79). Fructose-6-phosphate (F-6-P) and S-7-P also can serve as donors of the ketol group, while R-5-P, glyceraldehyde-3-phosphate (C3P), glyceraldehyde, glycolaldehyde and E-4-P can act as acceptors (78). With F-6-P as the donor and C3P as the acceptor in the transketolase reaction, Xu-5-P and E-4-P are the products (80).

The second reaction of the pathway is catalyzed by the enzyme transaldolase. In this reaction the dihydroxy acetone group of S-7-P is transferred to C3P to produce F-6-P and E-4-P.

Horecker and Smyrniotis (81) purified transaldolase from yeast. The enzyme apparently requires no specific cofactor. It appears to catalyze specifically the transfer of the dihydroxy acetone group of S-7-P or F-6-P to the acceptors which are C3P, E-4-P or R-5-P (81).

The third reaction of the pathway is catalyzed also by the transketolase with the transfer of a ketol group from Xu-5-P to E-4-P to form F-6-P and C3P.

Thus in these reactions three molecules of pentose phosphates yield two molecules of F-6-P and one molecule of C3P. Both of the products are intermediates of the Embden-Meyerhof pathway.

The reactions of the pentose phosphate pathway are indicated in figure 1.

Experiments with extracts from rat liver (82), pea-root and pea-leaves (83), along with added labelled pentoses, have confirmed the validity of the postulated formation of hexose from pentoses in the transketolase-transaldolase catalyzed sequence. The activity of the pentose pathway is shown also by the fact that, in E. coli, the ribose moiety of RNA is formed by loss of the carbon atom-1 (C_1) of glucose (84, 85). There is evidence that

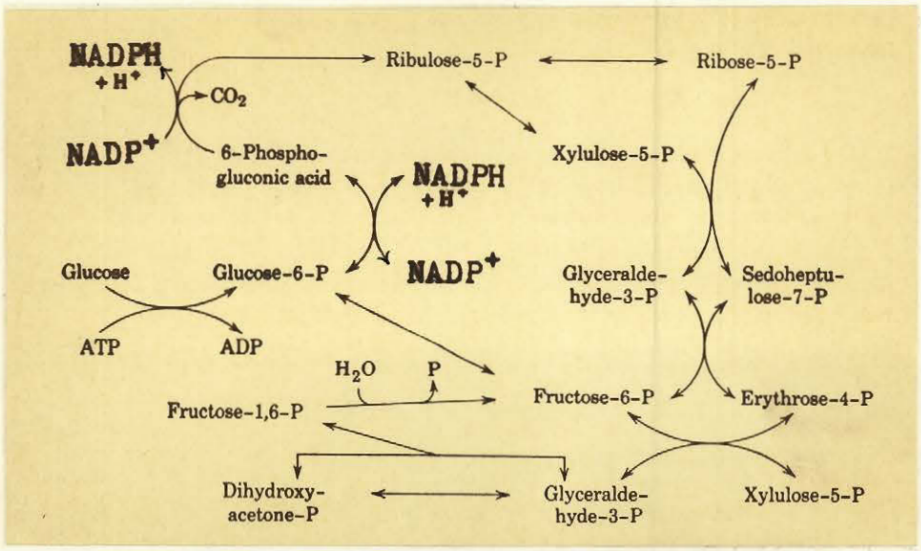


FIGURE 1

SCHEMATIC REPRESENTATION OF THE REACTIONS OF PENTOSE PHOSPHATE PATHWAY

this pathway may play a relatively more important metabolic role in plant tissue, especially in leaves, than in animal tissues (86).

The Embden-Meyerhof pathway, in conjunction with the Krebs cycle, is the exclusive metabolic route for the utilization of glucose in muscle (87) while 90 per cent of whatever amount of glucose metabolised by liver is utilised by this pathway (88, 89, 90). In some mammalian tissues for example, the lactating mammary gland (91) and leucocytes (92), the pentose phosphate pathway may be more active than the glycolytic pathway. In the erythrocyte, the Embden-Meyerhof pathway is the most important route of glucose utilization (93, 94, 95, 96).

Long before the existence of the reactions of the pentose phosphate pathway, or "shunt", was known, Harrop and Barron (97) investigated the respiratory activity of the red cell and found the oxygen consumption and CO_2 production of the mature cell to be very small and that it can be greatly increased by the addition of methylene blue. Other workers have confirmed the observation (98, 99). Brin and Yonemoto (100) showed that in this oxidation no CO_2 is obtained from carbon atom-6 (C_6) of glucose while carbons-1 and 2 accounted for about 95 per cent of CO_2. C_1 always gave more CO_2 than did carbon-2 (C_2). The ratio of the volumes of CO_2 produced from C_1 and C_2 was practically always constant.

Murphy (101) has shown that about 11 per cent of total glucose

utilized by erythrocytes is oxidized through the pentose phosphate pathway. He trapped and estimated the radioactive CO_2 evolved from the metabolism of uniformly labelled glucose-C-14 by absorbing it in sodium hydroxide. The percentage of glucose utilized through the "shunt" was calculated from the amount of radioactivity of trapped CO_2.

As the cytochrome system for the transport of hydrogen to oxygen is not present in the mature erythrocyte it has been suggested that the observed uptake of oxygen by these cells may be due to the contaminating white blood cells or reticulocytes or immature normocytes which are extremely difficult to remove from erythrocytes.

The presence of G-6-P dehydrogenase (Zwischenferment) in the erythrocyte was shown by Warburg et al. (62, 63) in 1935. They described also the presence of NADP (TPN) in erythrocytes. NADP is reduced when G-6-P is oxidized to 6-phosphogluconic acid and again when the latter is converted to Ru-5-P. There is no mechanism for the reoxidation of NADPH in the erythrocyte since the cytochrome system is absent. The observation of Harrop and Barron (97) that methylene blue, added to human erythrocytes, increased the oxygen consumption and CO_2 production is now satisfactorily explained by the knowledge that methylene blue can serve as an acceptor of hydrogen from reduced NADP and then transfer it to

atmospheric oxygen. Thus, under aerobic conditions, the addition of methylene blue or other acceptor of hydrogen transitorily can divert the oxidation of glucose. The oxidation of reduced NADP formed in the two dehydrogenase catalyzed reactions also is supposed to be coupled with a NADP-linked methemoglobin reductase (102) and a NADP-linked glutathione reductase (103,104).

The presence in the erythrocyte of various enzymes required for the conversion of pentose phosphates to hexose phosphate through S-7-P and C3P as intermediates has been demonstrated by many workers. Dische (36) has shown that R-5-P, added to hemolyzates, is converted to hexose-6-P. In 1957 Dische and Shigeura (105) demonstrated also the presence of phosphoribose isomerase and phosphoketopentose epimerase in human hemolyzates. Dickens and Williamson (106, 107) found the two enzymes to be present also in horse erythrocyte. Activity of enzymes converting R-5-P to Ketopentose-5-phosphate in human erythrocytes was studied by Brownstone and Denstedt (108).

The presence of transketolase activity in erythrocytes was described by Dische and Pollaczek (109) who demonstrated the formation of S-7-P from R-5-P in ATP-free hemolyzates. The presence of transketolase in human erythrocytes has been demonstrated also by Brownstone and Denstedt (110). They observed that no cofactors were required for transketolase activity of the dialysed stroma-

free hemolyzate. This is contrary to the observation with the transketolase from liver, yeast or spinach which appear to have a cofactor requirement for thiamine pyrophosphate and Mg^{++} ions. Brownstone and Denstedt (110) suggest that in erythrocyte these cofactors probably are so firmly bound to the enzyme that they are not detachable by dialysis.

Brownstone and Denstedt (110) demonstrated also the presence of transaldolase in erythrocytes. They measured the transaldolase activity by the amount of hexose-phosphate formed from S-7-P and C3P as substrates.

Thus all the enzymes required for the conversion of glucose to C3P through the pentose phosphate pathway are present in red blood cell. However, the cell contains no oxidant (hydrogen acceptor) for the oxidation of reduced NADP. Hence the NADP in the normal red cell remains in the reduced state. Though it may be oxidized through the NADPH-linked methemoglobin reductase or the NADPH-linked glutathione reductase, the magnitude of these reactions would be very small.

The purely artificial stimulation of the oxidation of glucose through the pentose phosphate pathway by addition of methylene blue or analogous hydrogen acceptor dyes, does not represent a true respiratory activity. Any energy liberated in the oxidation of glucose under these conditions is not collected in the form of high energy phosphate but is dissipated as heat.

Glucuronic acid pathway

Another possible route for the formation of a pentose or pentose-phosphate from glucose is called the 'glucuronic acid' pathway. The conversion of glucose to pentose by this route involves first, the conversion of glucose to glucuronic acid as follows (111, 112):

Glucose → Glucose-6-phosphate → Glucose-1-phosphate → UDP-Glucose
→ UDP-Glucuronic acid → Glucuronic acid

The conversion of UDP-Glucuronic acid to UDP-Glucuronic acid-1-phosphate and then to glucuronic acid has been demonstrated in rat kidney (113) and liver (114). Mano et al. (115) have purified, from rat liver, a NADPH-linked dehydrogenase which catalyzes the reduction of D-glucuronate to L-gulonate. A similar NADP specific dehydrogenase which oxidizes L-gulonate to D-glucuronate has been purified from mammalian kidney (116).

L-gulonate has either of the two metabolic fates: (a) lactonization to L-gulonolactone followed by oxidation to L-ascorbic acid, or (b) oxidation by a NAD dependent L-gulonate dehydrogenase to yield L-xylulose.

The NAD dependent conversion of L-gulonate to L-xylulose occurs in mammalian kidney in two steps. In the first step β-keto-L-gulonic acid is formed (117) which is then followed by the form-

ation of L-xylulose with the liberation of CO_2 from the carbon atom-6 of the original glucose molecule. L-xylulose is excreted in the urine of pentosuric subjects.

The metabolism of L-xylulose involves its conversion to xylitol. Mammalian liver contains a NADP dependent enzyme for this reversible conversion (118). Mammalian liver contains also a NADH dependent enzyme for the conversion of xylitol to D-xylulose (119). A kinase which, with ATP, effects the phosphorylation of D-xylulose to D-xylulose-5-phosphate is known to be present in mammalian liver (168).

In pentosuric subjects the normal metabolism of D-xylulose (or D-xylulose-5-phosphate) by way of the hexose-6-phosphate is blocked and this leads to the accumulation and excretion of L-xylulose. Xylulose-5-phosphate formed normally in the glucuronic acid pathway is converted to ribulose-5-phosphate and then to ribose-5-phosphate by the epimerase and isomerase, respectively.

The existence in the red cells of the enzymes essential for the conversion of glucose to pentose-phosphate through the glucuronic acid pathway has not been studied in any detail. However, it is evident that such a conversion will necessitate the formation of CO_2 from the oxidation of C_6 of glucose. The formation of CO_2 by red cells is known to be very small and all of it appears to arise from the C_1 of glucose. Therefore, it is reason-

able to assume that the glucuronic acid pathway is not operational in the red cell. Moreover, exogenous pentoses are not found to be utilized by the red cells and thus phosphorylation of pentoses cannot occur in these cells. As the presence of a kinase to phosphorylate xylulose to xylulose-5-phosphate is essential for the operation of the glucuronic acid pathway, it seems unlikely, from this consideration also, that pentose phosphates can be formed by this route.

It is evident from various routes of pentose phosphate formation from glucose that the various carbon atoms in the pentose are derived from different carbon atoms of glucose in each case. Thus it is apparent that when a pentose phosphate is formed through the hexose monophosphate shunt, the C_1 of glucose is not incorporated into pentose at all. On the other hand, in the formation of a pentose phosphate through the glucuronic acid pathway, C_6 of glucose is given off as CO_2 and thus does not appear in the pentose. In the transketolase catalyzed formation of pentose phosphates, two carbon atoms of fructose-6-phosphate and a molecule of glyceraldehyde-3-phosphate combine to form a pentose phosphate. In this type of formation of pentose phosphates, all the carbon atoms of glucose can be traced in the pentose. Therefore, if the carbon atoms of glucose - particularly C_1 and C_6 - are labelled, the extent of the incorporation of these into the pentose formed will give a clue as to the major route of formation of pentose.

