ENZYMES OF THE MAMMALIAN RETICULOCYTE

A Thesis

by

Paul Ottolenghi

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ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BAL	2, 3-dimercaptopropanol
CoA, CoASH, ~SCoA,	coenzyme A
DNP	2, 4-dinitrophenol
DPN, DPNH	diphosphopyridine nucleotide
DPG	diphosphoglycerate
e-o-t	electron-to-oxygen transfer system
FPH_2	flavoprotein
GDP	guanosine diphosphate
GTP	guanosine triphosphate
G-6-P	glucose-6-phosphate
Hb	hemoglobin
HbOH	methemoglobin
IDP	inosine diphosphate
ITP	inosine triphosphate
$Lip(SH)_2$, $LipS_2$, ~SLipSH	α-lipoic acid
LTPP	lipothiamide pyrophosphate
NMN	nicotinamide mononucleotide
O.D.	optical density
P	phosphate
6-P-G	6-phosphogluconate
PG	phosphoglycerate
P_i	inorganic phosphate

PP	inorganic pyrophosphate
RNA	ribonucleic acid
R.Q.	respiratory quotient
SFH	stroma-free hemolysate
TPN, TPNH	triphosphopyridine nucleotide
TPP	thiamine pyrophosphate
TRIS	tris (hydroxymethyl) aminomethane
W.H.	whole hemolysate

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INTRODUCTION

With the outbreak of World War II, work was initiated in the Department of Biochemistry at McGill University, at the request of the National Research Council, under the direction of Dr. O. F. Denstedt, with the express aim of improving the existing methods of blood preservation. Because of the great importance of maintaining a supply of blood for the treatment of battle and civilian casualties, researches on blood preservation were carried out not only in this laboratory, but in many others throughout the world.

At the end of the war most of the laboratories discontinued research on blood storage. The McGill group was among the very few, if not the only one, that continued a comprehensive programme of research in this problem. During the past ten years numerous aspects of the enzymology and metabolism of the mammalian erythrocyte have been studied in a fundamental way. These basic studies have been considered essential to gain an understanding of the changes that occur in the red cell during progressive aging and ultimate failure when stored in vitro. It is altogether likely that any improvement in the current techniques of preservation will depend on a better understanding of what happens during

failure. The findings of our group and of others which have resumed the study of this problem since 1949, confirm our original belief that the maintenance of the viability of the cell during storage depends on the successful maintenance of the energy metabolism of the cells.

The thought occurred to us that perhaps the gradual and inevitable failure of the erythrocyte during storage and even in the circulation, may be but the terminal phase of a slowing down of the metabolic activity of the cell which is a continuation of a process which begins in an early stage of the cell's development. This decline proceeds at a progressively decreasing rate and finally terminates in metabolic failure and loss of viability of the cell after it has completed its normal life span in the circulation, in about 125 days.

The writer's part of the larger investigation in progress in our laboratory is a study of the metabolism of the reticulocyte.

I

ERYTHROPOIESIS

a) Embryological Development of the Erythropoietic Organs

In the normal adult mammal, the formation of the red cells of the blood takes place exclusively in the red bone marrow, where the most primitive cells are undifferentiated mesenchymal cells, also known as primitive reticulated cells. These cells, by division and differentiation, give rise to the normal erythrocytes in the circulation. In the embryo, on the other hand, before the marrow has developed, and hence before it can take part in erythropoietic activity, the red cells are produced in several organs, the exact site depending on the stage of embryonic development.

The sequence of events in the development of erythropoietic tissues is the same in all mammals, but the onset and duration of the various stages varies from species to species. Taking the human embryo as an example to illustrate the changes that take place in this development during embryonic life, the first signs of erythropoietic activity appear between the 13th. and 18th. day (1). The first hemopoietic tissue develops from the endoderm of the yolk sac which gives rise to mesenchyme. The mesenchymal cells become free and form groups of spherical cells, the blood islands, which are joined to one another by strands of elongated cells. These,

together with the peripheral cells of the blood islands eventually form endothelial tubes, the endothelium of which secretes the plasma which fills the tubes. These tubes are the blood vessels of the yolk sac and contain primitive blood cells which arise from the differentiation of the central cells of the blood islands (2). The endothelial cells of the blood vessels, at this stage, still retain all the blood cell forming potentialities of the mesenchymal cells, and some of them become free and develop into primitive erythrocytes. Most of the primitive blood cells that appear at this stage form hemoglobin (3) and are nucleated. In this first stage in which erythropoiesis is extraembryonic (in the yolk sac), it is also intravascular (44).

During the 6th. week, the liver, which has developed by a budding of the epithelium of the intestinal tract, participates in erythropoiesis. The liver, in its early stage of formation, contains large, thin walled blood vessels, and the extravascular portion consists of thin layers of mesenchyme. This mesenchyme gives rise to groups of erythroblasts which undergo maturation into normocytes (1). By the 9th. week, erythropoiesis in the yolk sac ceases, and the primitive blood cells which have been produced by it gradually disappear, and are completely eliminated by the 4th. month (3). During the 4th. month the spleen also participates in erythropoiesis, and may continue to do so, though usually in diminishing degree, up to the end of pregnancy. The thymus also,

for a short time during the prenatal period, may produce some red cells (4). During the latter part of pregnancy, the extent of blood cell formation in the liver gradually decreases to a very small magnitude. During the 5th. month, when placental circulation is established (5), erythropoietic tissue develops in the myeloid tissue, which normally is present exclusively in the marrow cavities of bones. Fetal bone marrow at first contains many primitive reticulated cells which are multiplied to compensate for losses incurred by the differentiation, which many of them undergo into other types of cells, for example, erythrocyte stem cells (6) of the normoblastic series. As the fetus continues to develop the primitive reticulated cells decrease in number, and only a few remain after birth. However, those that remain in the bone marrow or other tissues, retain all their original potentialities, and, if an extreme situation should arise, for example, in severe blood loss, these primitive remnants, or nuclei, may undergo differentiation and become active hemopoietic centres. In the fetus, erythropoietically active bone marrow (red marrow), is present in most of the long bones. With growth, however, more and more of the red marrow is invaded by yellow fat cells, and it becomes inactive. In the adult, the red marrow is found in the ribs and sternum, part of the skull, the cancellous portion of some of the short bones, and in the head of the long bones.

b) Development of the Erythrocyte

(i) Morphology of the Erythrocyte

Two different series, or generations of erythrocytes are recognised, namely (a) the primitive, or megaloblastic generation, and (b) definite, or normoblastic generation.