Biosynthesis of nucleotides

In addition to the de novo synthesis of nucleotides from the metabolic intermediates of carbohydrates, proteins and fats, there are other biochemical processes by which various nucleotides can be synthesized.

Several enzymes are known which can catalyze reversible transphosphorylation reactions between a nucleoside triphosphate and a nucleoside monophosphate to form nucleoside diphosphates (120). These enzymes, isolated from yeast, are distinct from yeast-adenylate kinase which catalyzes specifically the transphosphorylation between ATP and AMP. Strominger et al. (121) provided evidence of transphosphorylation between ATP and UMP, ATP and AMP, UTP and AMP, ATP and CMP and between CTP andd AMP, in a preparation from calf liver. Krebs and Hems (122) demonstrated the presence, in sheep brain and liver, of an enzyme which catalyzes the following reaction:

$$AMP + ITP \rightleftharpoons ADP + IDP$$

An enzyme called diphosphokinase, purified by Berg and Joklik (123) from autolyzates of brewer's yeast and from extracts of rabbit muscle, is known to catalyze transphosphorylation between a nucleoside diphosphate and a nucleoside-triphosphate

$$ITP \text{ (or } UTP) + ADP \rightleftharpoons IDP \text{ (or } UDP) + ATP$$

Nucleotides also can be formed by transphosphorylation reactions involving nucleosides. Kornberg and Pricer (124) demonstrated the presence of an enzyme in yeast which phosphorylates adenosine to form AMP in the following manner:

$$\text{Adenosine} + \text{ATP} \rightleftharpoons \text{AMP} + \text{ADP}$$

2-amino adenosine also can serve as the substrate for this reaction.

Purine nucleosides, on the other hand, can be synthesized in the reactions catalyzed by the enzymes called nucleoside phosphorylases purified from rat (125, 126) and beef (127) liver. In these reactions a purine base and ribose-1-phosphate react reversibly to form a purine nucleoside and inorganic phosphate (Pi) as shown below:

$$\text{Hypoxanthine} + \text{R-1-P} \rightleftharpoons \text{inosine} + \text{Pi}$$

In another reaction, discovered by Kornberg *et al.* (128, 129, 130, 131), in a preparation from yeast, cleavage of a variety of nucleoside-5'-phosphates is brought about by pyrophosphate (P-P) giving rise to a nitrogenous base and 5-phosphoriboxyl pyrophosphate (PRPP)

$$\text{AMP} + \text{P-P} \rightleftharpoons \text{Adenine} + \text{PRPP}$$

This reaction is reversible and hence the same enzyme can synth-

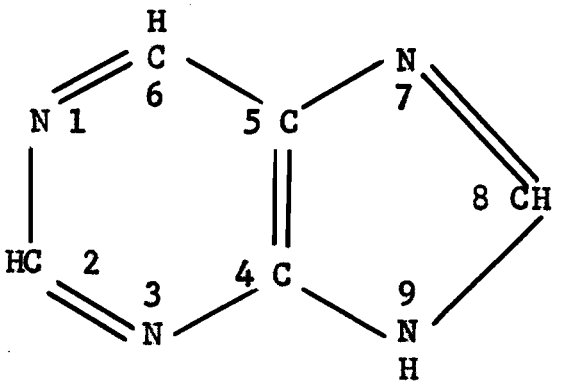
esize a mononucleotide from the nitrogenous base and PRPP.

PRPP is shown to arise also from the enzymic transfer of a pyrophosphate group from ATP to ribose-5-phosphate (128, 129)

$$ATP + R\text{-}5\text{-}P \rightleftharpoons AMP + PRPP$$

The de novo synthesis of purine nucleotides on first thought might be expected to begin with the synthesis of the purine ring and be followed by substitution with other groups. However, it is now known that the biosynthesis of purine ring starts at the nucleotide level.

It is known that the uric acid, which has the purine skeleton, is the principal nitrogenous excretion product of reptiles and birds. It is formed as a terminal metabolic product in the liver and the kidney of these species. It follows that a study of the distribution of isotopes of nitrogen and carbon in the purine ring of the uric acid excreted by these animals when fed with various isotopically labelled precursor compounds, may yield information about the mechanism of the synthesis of the purine skeleton. The system of numbering the atoms in the purine ring is indicated below:



Purine

The metabolic origin of the carbon atoms in the purine skeleton was established by administering labelled carbon compounds to pigeons. In 1948, Sonne et al. (132) showed that the carbon atoms of formate may be used as C_2 and C_8 of the uric acid. Buchanan et al. (133) demonstrated that the C_6 of the uric acid is derived from CO_2, the carboxyl carbon of glycine is the source of C_4, and that the α-carbon of glycine is the source of C_5. Elwyn and Sprinson (134) showed that the β-carbon of serine is incorporated into C_2 and C_8 of uric acid in the pigeon.

In 1956, Sonne et al. (135) showed with pigeon liver extracts that the N_7 of uric acid comes from the amino-nitrogen of glycine and N_9 is derived from the amide-nitrogen of glutamine. Levenberg et al. (136) demonstrated with similar enzyme preparations that N_1 is derived from aspartic acid and both N_9 and N_3 are derived in equal amounts from the amide-nitrogen of glutamine. Shemin and Rittenberg (137) also have demonstrated in human subjects that the amino-nitrogen of glycine is incorporated into N_7 of uric acid. Abrams et al. (138) obtained similar labelling of adenine and guanine of nucleic acids in yeast with N^{15} glycine.

The metabolism of the bird differs from that of the mammal in that the bird excretes uric acid as the chief end product of nitrogen metabolism while the mammal excretes urea. In the mammal the excretory product of purines, i.e. allantoin in most animals

and uric acid in man, constitutes a very small part of the total nitrogen excretion. The difference between the main products of nitrogen excretion among these species suggests that the pathways of metabolism and the precursors of purines may differ with the species. However, when rats were fed labelled carbon compounds, and guanine and adenine were isolated from the nucleic acids, it was found that, as in the experiments with the pigeon, the carbon of CO_2 is incorporated into C_6, the carboxyl carbon of glycine into C_4, the α-carbon of glycine into C_5, and the formate carbon into C_2 and C_8 (139).

The pigeon is unique among birds in that its liver contains no xanthine oxidase and thus hypoxanthine tends to accumulate in the isolated tissue. Greenberg (140) used carbon-labelled formate added to the pigeon liver homogenate and isolated labelled hypoxanthine and also some inosinic acid (IMP). From the respective specific activities of these compounds at various times during incubation, he concluded that IMP is a precursor of hypoxanthine. He suggested, therefore, that ribose and phosphate are added to the precursor of the purine before ring closure takes place. Dietary hypoxanthine also was found to be ineffective as a precursor of the nucleic acid purines in rats (141).

When the growth of certain bacteria is inhibited with bacteriostatic concentrations of sulphonamide drugs, a diazotizable amine

is found to accumulate in the medium. Stetten and Fox (142) suggested that it may be a normal intermediate in some metabolic reaction which is blocked by the drug, or be an abnormal product formed by the organisms under the influence of the drug. This compound has no dramatic effect on the growth of normal bacteria. Shiue et al. (143) identified the amine as 5-aminoimidazole-4-carboxamide and indicated that either it functions as a precursor of the purine bases or is formed from a precursor of the bases. Ravel et al. (144) found that in E. coli the synthesis of 5-aminoimidazole-4-carboxamide is stimulated by glycine. They suggested that it may be a precursor of hypoxanthine in biological systems.

Bergman et al. (145, 146) found that in a mutant strain of E. coli 5-formaminoimidazole-4-carboxamide is twice as effective as the 5-amino compound. However, isotopic studies with liver homogenates have shown that the imidazole compound is not a normal intermediate in the biosynthesis of purines (147).

Pigeon liver systems have been shown to catalyze the transfer of a formyl group to 5-aminoimidazole-4-carboxamide ribotide and also the closure of the ring to yield IMP (140, 148). This reaction occurs also in the presence of serine, and is reversible. A folic acid derivative is found to be essential for this reaction (149). This derivative has been shown to be N^{10}-formyl tetrahydrofolic acid (150).

The synthesis of 5-aminoimidazole-4-carboxamide ribotide begins with the introduction of N_9 which is known to be derived from the amide nitrogen of glutamine. Pigeon liver extract is known to contain an enzyme which converts PRPP and glutamine to 5-phosphoribosylamine, glutamic acid and pyrophosphate (151). Goldthwait et al. (152, 153) showed that in the pigeon liver extract, 5-phosphoribosylamine gives glycinamide ribotide in presence of glycine and ATP. They showed further that the product of the reaction if formylglycinamide ribotide if a folic acid derivative also is present. This folic acid derivative is shown to be N^5,N^{10}-anhydroformyltetrahydrofolic acid (150).

Levenberg and Buchanan (154) have shown that formylglycinamide ribotide is converted to formylglycinamide ribotide which, in turn, is converted to 5-aminoimidazole ribotide (155). They also showed that (155) 5-aminoimidazole ribotide can be converted to 5-aminoimidazole-4-carboxamide ribotide in the presence of ATP, aspartic acid and CO_2.

Lukens and Buchanan (156, 157) demonstrated that 5-aminoimidazole-4-carboxylic acid ribotide and 5-aminoimidazole-4(N-succinyl carboxamide) ribotide are intermediates between 5-aminoimidazole ribotide and 5-aminoimidazole-4-carboxamide ribotide. The conversion of 5-aminoimidazole-4-carboxamide ribotide to IMP in the presence of N^{10}-formyltetrahydrofolic acid has already been discussed.

The conversion of IMP to AMP is initiated by an enzyme - adenylosuccinate synthetase, partially purified from E. coli by Lieberman (158). This enzyme, in the presence of guanosinetriphosphate (GTP), converts IMP and aspartic acid to adenylosuccinic acid. Adenylosuccinic acid, in turn, is cleaved by the enzyme - adenylosuccinase to yield AMP and fumaric acid. This enzyme has been purified from yeast by Carter and Cohen (159).

A similar process probably operates in animal tissues. Adenylosuccinic acid and the corresponding purine (6 succinylaminopurine);have been found in mammalian liver (160). Abrams and Bentley (161) have obtained the conversion of FMP to AMP in rabbit bone marrow extract in the presence of L-aspartic acid and a source of high energy phosphate.

For the synthesis of guanosine monophosphate (GMP) from IMP in the rabbit bone marrow extract, the IMP first undergoes oxidation to xanthosine-5^1-phosphate with IMP dehydrogenase and NAD (161). The xanthosine-5^1-phosphate is then aminated to GMP in the presence of L-glutamic acid or L-glutamine, ATP and Mg^{++} ions. Aerobacter aerogenes also contains a NAD dependent dehydrogenase which converts IMP to xanthosine-5'-phosphate (162), and an ATP dependent enzyme which converts xanthosine-5'-phosphate to GMP in the presence of ammonia (163).

Purine nucleotide biosynthesis de novo in erythrocytes

Lowy et al. (164) have shown that rabbit reticulocytes obtained from animals made anemic by administration of acetyl - phenylhydrazine or by bleeding can, in vitro, synthesize purine nucleotides de novo. However, mature rabbit erythrocytes are unable to utilize glycine or sodium formate in vitro for the synthesis of purine nucleotides when these substances are supplied together with other small molecule purine ring precursors (165).

Despite the inability to carry out the de novo biosynthesis of purine nucleotides in vitro, the mature rabbit erythrocytes have been shown to be capable of effecting the final reaction required for the completion of the purine ring - namely the conversion of 5-aminoimidazole-4-carboxamide ribotide to IMP in the presence of formate (166). The rabbit mature erythrocyte also is capable of converting IMP to ATP and GTP in vitro by the metabolic routes described before (167). The mature rabbit erythrocyte has been shown to utilize various purines and their ribosyl derivatives for the biosynthesis of ATP and GTP (167).

The human mature erythrocyte resembles the rabbit cell in its inability to synthesize the purine nucleotides de novo from purine ring precursors in vitro. It is able to carry out the final step in the purine ring formation as is the rabbit cell (35). In contrast to the rabbit erythrocyte, the human red cell has little, if

any, capacity to convert IMP to AMP. The conversion of IMP to GTP, however, can occur in the human red cell (35).