The megaloblastic generation of red blood cells is the first process to make an appearance in the embryo. These cells are produced in the yolk sac, in progressively diminishing number during the first three months, and by the 4th. month their production becomes insignificant. In the adult these cells may be produced in conditions such as pernicious anemia (44), in which hemopoiesis is tremendously increased, but in this case the cells are produced in the bone marrow. The normoblastic blood cell generation which appears about the 6th week of embryonic life, persists throughout the individual's lifetime. The two classes of red cell are similar in many respects and have corresponding stages of differentiation, but there are certain differences between them. Thus, the megaloblasts as occur in the embryo, do not lose their nucleus (3); in cases of pernicious anemia the nucleus is lost, and this gives rise to the formation of megalocytes. The megaloblastic cells in all the stages of their development, are larger than the normoblastic analogues. The nucleus of the megaloblasts, in contrast with that of the normoblasts, displays very little tendency of the nuclear

material to undergo clumping in any of its stages (10), and the ratio of the amount of cytoplasm to nuclear material is higher in the megaloblasts. Hemoglobin formation is believed to occur at a later stage in the normoblast than in the megaloblast (11).

The following conventional description of the erythrocyte, is supplemented by information obtained in the microspectrophotometric studies of Thorell (7, 8), and others.

In hemopoiesis the primitive reticulated cell is the most primitive type of cell. Its cytoplasm is weakly basophilic, and there is relatively little chromatin in its nucleus. The cell contains no other characteristically stainable material (7). These cells may become free, and become transformed morphologically into the myeloblast, which is the stem cell of the erythrocytic series. The changes involved in this transformation include the appearance of mitochondria in the cytoplasm, and the taking on of more intense basophilia (8). The myeloblast is a large, round cell (15 to 20 μ in diameter) with a nucleus containing finely divided chromatin material. The cell contains from one to five nucleoli (6). The myeloblast undergoes differentiation to give rise to the proerythroblast, which, strictly speaking, is the first of the erythrocytic cells. In this first transformation the general tendency of the cells to become smaller in size as they undergo successive differentiations is already apparent. In the proerythroblast the ratio of the volume of

the nucleus to that of the cytoplasm is high, and the cytoplasm and nucleoli contain a high concentration of ribonucleic acid. The nucleus contains dense chromatin material which tends to undergo fusion. The basophilic staining property of the nucleus is due to its content of desoxyribonucleic acid, as can be shown by staining with the Feulgen reagent. Further evolution of the cell to the basophilic normoblast stage, is attended by a progressive decrease in the size, and increase in the basophilia, both of the nucleus and the cytoplasm. The nucleoli of this cell are much smaller than in the previous stages (7). Recently Carvalho (9), with the aid of microspectrophotometric methods, employing two different wavelengths (2650 Å for nucleic acids, and 4150 Å for heme), has shown that the nucleus of the basophilic normoblast contains a high concentration of heme. By the superimposition of photomicrographs obtained at the two wavelengths, he demonstrated that the heme is distributed chiefly in the spaces in between the masses of material that absorbs at 2650 Å. It was also shown that the concentration of heme in the nucleus decreased as the size of the nucleus decreased, i.e., with the maturation of the cell.

The polychromatic erythroblast represents the next stage in the development. This cell can be stained with both acidophilic and basophilic dyes, a property which is acquired with the appearance of hemoglobin in the cytoplasm. In this cell form the nucleoli are no longer present. The nucleus is

very small and stains very densely, while the mitochondria become sparse in distribution and later disappear (21).

The final erythroblastic stage is represented in the orthochromatic erythroblast, a cell in which the structure inside the nucleus is no longer discernible (i.e., the nucleus is pyknotic), and the cell is highly hemoglobinised. Soon the nucleus is lost and the cell becomes a reticulocyte. The latter is larger than a normocyte, and still contains some basophilic material, often in the form of a network which varies in the intensity of staining. The material sometimes appears as a series of granules dispersed throughout the cell, or it may be confined to small areas. The reticulum also may be distributed uniformly, or may form a thick band across the cell. The amount of stainable material varies widely from cell to cell. After a short time the reticulocyte loses its basophilic material and contracts further in size attaining the biconcave shape characteristic of the normocyte. Thus, the cell, after many transitions, becomes the mature erythrocyte which is believed to have a life span in the circulation, of about 125 days. The normocyte has no stainable material in the cytoplasm which is known to consist mainly of hemoglobin along with soluble enzymes.

(ii) Detailed Morphology of the Reticulocyte

Reticulocytes, compared with normocytes, are larger, have a lower specific gravity, and although having a full

complement of hemoglobin are hypochromic (7, 10). In 1921 Key (12) pointed out that reticulocytes can be recognised in unstained preparations, since the reticulum is visible in the form of granules attached to the stroma. The reticulum which is stainable with basic dyes, can be stained even after the cells had been laked, and the stainable material remains with the stroma. The same result was obtained whether the cells were first stained and then laked, or vice versa (12). These observations have been substantiated in more studies with the aid of the electron microscope (13, 14). It was shown that the reticulocytes are readily distinguishable from normocytes by the characteristic structure of the stroma of the former which exhibited certain bubble- or crater-like irregularities, demonstrable by shading techniques. These irregularities on the stroma vary in number from 4 to 50, the number tending to vary inversely with the size (15). These observations have been further corroborated with the aid of phase microscopy (16).

Burt et al. (17) have shown that the chemical composition of the stainable material of the reticulocyte is ribonucleic acid. This conclusion is based on the finding that the ribonucleic acid content of blood specimens runs parallel with the number of reticulocytes. Furthermore, the basophilia disappears on treatment of the cells with RNAase (22). The treatment with DNAase, caused no change, suggesting that the reticulum is not of nuclear origin. On the other hand

the widely accepted notion that the reticulum is of cytoplasmic origin is not very consistent with the evidence that the reticulum is part of, or strongly bound to the stroma.

(iii) Multiplication and Differentiation of the Normoblasts

The primitive reticulated cells have two functions, namely, multiplication and differentiation. Each of these functions is possessed in different degree, by the various types of cell in the process of development from the stem cell.

In erythropoiesis, with progressive differentiation of the various cell stages there is the tendency towards decreasing size of the cell, and the decreasing necessity for a nucleus, nucleoli and cytoplasmic organelles with increasing maturation of the cell, leads to the gradual disappearance of all these bodies, finally yielding the mature cell containing homogeneous material. The overall process follows a series of recognisable stages which are roughly related to the morphological stages previously described. The material reported here is taken largely from Thorell's observations (7).

The erythroblasts in all stages up to and including the orthochromatic normoblasts are capable of undergoing mitosis, as long as the nucleus is not pyknotic (18, 44). According to Dacie and White (18), the very primitive erythropoietic cells only very rarely undergo mitosis. The frequency of mitosis increases as the cells mature, being highest during

the early developmental stages of the polychromatic erythroblast, and decreasing in the later stages of maturation. From these observations it would seem that the polychromatic erythroblasts not only undergo mitosis at a comparatively high rate, but must also undergo differentiation into the more mature stages, in order to maintain a constant proportion among the various types of cells.

Thorell's observations (7) on bone marrow cells, show that the concentration of cytoplasmic nucleic acid begins to fall from the commencement of the proerythroblast stage and falls off rapidly until the early polychromatic erythroblast is reached, when the cytoplasm no longer contains any pentosenucleic acid. Concurrently with the decrease in the cytoplasmic RNA, the nucleoli also diminish in size until they can no longer be demonstrated in the polychromatic erythroblast.

According to Brachet (19), the synthesis of proteins within a cell is controlled by the nucleoli, and takes place in the microsomes which constitute the basophilic material of the ground substance of the cells (20). The synthesis of protein material in the erythroblast is most intensive during the proerythroblast stage, and this may be the reason for the increase in the basophilia associated with the earliest stages, and which becomes most intense on reaching the basophilic erythroblast stage. In the polychromatic erythroblast the synthesis of protein is complete, and heme which has been accumulated in the nucleus (9) is liberated and combines with

protein in the cytoplasm to form hemoglobin, or a precursor thereof. Hemoglobin synthesis is complete by the final stage of the polychromatic erythroblast, but in this cell the hemoglobin concentration is still less than in the circulating normocyte, the difference being accounted for by the further decrease in the volume of the cell which occurs after the loss of the nucleus (7). These changes are summarised in Figure 1, (page 14) after Thorell (7).

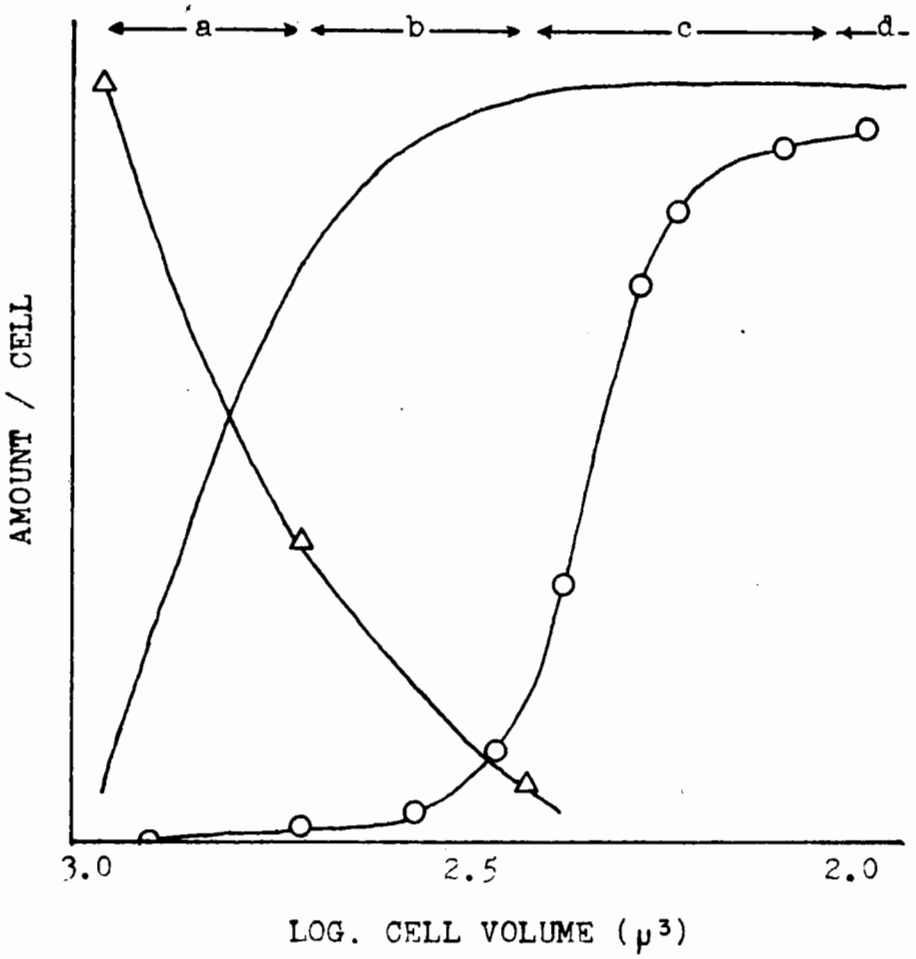
The final stages in the development of the erythrocyte are characterised by the loss of the mitochondria during the polychromatic stage (21), and finally, by the loss of the nucleus.