The human erythrocyte is incapable of utilizing the purine ring of adenosine for the formation of adenine nucleotides (35). The rabbit erythrocyte, on the contrary, can perform this synthesis (167). However, both the human and the rabbit erythrocytes can readily incorporate the purine ring of adenine into ATP (35).

The inability of the mature human erythrocyte to convert IMP to AMP was thought to be attributable to the lack in the cell of GTP, which is an essential cofactor in the reaction. The concentration of GTP in the rabbit erythrocyte is found to be about 10 times that in human red cell. Notwithstanding this finding, the inability of the human erythrocyte to convert IMP to AMP appears to be attributable to the lack of cellular enzymes. In an experiment in which considerable net synthesis of GTP was first brought about by incubating human red cells with guanine and guanosine, no evidence was obtained of the conversion of IMP to AMP (35). Hence, the concentration of GTP does not appear to be the deciding factor in the reaction. It appears that the human red cell lacks either adenylosuccinic synthetase or adenylosuccinase, or both. If it is not known whether one or both of these enzymes are lost during the maturation of the human reticulocyte, as is known to be responsible for the loss of capacity for purine nucleotide

synthesis de novo in the rabbit cell.

Thus, of the three possible mechanisms of the renewal of adenine moiety of ATP in stored erythrocytes, namely, (1) overall synthesis de novo, (2) exchange of ATP between the plasma and the cell and (3) utilization of preformed purine, only the last appears to have any experimental support.

The investigation reported in this thesis was undertaken mainly to determine whether glucose serves as a substrate in the reactions by which preformed adenine is incorporated into ATP and, if so, to determine the probable mechanism by which glucose is utilized in the reactions.

EXPERIMENTAL

Materials and Reagents

Adenine

The preparation used in this study was crystalline adenine purchased from Sigma Chemical Company. A solution of the purine, in concentration of 2 μM, per ml. was prepared in Krebs-Ringer phosphate solution, pH 7.4. From this, various dilutions ranging in concentrations from 2.0 to 0.02 μM per ml. were prepared by dilution with the buffer.

Radioactive glucose

D-Glucose-U-C^{14}, D-Glucose-1-C^{14} and D-Glucose-6-C^{14} were obtained in crystalline form from Merck, Sharp and Dohme of Canada Ltd. and of activity either 0.1 or 0.2 mc. The glucose of 0.1 mc. activity was dissolved in 5.0 ml. of physiological saline and of 0.2 mc in 10.0 ml. A portion (0.1 ml.) of these solutions, applied to chromatographic and electrophoresis paper pieces and counted, gave about 2,000,000 c.p.m. The count for each of the solutions was determined, and the solutions then stored in frozen state.

Activated charcoal

Norite Sx30 SG Extra activated charcoal obtained from Fisher Scientific, Ltd., was used in the experiments.

Krebs-Ringer phosphate solution

This solution of pH 7.4 was prepared according to specifications of Umbreit et al.(169) Calcium chloride was omitted from the solution.

Citrate buffer for paper electrophoresis

Citrate buffer 0.045M pH 4.8, for paper electrophoresis, was prepared as follows:

Solution A	Crystalline Citric Acid	21.008 g
	IN NaOH	200.0 ml
	Distilled water to make 1000 ml	
Solution B	0.1N HCl	

The buffer solution was a mixture of the following:

9 parts of solution A

1 part of solution B

10 parts of distilled water

The pH was adjusted to 4.8

Solvent mixture for paper chromatography

This solution is a mixture of the following

Isobutyric acid	300 ml
1M NH_4OH	180 ml
3.8% Ethylenediamine-tetraacetate (sodium salt)	4.8 ml

The chromatographic paper used for separation of nucleotides was Whatman 3 mm grade.

The "Phosphor" or the scintillation counting fluid was prepared as follows:

PPO (2,5-diphenyloxazole, scintillation grade)	12.0 g
POPOP (1,4-bis-2-(5-phenyloxazolyl)-benzene, scintillation grade)	0.4 g
Toluene	4 litres

PPO and POPOP were obtained from the Packard Instrument Company.

Eluting solution for nucleotides

The solution for eluting the adsorbed nucleotides from the activated charcoal was a mixture of the following:

Ammonium Hydroxide 28-29%	5.0 ml
Distilled water	200.0 ml
95% Ethyl alcohol	95.0 ml.

Methods

Preparation of rabbit erythrocytes

The blood was collected in centrifuge tube from the marginal ear vein into 3.2 per cent sodium citrate soultion. The specimen was centrifuged and the plasma, the "buffy coat" and the surface alyer of erythrocytes were removed by means of a Pasteur pipette. The cells were washed three times with five volumes of physiological sodium-chloride solution, each time removing the surface white-cell layer together with a little of surface red cells. The cells then were washed once with five volumes of Krebs-Ringer phosphate solution and suspended in 4 volumes of the latter. This suspension contained about 20 per cent of erythrocytes by volume.

Estimation of hemoglobin

The hemoglobin content of the red cell suspension or the hemolyzate was determined by the modified cyanmethemoglobin method of King (170) as further modified by Brownstone (108).

Estimation of phosphate

Inorganic orthophosphate was determined by the method of Fiske and Subbarow (171). The "labile" or 7-minute hydrolyzable phosphate was determined from the increase in the inorganic phosphate concentration after hydrolysis of the solution in IN HCl for 7 minutes in a boiling water bath.

Quantitative recovery of nucleotides adsorbed on active charcoal

The purine moiety of nucleotides absorbs light strongly at 260 mμ. The optical density of a solution of nucleotide at any fixed wave length in ultraviolet region is proportional to the concentration of nucleotide. If the solvent itself absorbs light at that particular wave length, then the concentration of nucleotide will truly be proportional to the difference in optical densities of the solution and that of the solvent. In the experiments to be described, the solvents in which the nucleotides were in the dissolved state were 2.5 per cent trichloracetic acid and 0.2N NH_4OH in 30 per cent ethyl alcohol. Trichloracetic acid (2.5 per cent solution) shows some absorption at the wave langth (260 mμ) at which the optical density was measured. NH_4OH (0.2N) in 30% ethyl alcohol has negligible absorption at 260 mμ. In the calculation of the concentration of nucleotide in solution the value of the optical density of the solvent was deducted from that of the solution.

When nucleotides are completely adsorbed on the activated charcoal, the optical density of the clear supernatant falls to that of the clear supernatant obtained from blank solvent treated in the similar way with the charcoal on elution, the portion of nucleotides eluted can again be calculated from the optical density of the eluate. Again, the optical density of the eluate from

the blank experiment must be deducted from that of the "test" experiment.

In determining the portion of nucleotides eluted from the charcoal, the volumes of nucleotide solution before and after the charcoal treatment must be taken into consideration. In the experiments to be described, the recovery of nucleotides by the method used (see below) was always about 55-60 per cent.

Separation of ATP, ADP and AMP by electrophoresis

Paper electrophoresis was carried out with a Spinco Paper Electrophoresis cell - Durrum Type, Model R, Series D. The apparatus takes eight paper strips at a time each about one inch wide. The quantity of nucleotide extract generally applied to each strip was 0.1 ml. This was applied at the cathode end across the whole width of the paper strip in a narrow band. The solutions of pure ATP, ADP and AMP together and individually were likewise applied to other strips which served as 'reference' strips. When the applied solutions were completely dried, the strips were quickly wetted with the citrate buffer, blotted lightly and placed in the apparatus. The separation of nucleotides was carried out at 300 volts for five hours. The distances travelled by the ATP, ADP and AMP towards the anode under these conditions in a typical run were 16, 14 and 10.5 cm respectively.

Separation of ATP, ADP and AMP by chromatography

The chromatographic separation was carried out by ascending paper chromatography in a cylindrical glass container 45 cm high and 15 cm in diameter. The nucleotide extract was applied as a narrow band 2.5 cm in length and 5 cm from the bottom edge of the paper. Pure solutions of ATP, ADP and AMP also were applied at different positions on the same paper to serve as 'references'. After application of the solutions the paper is dried in air and then the sides of the sheet stapled together to form a cylinder 40 cm in height. This paper cylinder is then placed in the glass jar containing about 250 ml of the chromatographic liquid mixture with the paper standing in the liquid to a depth of about 2.5 cm. The glass jar is covered with a glass place sealed by a layer of grease applied to the top edge of the jar. The separation of nucleotides is carried out for 15 hours at the room temperature. The R_F values of ATP, ADP and AMP were found to be 0.40, 0.46 and 0.60, respectively.

Locating the positions of nucleotides on paper

At the conclusion of the run the chromatographic paper and electrophoresis strips were dried in air. The positions of the various nucleotides on the paper were located by exposing the paper to ultraviolet light from a mercury vapour lamp in a dark room and the dark bands or spots of the nucleotides on fluor-

escent background of paper were marked with a pencil. Sometimes the position of the AMP band was only difficultly visible because of the very low concentration. In such cases its position is fixed by comparing the position of pure AMP on the accompanying 'reference' strip of paper.

Estimation of radioactivity of the isolated nucleotides

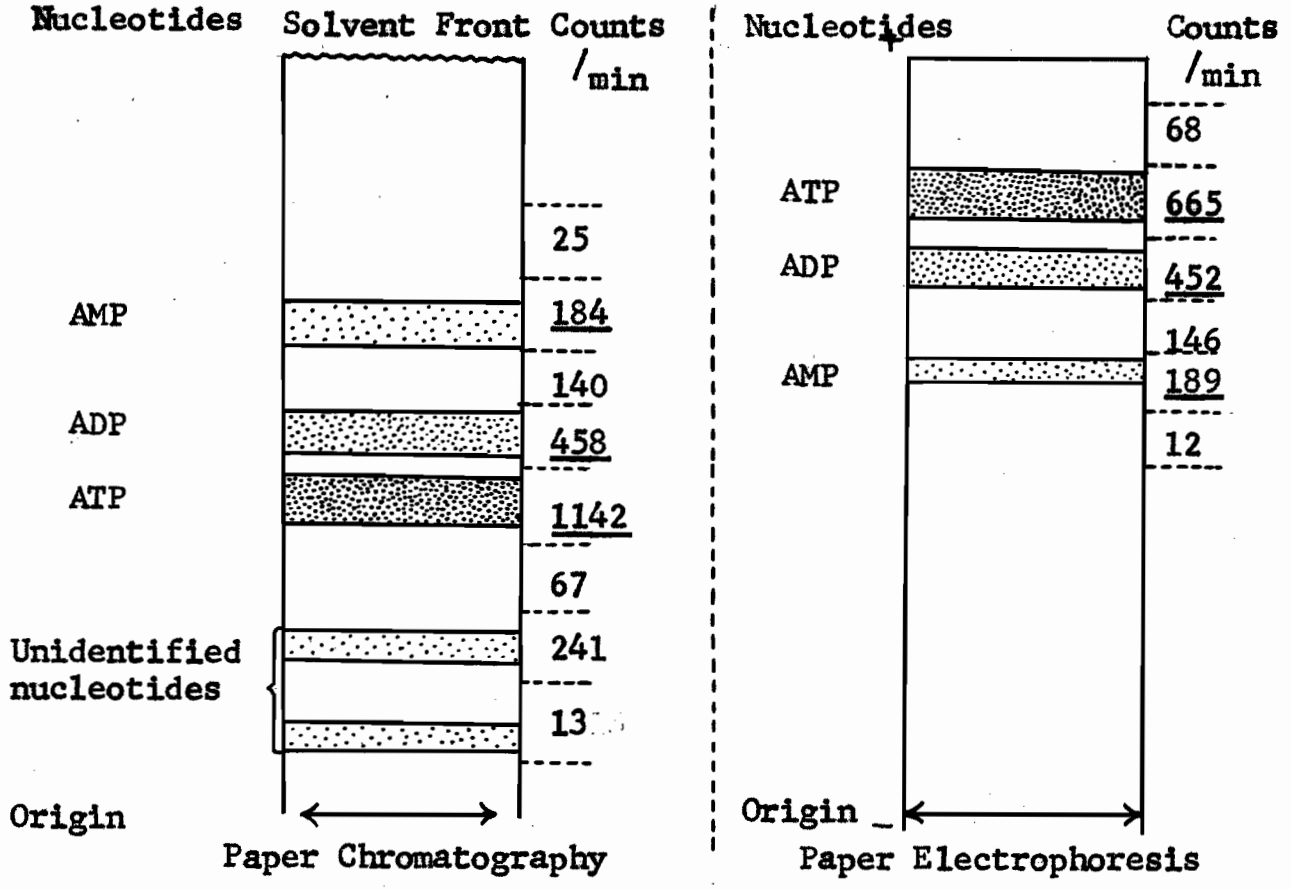
The method used for this purpose was essentially that of Wang and Jones (178). The pencilled areas were cut out of the paper. Each of the pieces usually was about 2.5 to 4.0 cm long and 1.5 cm in width. Each piece of paper was then put in a separate scintillation counter vial and the vials then filled with "phosphor", i.e. the scintillations counting fluid, so that the paper specimen is completely immersed. A blank piece of paper of the same type was used to determine the nonspecific background radiation. The radioactivity in each specimen was then determined by placing the vials in the Packard Tri-Carb Liquid Scintillation spectrometer. The efficiency of the estimation of radioactivity of carbon-C^{14} in this prodedure was about 60 per cent.