(iv) Loss of the Nucleus

The manner by which this process is accomplished is still unknown. Immediately preceding this event the cell is orthochromatic, has its full complement of hemoglobin, a pyknotic nucleus, but no visible nucleoli or mitochondria. Some hematologists believe that the nucleus is disposed of by a karyolytic process, that is, an enzymatic digestion; others maintain that the nucleus is eliminated by "extrusion". The sudden and complete disappearance of the nuclear material does not lend support to the theory of karyolysis. Nor does the paucity of free nuclei in the bone marrow (18) support the extrusion theory, since one should expect to find a considerable

FIGURE 1

Changes in Cytoplasmic Nucleic Acid, Total Protein and Hemoglobin, During the Maturation of the Erythroblast.



a proerythroblast
b basophilic erythroblast
c polychromatic erythroblast
d orthochromatic erythroblast

△——△ cytoplasmic nucleic acid
——— total protein
○——○ hemoglobin

number of nuclei in the regions where hemopoiesis takes place.[#]

According to Albrecht (23), the mature erythroblasts formed in bone-marrow tissue cultures, expel their nuclei by active, contractile movements which can be demonstrated with the aid of photomicrocinematography. Bessis (15), and Bessis and Bricka (24), also, have obtained photocinematographic evidence for the extrusion of the nucleus. According to these authors the cell, after loss of the nucleus, becomes a reticulocyte. These observations were made on bone-marrow cells, and the process of extrusion, which under the conditions used requires about ten minutes for completion, can be accelerated by raising the temperature.

Boström, in 1948 (25), proposed a new hypothesis for the loss of the nucleus by the normoblast. According to her view, the nucleus is not eliminated by extrusion. Instead, the normoblast forms a pseudopod which splits off, giving rise to a reticulocyte, or a normocyte, and leaving a portion of the parent cell, which retains the nucleus and a thin band of

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[#]The volume of available marrow in the human body has been estimated at about 1500 ml. (67), but only about 20 percent of this is active at any one time (168) in normal health. Hence, in the average man there is about 300 ml. or 3×10^5 mm.^{3} of active bone marrow.

The body contains approximately 3×10^{13} erythrocytes. Approximately 1 percent of these, or 3×10^{11} cells are renewed daily. Therefore it follows that about 10^6 cells are produced each day per mm.^{3} of active bone marrow, which means that there is a formation of 12 mature cells, per second, per mm.^{3} of active bone marrow.

cytoplasm. The latter cell is capable of growth and of formation of further pseudopoda, or buds, which in turn give rise to more reticulocytes or normocytes. Bostrom gives photographic evidence for her hypothesis. The results obtained by Bessis (15), and by Bessis and Bricka (24), could easily be explained by the formation of pseudopoda, since the extruded nuclei shown in their diagrams appear to have a thin band of cytoplasm around the periphery.

Whatever the process of elimination of the nucleus may be, authorities are generally agreed that the cell, after the loss of the nucleus becomes a reticulocyte.

(v) Maturation of the Reticulocyte

Nizet, in 1946 (26), obtained some evidence that all the non-nucleated red cells of the bone marrow are reticulocytes, and that the marrow contains no normocytes. The proportion of the different types of cell in the marrow is difficult to determine since samples of marrow obtained by aspiration always contain a significant number of blood cells together with the marrow cells. Nizet obtained his information by producing red cells containing Heinz bodies. He administered to a dog a dose of phenylhydrazine, which gives rise to the formation of Heinz bodies in a large percentage of the mature red cells, without affecting the reticulocytes (30, 229). Having thus labelled the normocytes of one dog, a cross circulation

with a second, normal dog was established, and maintained for a sufficiently long period to permit complete mixing of the circulating bloods. Counts were made on the cells of the peripheral circulation and the bone marrow, and the proportion of cells with and without Heinz bodies was determined. It was found that the ratios of the marked to the unmarked cells in the peripheral blood and the bone marrow were identical. This indicates that the mature cells present in the marrow samples originated from the blood circulating in the vessels in the marrow, rather than in the hemopoietic marrow tissue itself. Even if these results indicate that normocytes do not occur in the bone marrow, they throw no light on the question whether the reticulocyte is invariably a precursor of the circulating normocyte.

Efforts to determine the maturation time of the reticulocyte have yielded variable results. The results obtained from in vivo and in vitro studies, generally have shown good agreement when the experiments have been carried out simultaneously. No such agreement, however, has been obtained by different workers, presumably because the experimental conditions were not the same. Baar and Lloyd (237) found the maturation time, in vivo, to be ten hours, and possibly less, while in experiments on bone-marrow cultures (27), the ripening time in vitro was about eleven hours. A sudden increase in the reticulocyte count was believed to be associated with an increase in the ripening time, although no correlation between these two processes could be established.