Comparison of the counts obtained from the ATP, ADP and AMP separated by paper chromatography and by paper electrophoresis with identical amounts of nucleotide extracts always gave close agreement with the two methods with ADP and AMP. However, the count obtained with the ATP on the chromatogram always appeared to be much higher than that with the ATP on the electrophoresis

strip. This discrepancy is attributable to the interference by IMP and other compounds of unknown identity which travel with the ATP on paper chromatogram.

Having determined the number of counts for the various nucleotides by the two methods the lower of the two values for each compound was accepted as final value.

The counts obtained in AMP, ADP and ATP in one of the experiments are shown in the model strips. Shaded areas show the positions of the nucleotides as marked by exposing the strips to the ultraviolet light.



Design of experiments and results

Influence of adenine on the 7-minute hydrolysable phosphate content of fresh rabbit erythrocytes.

In a 10 ml flask 0.5 ml of washed rabbit red cells were suspended in 2.5 ml of Krebs-Ringer phosphate solution containing 28 μmoles of glucose (i.e. 5 mg) per ml.

In another flask 0.5 ml of cells were suspended in 2.5 ml of Krebs-Ringer phosphate solution containing 28 μmoles of glucose and 2 μmoles of adenine per ml.

Both flasks were incubated for one hour in a water bath at 37°C with occasional mixing of the contents. After incubation the erythrocytes were packed by centrifugation in a hard glass centrifuge tube, and the supernatant discarded. The cells were washed twice with 5 ml of physiological sodium chloride solution. They were then hemolyzed by adding water to make a total volume of 2.5 ml. A small quantity (0.02 ml) of this hemolyzate is used for the determination of the hemoglobin content and then the proteins were immediately precipitated by adding 2.5 ml of 5 per cent trichloracetic acid and mixing the contents vigorously. The precipitated proteins were removed by centrifugation and 1 ml of the clear supernatant was used for the determination of the inorganic phosphate content. Another 1 ml of the supernatant was

used for the determination of the 7-minute hydrolysable phosphate together with the inorganic phosphate. The 7-minute hydrolyzable phosphate in 1 ml of the supernatant is represented by the difference of the two values. Table I indicates the values for the labile (7-minute hydrolyzable)phosphate from six experiments.

The results indicate that the value for the content of 7-minute hydrolyzable phosphate of the erythrocytes incubated with glucose and adenine in any one experiment is very slightly lower than the value for the cells incubated with glucose alone in the same experiment. The labile phosphate fraction is derived mainly from the two terminal phosphate groups of ATP and the terminal phosphate of ADP. The phosphate groups of glucose-1-phosphate and pentose-1-phosphate also are easily hydrolyzable but the amounts of these in the red cells, compared with that of the nucleotide phosphates, must be negligibly small.

Influence of adenine on the incorporation of the carbon atoms of uniformly labelled glucose into ATP, ADP and AMP of fresh rabbit erythrocytes.

In a 10 ml flask 1 ml of washed rabbit erythrocytes were suspended in 2.6 ml of Krebs-Ringer phosphate solution, containing 5.6 μmoles of glucose per ml (1 mg/ml), together with 0.4 ml of a solution of glucose-U-C^{14} containing 2,000,000 counts per 0.1 ml per minute.

TABLE I

Content of Labile Phosphate Obtained from Rabbit Erythrocytes Incubated with Glucose or Glucose and Adenine

Experiment	Substrate	
	Glucose	Glucose and Adenine
	Labile Phosphate as µg P per 100 mg Hb.	
1	28.1	24.4
2	25.0	22.1
3	30.0	29.6
	Labile Phosphate as µg P per ml of cells.	
4	92.0	85.0
5	76.0	74.0
6	98.0	92.0

In another flask 1 ml of cells were suspended in 2.6 ml of Krebs-Ringer phosphate solution, containing 5.6 µmoles of glucose and 2 µmoles of adenine per ml, together with 0.4 ml of the glucose-U-C^{14} solution as described above.

The hemoglobin content of both of the suspensions is ident-

ical as identical amounts (5.0 ml) of a stock erythrocyte suspension (20%) is used in each case. Both flasks were incubated in a water bath at 37°C for one hour with occasional mixing of the contents.

After incubation, the suspension in each flask was transferred to a centrifuge tube and the cells separated by centrifugation from the supernatant which was removed with a Pasteur pipette. The cells were resuspended in 10 ml of physiological sodium chloride solution. They were then centrifuged again and the supernatant removed. The cells were washed in this manner three times with physiological saline and finally hemolyzed with water to give a volume of 5.0 ml of hemolyzate in each case.

The proteins were precipitated immediately by adding 5.0 ml of 5% trichloracetic acid and vigorous mixing of the contents. After centrifuging down the proteins, the supernatant was filtered. A quantity (6 or 7 ml) of the protein-free filtrate was placed in a 12.0 ml thick-walled centrifuge tube and 0.2 g of activated charcoal added. After mixing for five minutes, during which time all the nucleotides in the protein-free filtrate become adsorbed on charcoal, two or three drops of 95% ethyl alcohol were layered on the top of the contents in the tube, and the tubes were centrifuged at high speed. The clear supernatant was discarded and charcoal is washed three times with 10 ml of distilled water and a

thin layer of 95% ethyl alcohol being added each time before centrifugation. The top layer of 95% ethyl alcohol reduces to a minimum the amount of charcoal that tends to float on the surface of the supernatant.

After the third washing of the charcoal, the adsorbed nucleotides were eluted with 5 ml of 0.2N NH_4OH in 30% ethyl alcohol. The charcoal was mixed with this solution for 10 minutes in the centrifuged tube, by means of a thin glass rod. Charcoal was then centrifuge down and the supernatant was carefully removed with a Pasteur pipette and placed in a tube with ground glass top. This nucleotide extract was evaporated to dryness in the cold and again redissolved in 0.3 to 0.5 ml of the eluting solution.

The fraction of the total nucleotides eluted from the charcoal is determined from a control experiment in which an equal amount of protein-free filtrate obtained from an amount of fresh cells same as that used in the test, was treated in the same manner as described above. The calculations of the eluted fraction of the total adsorbed nucleotides were done as described previously (vide methods, p.44).

The pH of the protein-free filtrate was about 1.4 and that of the eluate about 9.3. There was no noticeable difference between optical density of the protein-free filtrate at pH 1.4 and

that adjusted to pH 9,3 with the alkali. This fact indicates that most of the nucleotides in the protein-free filtrate were adenine nucleotides, since it is known that the adenine nucleotides have very much the same absorption in acid and alkaline solutions. Other nucleotides show variable difference in absorption at various pH values.

Equal quantities of final nucleotide extract (generally 0.1 ml of final 0.4 ml extract) were subjected to chromatographic and electrophoretic separation. At the end of the run, the positions of the nucleotide bands on the paper were located by exposure to ultraviolet light and pieces of paper of uniform size were cut around the pencilled boundaries of the various nucleotides. The radioactivity in the zones was measured with the scintillation counter. The true counts in the ATP, ADP and AMP were determined as previously described.

Total counts in these compounds in the whole of the cell population was then calculated by taking into account (1) the amount of protein-free filtrate used for extraction of nucleotides, (2) the fraction of total nucleotides eluted from charcoal, and (3) the amount of nucleotide extract subjected for nucleotides separation.

As the amount of cells in both of the suspensions was the

same as also was the amount of glucose and the radioactivity of glucose, the ratio of counts obtained for the adenine nucleotides of the cells incubated with glucose and adenine, to the counts with cells incubated with glucose alone should indicate the influence of adenine on the incorporation of carbon atoms of the glucose into the adenine nucleotides.

The observations from a typical experiment are indicated in Table II.

The counts obtained in three similar experiments, and the ratios of these counts, with cells incubated with and without adenine, are given in Table III.

These results indicate that the effect of adenine added to the erythrocyte suspension is to increase considerably the incorporation of carbon atoms of glucose into the adenine nucleotides of the cells. The ratio of the incorporation of radioactivity of glucose-U-C^{14} into the adenine nucleotides with and without the addition of adenine was about 7 to 1.

TABLE II

Radioactivity Obtained in Adenine Nucleotides with Two Incubation Media

Medium	Counts per minute			
	ATP	ADP	AMP	Total
Glucose alone	1618	980	438	3036
Glucose + Adenine	10540	7063	3465	21068

Conditions

Total amount of glucose in each flask 14.5 μmoles (2.6 mg)

Counts in Glucose-U-C^{14} added to each flask 8,000,000/min.

Hemoglobin content of cells in each flask 318.7 mg.

TABLE III

Total Number of Counts in Adenine Nucleotides

Experiment	Substrate		Ratio a/b
	Glucose + Adenine (a)	Glucose alone (b)	
	Counts per min.		
1	30840	3510	8.7
2	21068	3036	6.9
3	18528	2866	6.4

Conditions

Total amount of glucose in each flask 14.5 μmoles (2.6 mg)

Counts in Glucose-U-C^{14} added to each flask 8,000,000/min.

Influence of the concentration of glucose on the incorporation of its carbon atoms into adenine nucleotides of erythrocyte in presence of adenine

The procedure in these experiments was the same as described in the previous section. Four flasks were prepared each containing 1 ml of erythrocytes and 2.6 ml of Krebs-Ringer phosphate solution containing 2 μmoles of adenine and respectively 5.6, 11.2, 16.8 and 28.0 μmoles of glucose per ml. To each flask was added 0.4 ml of glucose-U-C^{14} solution with a total activity of 8,000,000 c.p.m.

Following the procedure described in the preceding experiment the total count from the adenine nucleotides in each case after one hour incubation was determined. The number of g-atoms of carbon of glucose incorporated into these nucleotides was calculated from the total count and from the initial specific activity of glucose in the suspension.

Figure 2 indicates the extent of incorporation of the carbon atoms of glucose into the adenine nucleotides with the various concentrations of glucose used in the Krebs-Ringer solution.

It is evident that the incorporation of glucose carbon into the nucleotides rose rapidly with the glucose concentration up to 11.2 μmoles per ml (i.e. 2 mg/ml) of Krebs-Ringer solution. Higher concentrations had little additional effect.

FIGURE 2

INFLUENCE OF THE CONCENTRATION OF GLUCOSE ON THE INCORPORATION OF ITS CARBON ATOMS INTO THE ADENINE NUCLEOTIDES

Conditions

Incubation mixture in each flask contained 1.0 ml of washed erythrocytes, 2.6 ml of Krebs-Ringer phosphate solution containing 2.0 μmoles of adenine per ml and varying concentrations of glucose, and 0.4 ml of a solution of G-U-C^{14} containing 8,000,000 counts per minute.

Incubation time was 1 hour and temperature 37°C.