Riddle (28) studied patients with pernicious anemia, undergoing liver therapy, who had up to 45 percent of reticulocytes in the blood, and found the maturation time of the reticulocytes to be about two days. This value was derived from the observation that at the beginning of treatment there was an increase in the total red cell count which was paralleled after two days by an increase in the number of non-reticulated cells. Heath and Daland (29) in in-vitro experiments, examined blood specimens containing up to 42 percent of reticulocytes, and observed that the maturation time varied between one and five days. The reticulocyte count in samples incubated at 37°C. was observed to decrease rapidly at first, and subsequently the rate of maturation progressively decreased until all the reticulocytes disappeared. The reticulocytes from rabbit and human bloods, produced in either species by different methods, e.g. removal of blood or administration of phenylhydrazine in the rabbit, or in hemolytic jaundice or pernicious anemia in human patients, behaved in the same general manner during maturation experiments in vitro. When reticulocytes were placed in the pleural cavity of rabbits, so as to maintain somewhat more physiological conditions, the maturation process followed the same pattern. When reticulocytes were kept at 10°C. or at 23°C. no change in the count was observed within 120 hours. That the disappearance of the reticulocytes occurred through their maturation and not as the result of hemolysis was established by demonstrating that the total red-cell count during the experimental period remained constant. Cruz (30)

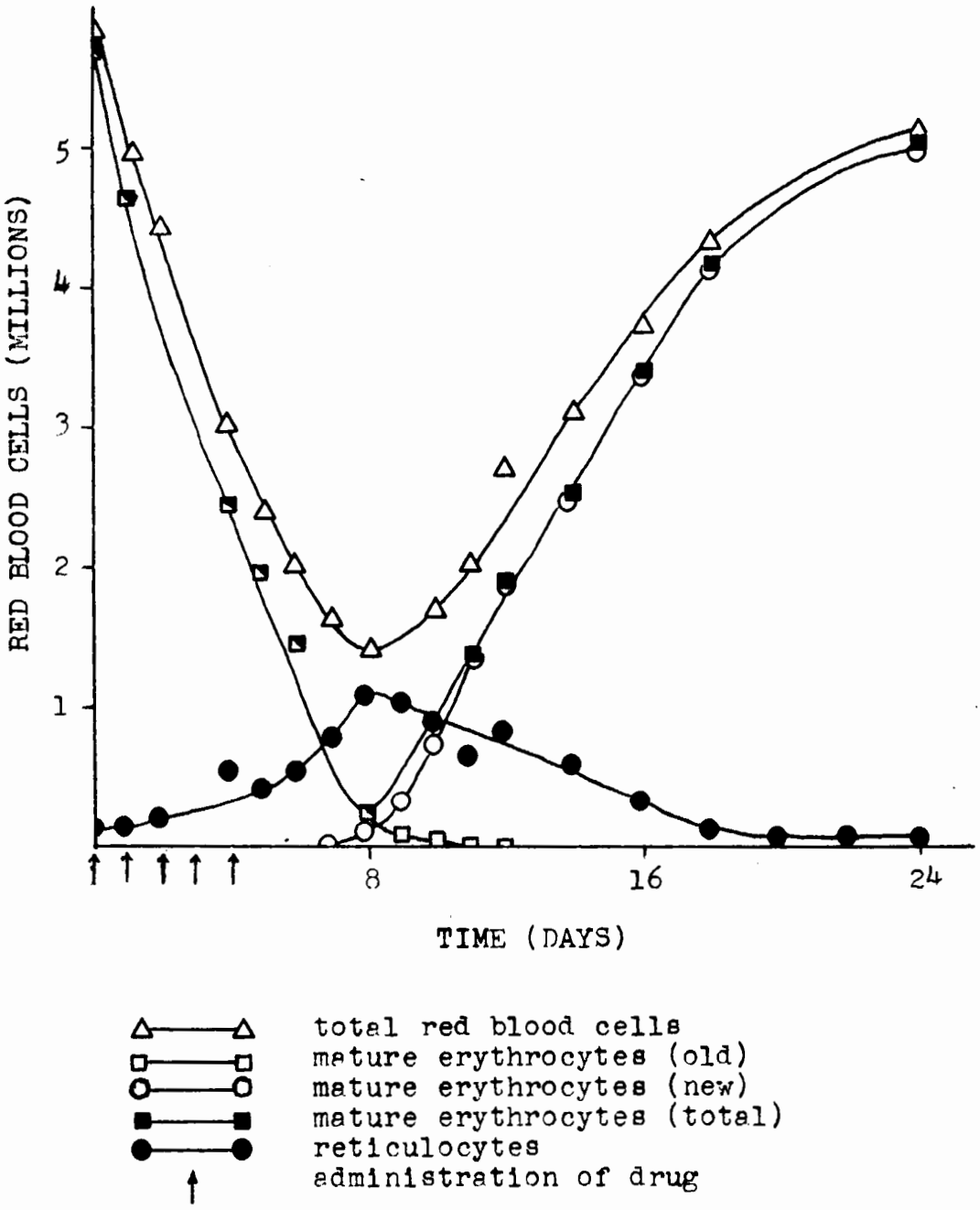
studied the changes in the erythrocytic picture following the administration of acetylphenylhydrazine to dogs. The results showed that the progressive anemia produced by the acetylphenylhydrazine was accompanied by an increase in the proportion of reticulocytes in the circulation. This trend continued for two days after the final injection of the drug, by which time the reticulocyte count began to decrease while the normocyte count increased. This is illustrated in Figure 2, on page 20.

Concurrently with the decrease in the reticulocyte count there was a net increase in the number of red cells, thus indicating that the maturation of the reticulocytes which were already in the circulation did not account for all the new erythrocytes found in the blood. It is suggested by Cruz that the two-day delay in the appearance of the normocytes may have been due to interferance by the drug, with the maturation processes, and that the animal requires two days to dispose of sufficient amounts of the drug to allow the maturation mechanisms to return to normal.

Young and Lawrence (31), studying the in-vitro maturation of reticulocytes in human cases of hemolytic anemia, found that the maturation time was about six days. The same maturation time was indicated in a in-vivo experiment performed by transfusing a sample of blood containing a high proportion of reticulocytes from a patient with hemolytic anemia, into a patient with aplastic anemia. It is important to note that

FIGURE 2

The Changes Produced in the Erythrocytic Picture by Acetylphenylhydrazine. (30).



in this experiment the red cells were not normal, and in fact, it is questionable whether reticulocytes produced in large quantities by any method should be considered normal. In the above mentioned case, furthermore, there was the additional possibility of an immunological complication.

About ten years ago, a series of reports by Plum and his associates with reference to substances which were supposed to accelerate the maturation of reticulocytes appeared in Scandinavian journals. Plum (32) observed that reticulocytes suspended in saline disappeared very slowly, even when the temperature was raised to 40°C., but if the cells were suspended in plasma, or on addition of liver extracts to the saline suspension, maturation was accelerated. Furthermore, the rate of maturation under the latter conditions became dependent on the temperature. At least, he observed that the maturation rate increased with increase in the temperature. Studies on bloods with different content of reticulocytes, showed that the quantity of the maturation-producing substances in the plasma appeared to vary inversely with the number of reticulocytes (33). Jacobsen and Plum (34) fractionated the ripening principle in liver, into a thermostable and a thermolabile fraction. The two fractions when present together were effective, but with either alone, little if any response was obtained. The stable principle was found to be tyrosine (35) the l-form being more active than the d-isomer. Changes in the chemical substituents in the aromatic ring or in the side chain altered the activity of the compound. Apparently the

hydroxyl in the para position in the ring is essential for the action of this factor in promoting maturation. Gad, Jacobsen and Plum (36), demonstrated further that if tyrosine be exposed to tyrosinase, the product of the oxydation was from fifty to one hundred times more active in its effect on the ripening of the reticulocytes. The ripening action of adrenaline was similarly enhanced by the action of tyrosinase, but with tyramine, the increase in activity was only about ten-fold. Gad et al. have found also that the red cell contains enzymes which increase the activity of tyrosine and of dihydroxyphenylalanine in stimulating the maturation of reticulocytes.

Gad et al. (37), also investigated the ripening activity of extracts of various organs, with respect to the thermolabile fraction which had been previously found in liver (34). This factor appeared to exist in two forms, depending on the source material. In some material (e. g. plasma), the factor is self sufficient, producing maturation without the addition of tyrosine. At least, when the latter is added, it produces no increase in the rate of maturation. Stomach tissue, especially the mucosa, but only very slightly the muscular portion, contains a high concentration of the principle, which, however, requires tyrosine for ripening activity. Tissues such as bone marrow, liver, striated muscle and spleen, contain both forms of the principle. Gastric juice supplemented with tyrosine, also was found to have considerable activity. The especially high concentration of the ripening

factor in stomach tissue suggests that it is probably synthesised in this organ, and particularly in the mucosa. After absorption into the blood it may be linked with some substance related to tyrosine, or to one of its metabolic oxydation products, to form the complete maturation principle which is found in the blood plasma. The distribution of the factor in the stomach agrees closely with the distribution of the intrinsic factor (38).

The respiratory activity of the reticulocyte is related to the maturation rate of the reticulocytes (39). Substances such as urethane, cyanide, carbon monoxide, and malonate, which lower the oxygen uptake of the immature cells, also cause a lengthening of the ripening time (39, 40).

II

METABOLISM OF THE ERYTHROCYTE

a) Introduction

The absence of a nucleus makes the mammalian erythrocyte unique among cells, and has given rise to doubt whether the erythrocyte is a "living" cell. For many years these cells were believed to be mere parcels of hemoglobin with a sole function of transporting oxygen. This view in recent years has yielded to the opinion that the erythrocyte, despite the lack of a nucleus has an energy metabolism to enable it to do osmotic and other work, and thus is a "living" cell.

The reticulocyte differs from the mature erythrocyte in its chemical make-up and activity, in that it contains ribonucleoprotein (17), and numerous additional enzymes, and is metabolically much more active. As early as 1909, Warburg (41) observed that the erythrocytes of mammalian blood appeared to show a feeble oxygen consumption, which later was found to vary with the number of reticulocytes in the blood sample. That the oxygen consumption is attributable to the reticulocytes was confirmed by Harrop (42) and Wright (43). It is now appreciated that the chemistry and activity of all the immature forms of the red cell differ with the stage of development.

Thus the reticulocyte is capable of synthetic processes which do not occur in the normocyte. The synthesis of protein (19), and heme (45), occur in the reticulocyte, but there is no incorporation of radioactive amino acids into proteins (46) or hemoglobin (45) in the mature red cell.

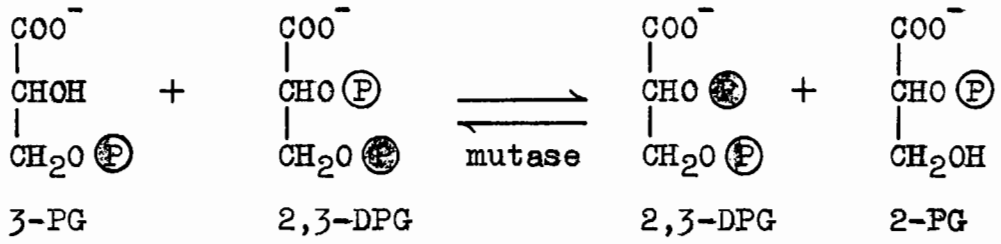
The reticulocyte, therefore, may be considered as an intermediate stage in the development of the mature erythrocyte and the process of maturation is attended by a progressive and extensive degeneration of the enzymatic processes which probably result from the loss of the nucleus.

b) Glycolysis

The most prominent metabolic activity of the erythrocyte is glycolysis. The term "glycolysis" implies the breakdown of glycogen or glucose to lactic acid. The process is a complex one involving several enzyme catalysed reactions. Our present understanding of the glycolytic process represents the contributions of pioneers including Meyerhof, Embden, and Parnas, and the subsequent contributions over the past years of many other workers whose studies not only have elucidated the mechanism of these reactions, but their relation to other metabolic processes.