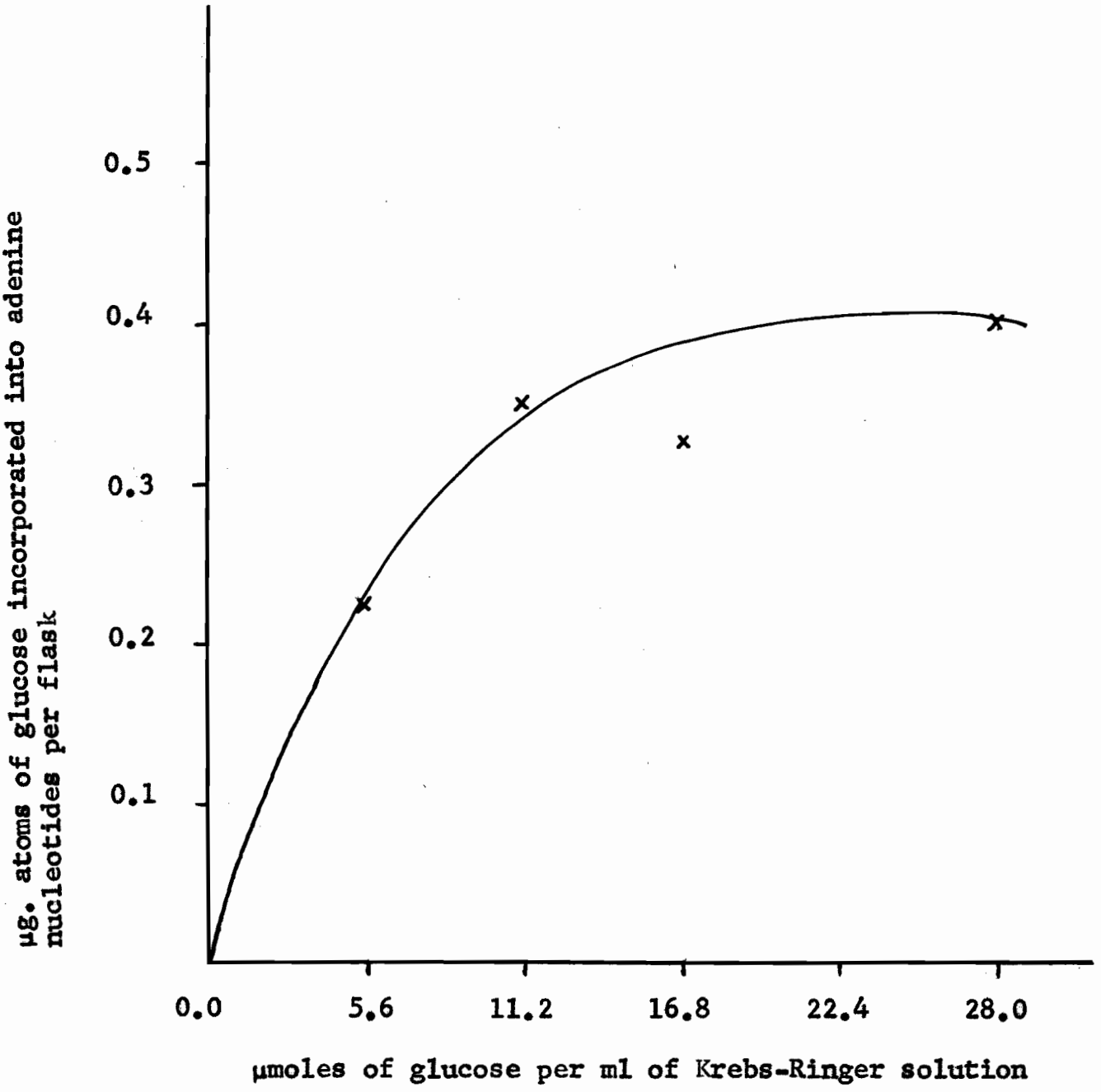

0.5
0.4
0.3
0.2
0.1
µg. atoms of glucose incorporated into adenine nucleotides per flask
0.0
5.6
11.2
16.8
22.4
28.0
µmoles of glucose per ml of Krebs-Ringer solution

FIGURE 2

Influence of concentration of adenine on the incorporation of glucose carbon into adenine nucleotides of red cells.

Following the procedure as described in the previous experiments, four flasks were prepared each containing 1 ml of red cells and 2.6 ml of Krebs-Ringer phosphate solution containing 11.2 μmoles of glucose (i.e. 2 mg/ml) and respectively 0.0, 0.02, 0.08 and 0.2 μmoles of adenine per ml. To each flask was added 0.4 ml of glucose-U-C^{14} solution with a total activity of 8,000,000 c.p.m.

The degree of incorporation of glucose carbon into the adenine nucleotides was determined as before.

Figure 3 indicates the effect of the adenine concentration on the incorporation of glucose carbon into the adenine nucleotides.

From this figure it is evident that the incorporation of the glucose carbon increased with the concentration of adenine until the concentration reached 0.2 μmoles per ml of Krebs-Ringer solution. Higher concentrations up to 2.0 μmoles/ml gave no further incorporation.

FIGURE 3

INFLUENCE OF THE CONCENTRATION OF ADENINE ON THE INCORPORATION OF GLUCOSE CARBON INTO ADENINE NUCLEOTIDES

Conditions

Incubation mixture in each flask contained 1.0 ml of washed erythrocytes, 2.6 ml of Krebs-Ringer phosphate solution containing 11.2 μmoles (2.0 mg) of glucose per ml and varying concentrations of adenine, and 0.4 ml of a solution of G-U-C^{14} containing 8,000,000 counts per minute.

Incubation time was 1 hour and temperature 37°C.

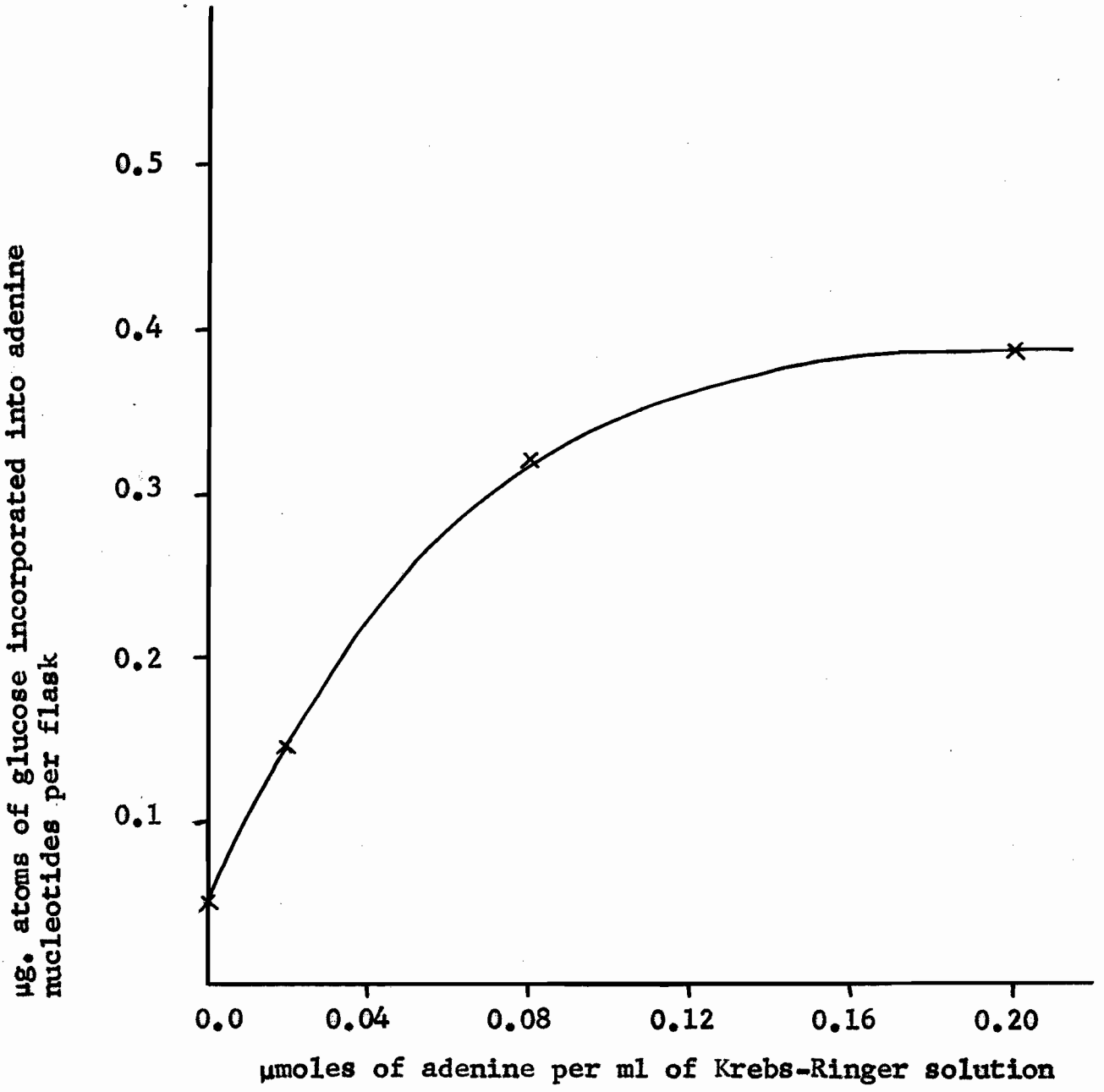

µg. atoms of glucose incorporated into adenine nucleotides per flask
0.5
0.4
0.3
0.2
0.1
0.0
0.04
0.08
0.12
0.16
0.20
µmoles of adenine per ml of Krebs-Ringer solution

FIGURE 3

Effect of labelling the glucose in carbon-1 and -6 on the radioactivity of the isolated adenine nucleotides of the red cell.

The results of the studies described in the preceding sections demonstrated that higher concentrations of glucose in the medium favor greater incorporation of the glucose carbon atoms into the adenine nucleotides. However, the greater concentrations of glucose also decrease the specific activity of it in the medium, hence also decrease the counts obtained in the isolated adenine nucleotides. In view of these circumstances a representative concentration of 11.2 μmoles of glucose per ml of Krebs-Ringer solution was used in the experiments described in this section. The concentration of adenine in this solution was 0.2 μmoles per ml, as the higher concentrations up to 2.0 μmoles per ml were found not to increase further the incorporation of glucose carbon into the adenine nucleotides.

Equal quantities of cells (1 ml) were placed in two flasks as in the previous studies. To one flask glucose-1-C^{14} and to the other glucose-6-C^{14} in equal amounts of radioactivity were added. After incubation of one hour the degree of radioactivity incorporated into the adenine nucleotides was determined.

The results given in Table IV indicate the number of counts obtained in the nucleotides in a typical experiment.

Table V represents the results of three such experiments,

and gives also the ratio of the radioactivity in the adenine nucleotides with glucose-1-C^{14} and glucose-6-C^{14}.

From these results it is apparent that the incorporation of radioactivity was always greater with glucose-1-C^{14} and hence that the carbon atom-1 of glucose is utilized more extensively than the carbon atom-6 for incorporation into the adenine nucleotides.

TABLE IV

Radioactivity Obtained in Adenine Nucleotides with G-1-C^{14} and G-6-C^{14}

Substrate	Counts per minute			
	ATP	ADP	AMP	Total
G-1-C^{14}	12760	7254	1130	21144
G-6-C^{14}	5360	3195	1159	9714

Conditions

Total amount of glucose in each flask 29.0 μmoles (5.2 mg)

Counts in labelled glucose in each flask 8,800,000/min.

TABLE V

Total Number of Counts in Adenine Nucleotides

Experiment	Substrate		Ratio C_1/C_6
	G-1-C^{14}	G-6-C^{14}	
	Counts per minute		
1	12,530	7,504	1.7
2	21,114	9,714	2.1
3	12,910	7,725	1.7

Conditions

Total amount of glucose in each flask 29.0 μmoles (5.2 mg)

Counts of labelled glucose in each flask 8,800,000/min.