The erythrocyte has long been known to utilise glucose as a substrate. In 1913, Rona and Arnheim (47) demonstrated for the first time that the red cells of the blood

possess glycolytic activity. It is now well known that the glycolytic mechanism in the erythrocyte is closely similar to that which occurs in yeast, muscle, and other cells. The system in the erythrocyte differs, however, in that it favors the accumulation of the intermediate 2, 3-Diphosphoglycerate (2, 3-DPG), which makes up from 30 to 50 percent of the acid-soluble phosphorus of the erythrocyte in most mammals (48). This compound was thought not to be present in other tissues until Sutherland et al., in 1949 (49), isolated it from muscle and demonstrated its role as coenzyme for the enzyme phosphoglyceromutase which catalyses the interconversion of 3-Phosphoglycerate (3-PG) and 2-Phosphoglycerate (2-PG). (Reaction 1)

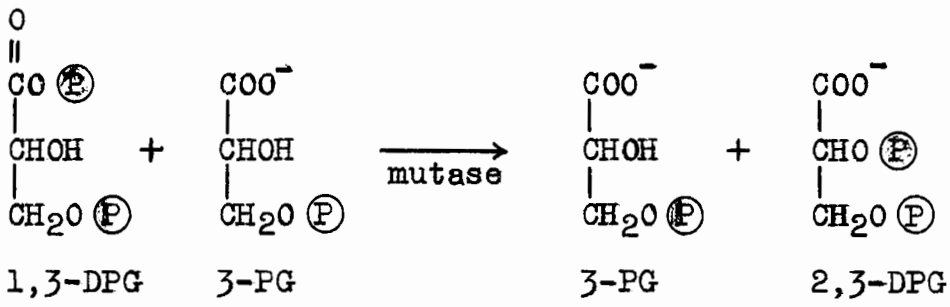
1) 

Studies by Hevesy and Aten (50), and by Gourley (238), with orthophophate containing P^{32}, and by Bartlett and Marlow (51), with C^{14} labelled glucose, demonstrated that the turnover of the 2, 3-DPG in the erythrocyte is rapid, and comparable with that of the other glycolytic intermediates. DPG therefore, must either be an intermediate or be in equilibrium with an intermediate of the glycolytic system.

As further evidence of the participation of 2, 3-DPG in glycolysis, Papoport and Luebering (52) demonstrated the existence of an enzyme, diphosphoglyceric acid-mutase, which

catalyses the conversion of 1, 3-DPG to 2, 3-DPG. These workers succeeded in partially purifying the enzyme, but under the conditions used, failed to demonstrate that it catalyses the reaction in the reverse direction also. The reversal of the mutase would be thermodynamically unlikely since it would involve the conversion of a low-energy phosphate into a high-energy one with a change in the free energy of the group of more than 10,000 calories per mole (52). In the following year the same authors described a phosphatase (53) which is present in both muscle and erythrocytes, and which produces the removal of the phosphate group at the 2-position in the 2, 3-DPG forming 3-PG and inorganic phosphate (P_i). The phosphatase in the erythrocyte being relatively inactive, Rapoport and Luebering (53) proposed that the 1, 3-DPG is converted, by the mutase, to the more stable 2, 3-isomer, which then, because of the weak action of the phosphatase tends to accumulate in the cell. These workers (239) postulate that since the activity of the mutase is increased by the addition of 3-PG, it is possible that this ester acts as a coenzyme in the following reaction:

2)



This is further substantiated in studies with radioactive phosphorus which showed that the relative specific activity of the phosphate group in the 3-position in 2, 3-DPG and in 3-DPG remains very low compared with that of the phosphate in the 2-position of 2, 3-DPG (56).

If the reaction catalysed by the mutase be irreversible, and the 2, 3-DPG formed must be dephosphorylated before it can undergo glycolysis to lactate, there will be a loss of two potential ATP molecules for every molecule of glucose metabolised by way of 2, 3-DPG. Glycolysis, under these conditions, therefore, would become a useless system, since no energy would be derived from it. Only two molecules of ATP would be formed, and two molecules would be used up for the phosphorylation of the hexose and the hexose monophosphate. If such a system were present in the erythrocyte it presumably would play a role analogous to that of the creatine-phosphocreatine system in muscle, since it would act solely as a reserve of ATP to be drawn upon when other energy yielding mechanisms fail.

Studies, in our laboratories, on blood during storage (54, 55), have shown that there is a good correlation between the amount of pyruvate accumulated and the 2, 3-DPG that disappears. Both types of change begin at about the same time, when the rate of glucose breakdown begins to slow down. From these changes, which also may be observed on incubating the

blood at 37°C. (54), it is apparent that the maintenance of the high level of 2, 3-DPG depends on glycolysis, and that when glycolysis begins to fail, the cell cannot maintain the 2, 3-DPG. The rate of breakdown, therefore, will exceed the rate of formation, and the concentration will fall.