DISCUSSION

As mentioned in the 'Introduction' of this thesis, Lowy et al. (35) showed that when adenine was added to the red blood cells from the rabbit or the human, it was incorporated into ATP. There are two plausible mechanisms by which this incorporation can occur. One involves the replacement of the adenine nucleus of the endogenous ATP with exogenous adenine without affecting the phosphoribosyl portion of the native ATP. This possibility had been suggested previously with regard to the introduction of pre-formed adenine into nucleic acids. Thus Furst et al. (172) has shown that after administration of adenine labelled in the N_1 and N_3 positions, to the rat, the isotope was found in the adenine of RNA and, to a small extent, of DNA of the liver. Brown (173) has suggested that the exogenous adenine may possibly be transferred from an active intermediate to a pre-existing polynucleotide by the transglycosidation (transpurination) reaction.

A second and less direct possibly route of incorporation of adenine into ATP involves the rupture of the N-glycosidic bond of AMP by pyrophosphate to liberate adenine and phosphoribosyl pyrophosphate (PRPP). The latter might then react with the exogenous adenine to regenerate AMP. This reversible reaction between a mononucleotide and pyrophosphate was first discovered by Kornberg

et al.(128-131) in the pigeon liver. The AMP formed from the exogenous adenine in this manner might then be converted to ADP and to ATP. An enzyme in the erythrocyte which catalyzes a general reaction between PRPP and a purine or pyrimidine base to form a mononucleotide also has been described by Preiss and Handler (41).

It is clear from the nature of these two routes of incorporation of adenine into ATP that they do not involve the utilization of any of the intermediates formed in the metabolism of glucose. The ribosyl portion in the newly formed ATP is derived from that of an endogenous nucleotide. The original purine moiety is replaced by exogenous adenine, and the original purine is set free in the reaction. It is clear also that in these reactions the adenine may likewise replace a pyrimidine base, or a purine other than adenine, in the original nucleotide.

There is a third way in which exogenous adenine may be incorporated into ATP, namely, by reaction with a PRPP molecule synthesized from sources other than nucleotides. In this case the synthesis of PRPP might be expected to take place by the interaction between ribose-5-phosphate and ATP. The enzyme necessary for this reaction is known to be present in the erythrocyte (41).

For the synthesis of PRPP by the above-mentioned reaction, ribose-5-phosphate is required. As glucose is the principal substrate utilized by the erythrocyte, it is logical to expect that

when exogenous adenine is utilized in the red cell, any ribose-5-phosphate, not of nucleotide origin must arise from the glucose. The validity of this hypothesis can be tested by using glucose-U-C^{14} and analyzing the isolated adenine nucleotides for the presence of the isotopic carbon.

The results of the experiments described in the foregoing chapter have shown that carbon atoms of glucose actually are incorporated into the adenine nucleotides of the erythrocyte. The extent of incorporation is increased about 7-8 times when a small quantity of adenine also is added to the red cell suspension.

The question then arises whether the carbon atoms of glucose are incorporated only into the ribose moiety or in the adenine or in both. Lowy et al. (165, 35) have shown that in the mature erythrocyte of the rabbit and the human, the purine ring cannot be synthesized de novo from the small purine ring precursors. Bartlett and Marlow (174) found none of the C^{14} in the adenine of ATP when human erythrocytes were incubated with glucose-U-C^{14}. It is evident, therefore, that the carbon atoms from the glucose found in the adenine nucleotides are confined to the ribose moiety.

It is reasonable to assume that ribose-5-phosphate is always present,at least in small concentration, in fresh red cells. The probable presence of this intermediate as a metabolic derivative

of glucose was demonstrated chromatographically by Bartlett and Marlow (174). The small C^{14} -content in the adenine nucleotides from the erythrocytes incubated with glucose-U-C^{14} in absence of adenine is probably attributable to the interaction between PRPP and hypoxanthine, first to form IMP, which then may be converted to AMP. Hypoxanthine is always present in small concentration in the erythrocyte since this purine derivative is the terminal degradation product of the purine nucleotides in this cell.

Adenine, added to the suspension of rabbit erythrocytes, reacts with PRPP to form AMP. PRPP is initially formed by reaction between ribose-5-phosphate and ATP. In the absence of adenine most of the ribose-5-phosphate is converted through the pentose phosphate pathway to hexose phosphates and triose phosphate which can be utilized through the glycolytic system to form lactic acid. When adenine is added to the cell suspension some of the available ribose-5-phosphate is diverted from conversion to hexose- and triose phosphates with the formation of adenine nucleotides.

There are three prominent routes for the conversion of glucose to ribose-5-phosphate: (1) by oxidative decarboxylation of the C_1 of glucose through the pentose phosphate pathway. This route has been shown to be operational to a greater or lesser

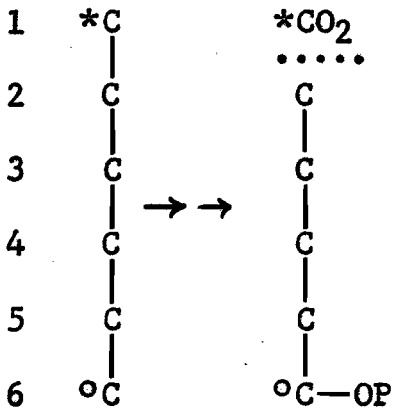
extent in many tissues. It is generally accepted that oxidation of glucose by this route occurs only to a slight extent in the mammalian erythrocyte. (2) The second route is the oxidation of C_6 of glucose to CO_2 by the glucuronic acid pathway. The possibility of this route being operational in erythrocytes is remote because some of the enzymes necessary for this pathway are absent in red cells. (3) The third route of formation of ribose-5-phosphate from glucose is through the catalytic action of the enzyme, transketolase, which transfers carbon atoms 1 and 2 of fructose-6-phosphate to triose phosphate to form xylulose-5-phosphate and erythrose-4-phosphate. Xylulose-5-phosphate can be converted to ribose-5-phosphate through epimerase and isomerase. Erythrose-4-phosphate does not accumulate. It is probably reconverted to hexose-6-phosphate, pentose-5-phosphates, and triose phosphate through the reactions catalyzed by the enzymes, transketolase and transaldolase. However, the most important step of this route is the first reaction in which fructose-6-phosphate and triose phosphate form xylulose-5-phosphate and erythrose-4-phosphate. Xylulose-5-phosphate formed in this reaction is important in this discussion because any additional pentose-5-phosphate formed from erythrose-4-phosphate will be small considering the fact that erythrose-4-phosphate can give rise to a variety of other products.

The formation of pentose-5-phosphates from fructose-6-phosphate and triose phosphate can occur in the erythrocyte as the enzymes transketolase and transaldolase are functional in these cells. Predominance of this mechanism of pentose formation has been shown in certain organisms. Szynkiewicz et al. (175) have found that in E. coli, ribose synthesis from transketolase route predominates under certain conditions. Bernstein (176) found that the transketolase-catalyzed formation of pentose phosphate (of visceral RNA) predominates in the intact chick, and that neither the oxidation of hexose through the pentose shunt nor the decarboxylation of uronic acid could account for the observed labelling of pentose phosphate. The results were consistent with the involvement of the transketolase route.

Incubation of erythrocytes with glucose labelled either in C_1 or C_6, and estimation of radioactivity incorporated into adenine nucleotides is a convenient way of determining the pathway which is predominant for the formation of pentose phosphates in these cells.

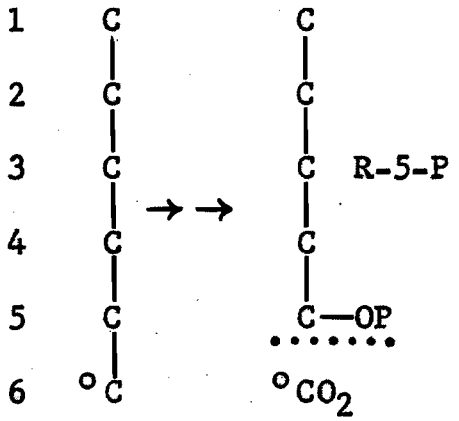
When glucose is labelled in C_1 or C_6 the ribose-5-phosphate formed by the three routes will be labelled in the respective pathways as follows:

1 By way of the pentose shunt



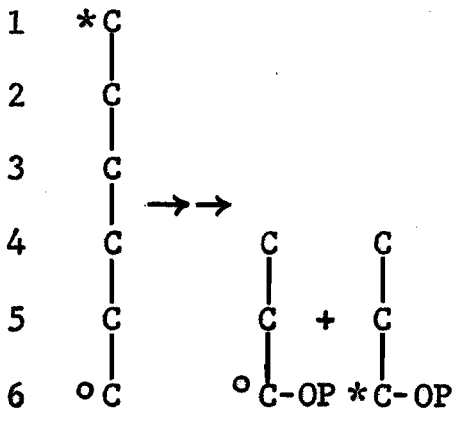
Glucose R-5-P

2. By way of the glucuronic acid pathway



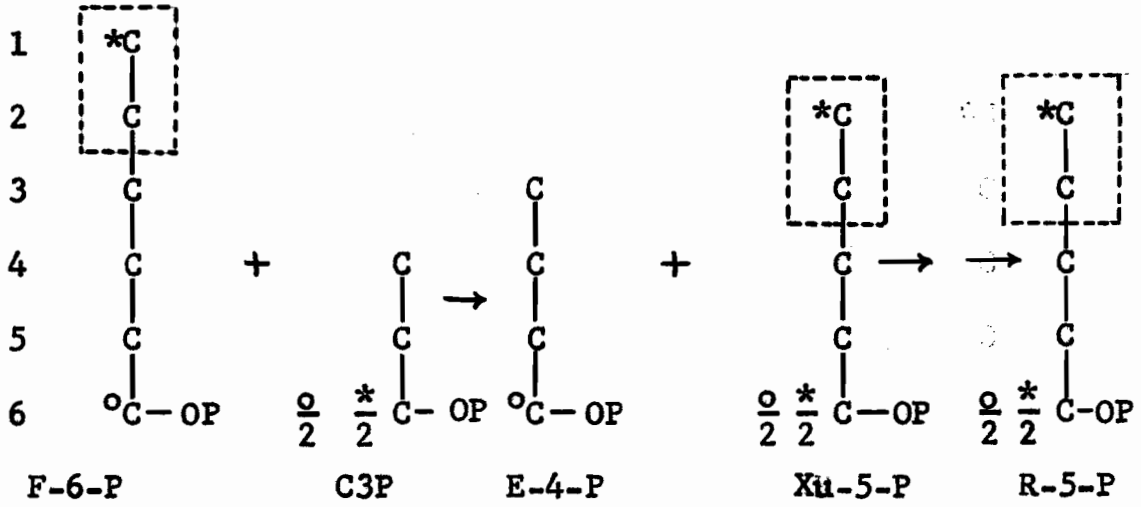
Glucose

3. By way of the transketolase catalyzed reaction



Glucose C3P

Continued on the next page



This scheme shows that glucose labelled only in C_1 gives unlabelled ribose-5-phosphate by the pentose shunt pathway. The glucuronic acid pathway gives rise to labelled ribose-5-phosphate as C_1 of glucose is retained in the pentose formed. As xylitol, which is a symmetrical molecule, is formed as an intermediate in this pathway, the ribose-5-phosphate formed in this process may be labelled in C_1 and C_5 positions. Transketolase catalyzed reaction will give ribose-5-phosphate labelled in C_1 and C_5 of it.

If glucose is labelled in C_6, the ribose-5-phosphate arising from the pentose shunt pathway will be labelled in C_5. An unlabelled ribose-5-phosphate will arise from the glucuronic acid pathway while in the transketolase catalyzed reaction it will be labelled in C_5, but the amount of radioactivity of the label

will be much less than through the pentose shunt pathway.

In view of these mechanisms of formation of ribose-5-phosphate, if glucose is separately and equally labelled in C_1 and C_6 in two parallel experiments, the ratio of radioactivity incorporated in adenine nucleotides in these two experiments will indicate the major route by which ribose-5-phosphate is formed from glucose.

It is evident from the mechanism of the pentose shunt that as the C_1 of glucose is given off as CO_2 and as the C_6 of glucose is totally incorporated into the C_5 of ribose, the ratio of radioactivity incorporated into adenine nucleotides with glucose-1-C^{14} and glucose-6-C^{14} will be $\frac{0}{1}$ which is 0.

The ratio with the glucuronic acid pathway will be $\frac{1}{0}$ which is ∞.

It is clear from the pattern of labelling of the ribose synthesized through transketolase catalyzed reaction that the ratio of C_1 to C_6 of glucose incorporated into ribose is $\frac{1.5}{0.5}$ which is 3.

In actual experiments done with rabbit erythrocytes the ratio is found to vary from 1.7 to 2.1. When these experimental values are referred to the theoretical ones as described above it is evident that the experimental values are much closer to the theoretical value for the transketolase catalyzed route of formation

of ribose-5-phosphate from glucose. Considering that the erythrose-4-phosphate formed in these reactions also can be converted to ribose-5-phosphate to some extent by similar reactions, the theoretical ratio actually should be somewhat less than 3. Thus the experimental values confirm the validity of our interpretation that the major part of ribose-5-phosphate in the rabbit erythrocyte is synthesized by the transketolase catalyzed reaction.

While discussing the evaluation of pentose shunt and Embden-Meyerhof pathway of glucose metabolism Horecker and Mehler (177) have emphasized the need of great caution in interpreting the results from labelled precursors in view of the complicated pattern of carbohydrate interconversions which are possible by the transketolase and transaldolase catalyzed reactions. However, this may be, the experimental results in this case are so much closer to those theoretically expected for the transketolase catalyzed reaction than to the other two mechanisms, that there is little doubt about the interpretation.

The concentration of 7-minute hydrolyzable phosphate of the erythrocytes incubated with adenine was found to be no more than that of the cells incubated in the absence of adenine. In fact a slightly lower value for the labile phosphate (ATP) was obtained in the former case. When adenine is added to the red cell sus-

pension it is known to be incorporated in the adenine nucleotides (35). The reactions by which adenine is incorporated into the adenine nucleotides are as follows:

(1) ribose-5-phosphate + ATP $\rightleftharpoons$ PRPP + AMP

(2) Adenine + PRPP $\rightleftharpoons$ AMP + P-P

It is apparent from these reactions that for the introduction of one molecule of adenine into a nucleotide, one molecule of ribose-5-phosphate and both of the high energy phosphates of one molecule of ATP are utilized. It follows, therefore, that the molecule of ribose-5-phosphate becomes unavailable as a substrate for the metabolism in which high energy phosphates are produced. Similarly, it is evident that two moles of high energy phosphate are utilized in this process from a molecule of ATP already present in the cell. Therefore, it follows that for the introduction of adenine into a nucleotide some of the high energy phosphate of the cell is used. As the total of the 7-minute hydrolyzable phosphate is a measure of high energy phosphates, it is logical to suppose that it must decrease, at least slightly, when adenine is added to the erythrocyte suspension.

It is true that the amount of glucose-6-phosphate, converted to ribose-5-phosphate for the introduction of adenine into the nucleotides, may be replaced to certain extent by conversion of

additional amount of glucose to glucose-6-phosphate. In fact many workers have shown with fresh human erythrocytes, that if glucose-6-phosphate is removed through the pentose shunt system by addition of methylene blue more glucose can be utilized.

It may be supposed that, in the experiments reported in this thesis, the rate of removal of glucose-6-phosphate for the formation of ribose-5-phosphate may be more than the rate at which this loss is made up by the conversion of additional amounts of glucose to glucose-6-phosphate.

Simon et al. (57) and de Verdier et al. (59) have shown that with adenine the ATP levels are maintained continuously at a slightly higher level in the blood stored at $4^{o}C$. This beneficial effect of adenine in the blood stored for a longer time may be attributable to the possibility that added adenine may be replacing the purine moiety of the endogenous nucleotides, the concentration of which undoubtedly diminishes during storage.

SUMMARY

1. In the mature rabbit erythrocyte the carbon atoms of glucose can be incorporated into the adenine nucleotides.

2. Incorporation of glucose carbon atoms into the adenine nucleotides in the red cells increases with the adenine concentration in the incubation medium to a limit.

3. Incorporation of glucose carbon atoms into the adenine nucleotides in the red cells increases with the glucose concentration in the incubation medium to a limit.

4. The C_1 of glucose is incorporated into the adenine nucleotides to a greater extent than the C_6.

5. The ratio of the incorporation into adenine nucleotides of C_1 to C_6 suggests that the major route of formation of ribose-5-phosphate from glucose is through a reaction catalyzed by the enzyme transketolase.

6. Over a short period of time (1 hour) at 37°C, adenine has no favourable effect on the 7-minute hydrolyzable phosphate content of the rabbit erythrocyte.

BIBLIOGRAPHY

1. Mollison, P.L. and Young, I.M., Brit.Med.J., II:797, 1941.

2. Mollison, P.L. and Young, I.M., Quart.J.Exp.Physiol., 31:359, 1941-42.

3. Donohue, D.M. and Finch, C.A., Vox Sanguinis, 2:369, 1957.

4. Rous, P. and Turner, J.R., J.Exp.Med., 23:219, 1916.

5. Aylard, F.X., Mainwaring, R.S. and Wilkinson, J.F., The Lancet, 238:685, 1940.

6. Maizels, M., The Lancet, 240:722, 1941.

7. Bushby, S.R.M., Kekwick, A., Marriott, H.L. and Whitby, L.E.H., The Lancet, 239:414, 1940.

8. Loutit, J.F., Mollison, P.L. and Young, I.M., Quart.J.Exp.Physiol., 32:183, 1943-44.

9. Kashket, S., Rubinstein, D. and Denstedt, O.F., Can.J.Biochem. Physiol., 35:827, 1957.

10. Rapoport, S., J.Clin.Invest., 26:591, 1947.

11. Gabrio, B.W. and Finch, A., J.Clin.Invest., 33:242, 1954.

12. Lowy, B.A., Ramot, B. and London, I.M., Nature, 181:324, 1958.

13. Mollison, P.L. and Robinson, M.A., Brit.J.Haemat., 5:331, 1959.

14. Allbaum, H.G., Cayle, T. and Shapiro, A., J.Clin.Invest.,30:525,1951.

15. Klein, J.R. and Kohn, H.I., J.Biol.Chem., 136:177, 1940.

16. Gabrio, B.W. and Finch, C.A., Federation Proc., 13:51, 1954.

17. Gabrio, B.W., Hennesy, M., Thomasson, J. and Finch, C.A. J.Biol.Chem. 215:357, 1955.

18. Gabrio, B.W., Donohue, D.M. and Finch, C.A., J.Clin.Invest., 34:1509, 1955.

19. Gabrio, B.W. and Huennekens, F.M., Federation Proc., 14:217, 1955.

20. Prankerd, T.A.J., The Lancet, 270:469, 1956.

21. Donohue, D.M., Finch, C.A. and Gabrio, B.W., J.Clin.Invest., 35:562, 1956.

22. Donohue, D.M., Huennekens, F.M. and Finch, C.A., Gabrio, B.W., J.Clin.Invest., 35:657, 1956.

23. Lange, R.D., Crosby, W.H., Donohue, D.M., F nch, C.A., Gibson II, J.G., McManus, T.J. and Strumia, M.M., J.Clin.Invest., 37:1485, 1958.

24. Overgaard-Hansen, K., Jørgensen, S. and Praetorius, E., Nature, 179:152, 1957.

25. Jones, N.C.H., Mollison, P.L. and Robins, M.A., Proc.Roy.Soc., B 147:476, 1957.

26. Wittman, R., J.Physiol., 154:614, 1960.

27. Conway, E.J. and Cooke, R., Biochem.J., 33:457, 1939.

28. Rubinstein, D., Kashket, S. and Denstedt, O.F., Can.J.Biochem. Physiol., 34:61, 1956.

29. Sandberg, A.A., Lee, G.R., Cartwright, G.E. and Wintrobe, M.M., J.Clin.Invest., 34:1823, 1955.

30. Gabrio, B.W., Biochim.Biophys.Acta, 18:585, 1955.

31. Tsuboi, K.K. and Hudson, P.B., J.Biol.Chem., 224:879, 1957.

32. Tsuboi, K.K. and Hudson, P.B., J.Biol.Chem. 224:889, 1957.

33. Prankerd, T.A.J., Brit.J.Haemat., 1:406, 1955.

34. Rubinstein, D. and Denstedt, O.F., Can.J.Biochem.Physiol., 34:927, 1956.

35. Lowy, B.A., Williams, M.K. and London, I.M., J.Biol.Chem., 237:1622, 1962.

36. Dische, Z., in "Phorphorus Metabolism" Vol.1, page 171. Edited by McElroy and Glass, John Hopkins Press, Baltimore, 1951.

37. Rubinstein, D., Kashket, S. and Denstedt, O.F. Can.J.Biochem.Physiol., 36:1269, 1958.

38. Nakao, M., Nakao, T., Tatibana, M., Yoshikawa, H. and Abe, T., Biochim.Biophys.Acta, 32:564, 1959.

39. Nakao, M., Nakao, T., Tatibana, M., and H.Yoshikawa, J.Biochem., 47:661, 1960.

40. Nakao, M., Motegi, T., Nakao, T. and Yamazoe, S., Nature, 191:283, 1961.

41. Preiss, J. and Handler, P., J.Biol.Chem., 225:759, 1957.

42. Colowick, S.P. and Kalckar, H.M., J.Biol.Chem., 148:117, 1943.

43. Kalckar, H.M., J.Biol.Chem., 148:127, 1943.

44. Kotelnikova, A.V., Chemical Abstracts, 42:6388c, 1948.

45. Novikoff, A.B., Hecht, L., Podber, E. and Ryans, J., J.Biol.Chem., 194:153, 1952.

46. Kotelnikova, A.V., Chemical Abstracts, 43:6263 i, 1949.

47. Barkulis, S.S. and Lehninnger, J.Biol.Chem., 190:339, 1951.

48. Kashket, S. and Denstedt, O.F., Can.J.Biochem.Physiol., 36:1057, 1958.

49. Tatibana, M., Nakao, M. and Yoshikawa, H., J.Biochem., 45:1037, 1958.

50. Askari, A. and Fratantoni, J.C., Proc.Soc.Exp.Biol.Med., 116:751, 1964.

51. Cerletti, P. and Bucci, E., Biochim.Biophys.Acta, 38:45, 1960.

52. Sventsitskaya, M.B., Chemical Abstracts, 48:8284 h, 1954.

53. Surgeor, D.M., Hunter, M.J. and Brown, R.K., in "Blood Cells and Plasma Proteins", page 315; Academic Press Inc.,New York,1953.

54. Nakao, K., Wada, T. and Kamiyama, T., Nature, 194:877, 1962.

55. Wada, T., Takaku, F., Nakao, K., Nakao, M. and Yoshikawa, H., Proc.Japan.Acad., 36:618, 1960.

56. Nakao, M., Nakao, T., Arimatsu, Y., Kitamura, M. and Yoshikawa, H., Proc.Japan Acad., 36:43, 1960.

57. Simon, E., Chapman, R.G. and Finch, C.A., J.Clin.Invest., 41:351, 1962.

58. Simon, E., Blood, 20:485, 1962.

59. deVerdier, C.H., Garby, L., Hjelm, M. and Högman, C., Transfusion, 4;331, 1964.

60. Hakala, M.T. and Taylor, E., J.Biol.Chem.,234:126, 1959.

61. Aronow, L., Biochim.Biophys.Acta, 47:184, 1961.

62. Warburg, O., Christian, W. and Griese, A., Biochem.Z., 282:157, 1935.

63. Warburg, O., Christian, W. and Griese, A., Biochem.Z., 279:143, 1935.

64. Cori, O. and Lipmann, F., J.Biol.Chem., 194:417, 1952.

65. Brodie, A.F. and Lipmann, F., J.Biol.Chem., 212:677, 1955.

66. Glock, G.E. and McLean, P., Biochem.J., 56:171, 1954.

67. Kelly, T., Nielson, E.D., Johnson, R.B. and Vestling, C.S., J.Biol.Chem., 212:545, 1955.

68. Horecker, B.L. and Smyrniotis, P.Z., J.Biol.Chem., 193:371, 1951.

69. Horecker, B.L., Smyrniotis, P.Z. and Seegmiller, J.E., J.Biol.Chem., 193:383, 1951.

70. Axelrod, B. and Jang, R., J.Biol.Chem., 209:847, 1954.

71. Srere, P.A., Cooper, J.R., Klybas, V. and Racker, E., Arch.Biochem.Biophys., 59:535, 1955.

72. Hurwitz, J. and Horecker, B.L., J.Biol.Chem., 223:993, 1956.

73. Dickens, F. and Williamson, D.H., Biochem.J., 64:567, 1956.

74. Ashwell, G. and Hickman, J., J.Biol.Chem., 226:65, 1957.

75. Horecker, B.L. and Smyrniotis, P.Z., J.Biol.Chem.,196:135, 1952.

76. Horecker, B.L., Smyrniotis, P.Z. and Klenow, H., J.Biol.Chem., 205:661, 1953.

77. de la Haba, G., Leder, I.A. and Racker, E., J.Biol.Chem., 214:409, 1955.

78. Horecker, B.L. and Mehler, A.H., Annual Review of Biochemistry, 24:207, 1955.

79. Horecker, B.L., Hurwitz, J., and Smyrniotis, P.Z., J.Am.Chem.Soc., 78:692, 1956.

80. Horecker, B.L., Smyrniotis, P.Z., and Hurwitz, J., J.Biol.Chem., 223:1009, 1956.

81. Horecker, B.L. and Smyrniotis, P.Z., J.Biol.Chem.,212:811,1955.

82. Horecker, B.L., Gibbs, M., Klenow, H. and Smyrniotis, P.Z., J.Biol.Chem., 207:393, 1954.

83. Gibbs, M. and Horecker, B.L., J.Biol.Chem., 208:813, 1954.

84. Lanning, M.C. and Cohen, S.S., J.Biol.Chem., 207:193, 1954.

85. Bernstein, I.A., J.Biol.Chem., 221:873, 1956.

86. Axelrod, B. and Beevers, H., Ann.Rev.Plant.Physiol.,7:267, 1956.

87. Bloom, B., Stetten, M.R. and Stetten Jr., D., J.Biol.Chem., 204:681, 1953.

88. Katz, J., Abraham, S., Hill, R. and Chaikoff, I.L., J.Biol.Chem., 214:853, 1955.

89. Ashmore, J., Kinoshita, J.H., Nesbett, F.B. and Hastings, A.B., J.Biol.Chem., 220:619, 1956.

90. Ashmore, J., Cahill, G.F. Jr., Hastings, A B. and Zottu, S., J.Biol.Chem., 224:225, 1957.

91. Abraham, S., Hirsch, P.F. and Chaikoff, I.L., J.Biol.Chem., 211:31, 1954.

92. Coxon, R.V. and Robinson, R.J., Proc.Roy.Soc.,145B:232, 1956.

93. Denstedt, O.F., in "Blood Cells and Plasma Proteins", page 223, Edited by J. Tullis, Academic Press, N.Y., 1953.

94. Bartlett, G.R. and Marlow, A.A., J.Lab.Clin.Med., 42:178, 1953.

95. Prankerd, T.A.J., Brit.J.Haemat., 1:131, 1955.

96. Bartlett, G.R., J.Biol.Chem., 234:449, 1959.

97. Harrop Jr., G.A. and Barron,E.S.G., J.Exp.Med., 48:207, 1928.

98. Bird, R.M., J.Biol.Chem., 169;493, 1947.

99. Huennekens, F.M., Liu, L., Myers, H.A.P. and Gabrio, B.W., J.Biol.Chem., 227:253, 1957.

100. Brin, M. and Yonemoto, R.H., J.Biol.Chem., 230:307, 1958.

101. Murphy, J.R., J.Lab.Clin.Med., 55:281, 1960.

102. Huennekens, F.M., Caffrey, R.W., Basford, R.E. and Gabrio, B.W., J.Biol.Chem., 227:261, 1957.

103. Meldrum, N.U. and Tarr, H.L.A., Biochem.J., 29:108, 1935.

104. Francoeur, M. and Denstedt, O.F., Can.J.Biochem.Physiol., 32:663, 1954.

105. Dische, Z. and Shigeura, H., Biochim.Biophys.Acta,24:87, 1957.

106. Dickens, F. and Williamson, D.H., Nature, 176:400, 1955.

107. Dickens, F. and Williamson, D.H., Biochem.J., 64:567, 1956.

108. Brownstone, Y.S. and Denstedt, O.F., Can.J.Biochem.Physiol. 39:527, 1961.

109. Dische, Z. and Pollaczek, E., Abstr. 2nd International Biochemical Congress, Paris 1952, page 289.

110. Brownstone, Y.S. and Denstedt, O.F., Can.J.Biochem.Physiol., 39:533, 1961.

111. Eisenberg Jr., F. and Gurin, S., J.Biol.Chem.,195:317, 1952.

112. Eisenberg Jr., F., J.Biol.Chem., 212:501, 1955.

113. Ginsburg, V., Weissbach, A. and Maxwell, E.S., Biochim. Biophys.Acta, 28:649, 1958.

114. Pogel, B.M. and Krisman, C.R., Biochim.Biophys.Acta, 41:349, 1960.

115. Mano, Y., Suzuki, K., Yamada, K. and Shinazoho, N., J.Biochem., 49:618, 1961.

116. York, J., Grollman, A.P. and Bublitz, C., Biochim.Biophys. Acta, 47:298, 1961.

117. Smiley, J.D. and Ashwell, G., J.Biol.Chem., 236:357, 1961.

118. Touster, O., Reynolds, V.H. and Hutcheson, R.M., J.Biol.Chem., 221:697, 1956.

119. Hollman, S. and Touster, O., J.Biol.Chem., 225:87, 1957.

120. Lieberman, I., Kornberg, A. and Simms, E.S., J.Biol.Chem., 215:429, 1955.

121. Strominger, J.L., Heppel, L.A. and Maxwell, E.S., Arch. Biochem.Biophys., 52:488, 1955.

122. Krebs, H.A. and Hems, R., Biochem.J., 61:435, 1955.

123. Berg, P. and Joklik, W.K., J.Biol.Chem., 210:657, 1954.

124. Kornberg, A. and Pricer Jr., W.F., J.Biol.Chem., 193:481, 1951.

125. Kalckar, H.M., J.Biol.Chem., 158:723, 1945.

126. Kalckar, H.M., J.Biol.Chem., 167:477, 1947.

127. Korn, E.D. and Buchanan, J.M., J.Biol.Chem., 217:183, 1955.

128. Kornberg, A., Lieberman, I. and Simms, E.S., J.Am.Chem.Soc., 76:2027, 1954.

129. Kornberg, A., Lieberman, I. and Simms, E.S., J.Biol.Chem., 215:389, 1955.

130. Kornberg, A., Lieberman, I., and Simms, E.S., J.Biol.Chem., 215:403, 1955.

131. Kornberg, A., Lieberman, I. and Simms, E.S., J.Biol.Chem., 215:417, 1955.

132. Sonne, J.C., Buchanan, J.M. and Delluva, A.M., J.Biol.Chem., 173:69, 1948.

133. Buchanan, J.M., Sonne, J.C. and Delluva, A.M., J.Biol.Chem., 173:81, 1948.

134. Elwyn, D. and Sprinson, D..B., J.Biol.Chem., 184:465, 1950.

135. Sonne, J.C., Lin, I. and Buchanan, J.M., J.Biol.Chem., 220:369, 1956.

136. Levenberg, B., Hartman, S.C. and Buchanan, J.M., J.Biol.Chem., 220:379, 1956.

137. Shemin, D. and Rittenberg, D., J.Biol.Chem., 167:875, 1947.

138. Abrams, R., Hammarsten, E. and Shemin, D., J.Biol.Chem., 173:429, 1948.

139. Heinrich, M.R. and Wilson, D.W., J.Biol.Chem., 186:447, 1950.

140. Greenberg, G.R., J.Biol.Chem., 190:611, 1951.

141. Getler, H., Roll, P.M., Tinker, J.F. and Brown, G.B., J.Biol.Chem., 178:259, 1949.

142. Stetten, M.R. and Fox, C.L., Jr., J.Biol.Chem., 161;333, 1945.

143. Shive, W., Ackerman, W.W., Gordon, M., Getzendaner, M.E. and Eakin, R.E., J.Am.Chem.Soc., 69:725, 1947.

144. Ravel, J.M., Eakin, R.E. and Shive, W., J.Biol.Chem., 172:67, 1948.

145. Bergman, E.D., Volcani, B.E. and Ben-Ishai, R., J.Biol.Chem., 194:521, 1952.

146. Bergman, E.D., Ben-Ishai, R. and Volcani, B.E., J.Biol.Chem., 194:531, 1952.

147. Miller, C.S., Gurin, S. and Wilson, D.W., Science, 112:654, 1950.

148. Schulman, M.P. and Buchanan, J.M. , J.Biol.Chem., 196:513, 1952.

149. Flasks, J.G., Warren, L. and Buchanan, J.M., J.Biol.Chem., 228:215, 1957.

150. Hartman, S.C. and Buchanan, J.M., J.Biol.Chem., 234:1812,1959.

151. Goldthwait, D.A., J.Biol.Chem., 222:1051, 1956.

152. Goldthwait, D.A., Peabody, R.A. and Greenberg, G.R., J.Biol.Chem., 221:569, 1956.

153. Peabody, R.A., Goldthwait, D.A. and Greenberg, G.R., J.Biol.Chem., 221:1071, 1956.

154. Levenberg, B. and Buchanan, J.M., J.Biol.Chem., 224:1019, 1957.

155. Levenberg, B. and Buchanan, J.M., J.Biol.Chem., 224:1005, 1957.

156. Lukens, L.N. and Buchanan, J.M., J.Biol.Chem., 234:1799, 1959.

157. Lukens, L.N. and Buchanan, J.M., J.Biol.Chem., 234:1791, 1959.

158. Lieberman, I., J.Biol.Chem., 223:327, 1956.

159. Carter, C.E. and Cohen, L.H., J.Biol.Chem., 222:17, 1956.

160. Joklik, W.K., Biochem.J., 66:333, 1957.

161. Abrams, R. and Bentley, M., J.Am.Chem.Soc., 77:4179, 1955.

162. Magasanik, B., Moyed, H.S. and Gehring, L.B., J.Biol.Chem., 226:339, 1957.

163. Moyed, H.S. and Magasanik, B., J.Biol.Chem., 226:351, 1957.

164. Lowy, B.A., Cook, J.A. and London, I.M., J.Biol.Chem., 236:1442, 1961.

165. Lowy, B.A., Ramot, B., and London, I.M., J.Biol.Chem., 235:2920, 1960.

166. Lowy, B.A. and Williams, M., J.Biol.Chem., 235:2924, 1960.

167. Lowy, B A., Williams, M.K. and London, I.M., J.Biol.Chem., 236:1439, 1961.

168. Hickman, J., J.Am.Chem.Soc., 78:6209, 1956.

169. Umbreit, W.W., Burris, R.H. and Stauffer, J.F., in "Manometric Techniques", page 149, Published by Burgess Publishing Co. 1959.

170. King, E.J., in "Microanalysis in Medical Biochemistry", page 33, Edited by J.&.A. Churchill Ltd., London.

171. Fiske, C.H. and Subbarow, Y., J.Biol.Chem., 66:375, 1925.

172. Furst, S.S., Roll, P.M. and Brown, G.B., J.Biol.Chem., 183:251, 1950.

173. Brown, G.B., Federation Proc., 15:823, 1956.

174. Bartlett, G.R. and Marlow, A.A., J.Lab.clin.Med.,42:188, 1953.

175. Szynkiewicz, Z.M., Sable, H.Z., and Pflueger, E.M., J.Bacteriology, 81:837, 1961.

176. Bernstein, I.A., J.Biol.Chem., 205:317, 1953.

177. Horecker, B.L. and Mehler, A.H., Ann.Rev.Biochem., 24:207, 1955.

178. Wang, C.H. and Jones, D.E., Biochem.Biophys.Res.Comm., 1:203, 1959.