Prankerd and Altman (56), and Bartlett and Marlow (240), have obtained evidence that the uptake of inorganic phosphate occurs with the oxidative phosphorylation of 3-phosphoglyceraldehyde to 1, 3-DPG. That the phosphate taken up comes from the extracellular rather than the intracellular fluid, was shown by means of P^{32} labelled orthophosphate. Furthermore, even though the phosphate in the 3-position in 3-PG has very low relative specific activity, the 2, 3-DPG was found to be precursor of the cellular ATP and inorganic phosphate, as determined by the radioactivity measurements (56). Prankerd and Altman consider that the reaction catalysed by the diphosphoglyceric acid-mutase is freely reversible. As already mentioned, however, this is unlikely for thermodynamic reasons. The results could also be explained by considering the mutase to catalyse a side reaction of glycolysis. In this way the 1, 3-DPG would be converted partly to the 2, 3-isomer which then could lose only the radioactive phosphate and thus enrich the orthophosphate of the medium. The rest of the 1, 3-DPG would be converted to lactate by the usual way, with the formation of radioactive ATP rather than liberation

of inorganic phosphate. It is known that the relative specific activity of the inorganic phosphate inside the cell increases at almost the same rate as that of the ATP (56, 238).

The glycolytic mechanism is most probably the only source of energy possessed by the mature erythrocyte. Fishman, in our laboratories, has shown (241) that inhibition of glycolysis will increase the osmotic fragility of the cell and will also interfere with the maintenance of the normal ionic gradients existing between cell and plasma. The capacity of the erythrocyte to expel Na^+ and to take up K^+ is related to glycolysis in that this mechanism provides the energy necessary for the transfer of the ions across a membrane, against a concentration gradient.

c) Hexose Monophosphate Shunt

The enzymes of the hexose monophosphate shunt as elucidated by Warburg, Lipmann, Dickens and others, have been studied extensively during the last twenty years in many tissues including the erythrocyte (57, 58, 59). In 1931, Warburg and Christian (60, 61) demonstrated the presence of a system which oxidised glucose-6-phosphate to 6-phosphogluconic acid. It involved an enzyme which they called "Zwischenferment", later known as Glucose-6-phosphate dehydrogenase, and which requires TPN as a coenzyme. Dickens (62) then showed that the 6-phospho-

gluconic acid could be further oxidised by another TPN-linked dehydrogenase in horse red cell hemolysate, with the formation of a pentose ester. These results were confirmed by Horecker (63).

Dische (57) incubated hemolysates with ribose-5-phosphate, in an ATP-free medium, and recovered a mixture of glucose-6-phosphate, fructose-6-phosphate and fructose-1, 6-diphosphate. Under the conditions of the experiment there was no interconversion of the hexose monophosphates and the fructose diphosphate, thus indicating that these intermediates must be produced by different pathways. Furthermore, it also appears that the glucose-6-phosphate and the fructose-6-phosphate arise by independent pathways, since the equilibrium between these two compounds is established within one hour when ribose-5-phosphate is the starting material. Starting with either of the two hexose monophosphates, on the other hand, equilibrium is not reached until after four hours. Dische postulated the involvement of a 4-carbon atom intermediate.

Francoeur and Denstedt (68), observed the rapid breakdown of ribose-5-phosphate in the red cell catalysed by catalase, both anaerobically, in the presence of ferricyanide, and aerobically, with methylene blue. Anaerobically, the action can be reproduced by using purified catalase from beef liver. Under aerobic conditions, the action is due to the peroxidase activity of the catalase, and depends on the presence of a system

which slowly generates hydrogen peroxide. The reaction involves neither DPN nor TPN. The products of the reaction have not been identified, but the reaction can be linked, with the reduction of DPN by triose phosphate dehydrogenase (59).

The presence of at least a partially functional hexose monophosphate shunt in the erythrocyte has now been amply demonstrated, not only by the work of Dische already mentioned, but also by the significant observations made by Gabrio and Finch concerning the dramatic effect of adenosine in the restoration of the metabolic activity in stored erythrocytes.

Dische (57) had found that hemolysates could use nucleosides such as adenosine, inosine or guanosine, as a source of ribose-5-phosphate. Desoxyadenosine, and xanthosine also are utilisable, but the pyrimidine nucleotides cannot (76). Gabrio and Finch (76) found that the changes which occur during the storage of blood, such as the breakdown of organic phosphates, with the liberation of inorganic phosphate, the increased osmotic fragility, and others, cannot be arrested or reversed by the addition of amino acids, carbohydrate intermediates, ATP, ADP, ribose-5-phosphate, or adenine, to the preservative medium. AMP was found to produce a very slightly beneficial effect, but adenosine was found to produce a spectacular reversal of many of the deteriorative changes that occur during storage of blood in the cold. The addition of adenosine

to the ACD[#] medium for the storage of blood, induces a considerable improvement in the general physiological condition and viability of the cells compared with the preservation of the cells in the ACD medium alone (76). In the presence of adenosine the rate of increase in inorganic phosphate, that is, the rate of hydrolysis of organic phosphates, is greatly retarded and the concentration of 2,3-DPG and ATP are better maintained during storage. Likewise, the potassium to sodium ratio was better maintained, the osmotic fragility improved and the extent of spontaneous hemolysis greatly reduced (76, 77). When deteriorated cells were incubated with adenosine, a rapid resynthesis of organic phosphates and an equivalent decrease in the inorganic phosphate occurred, with an increase in the content of ATP and 2, 3-DPG (76, 78). The decided improvement in the viability of the cells which resulted from this reconstitution, was confirmed by clinical tests of the capacity of the cells to survive in the circulation after transfusion.

On incubating erythrocytes in a medium containing glucose and inorganic phosphate labelled with P^{32}, at 37°C., an increase in both free and esterified P^{32}-containing phosphate occurs inside the cell (56). At the end of twenty-four hours, however, even though glucose is still present in the medium, a progressive fall in the relative specific activity

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[#]Acidified citrate dextrose, consisting of an isotonic mixture of trisodium citrate, citric acid and glucose.

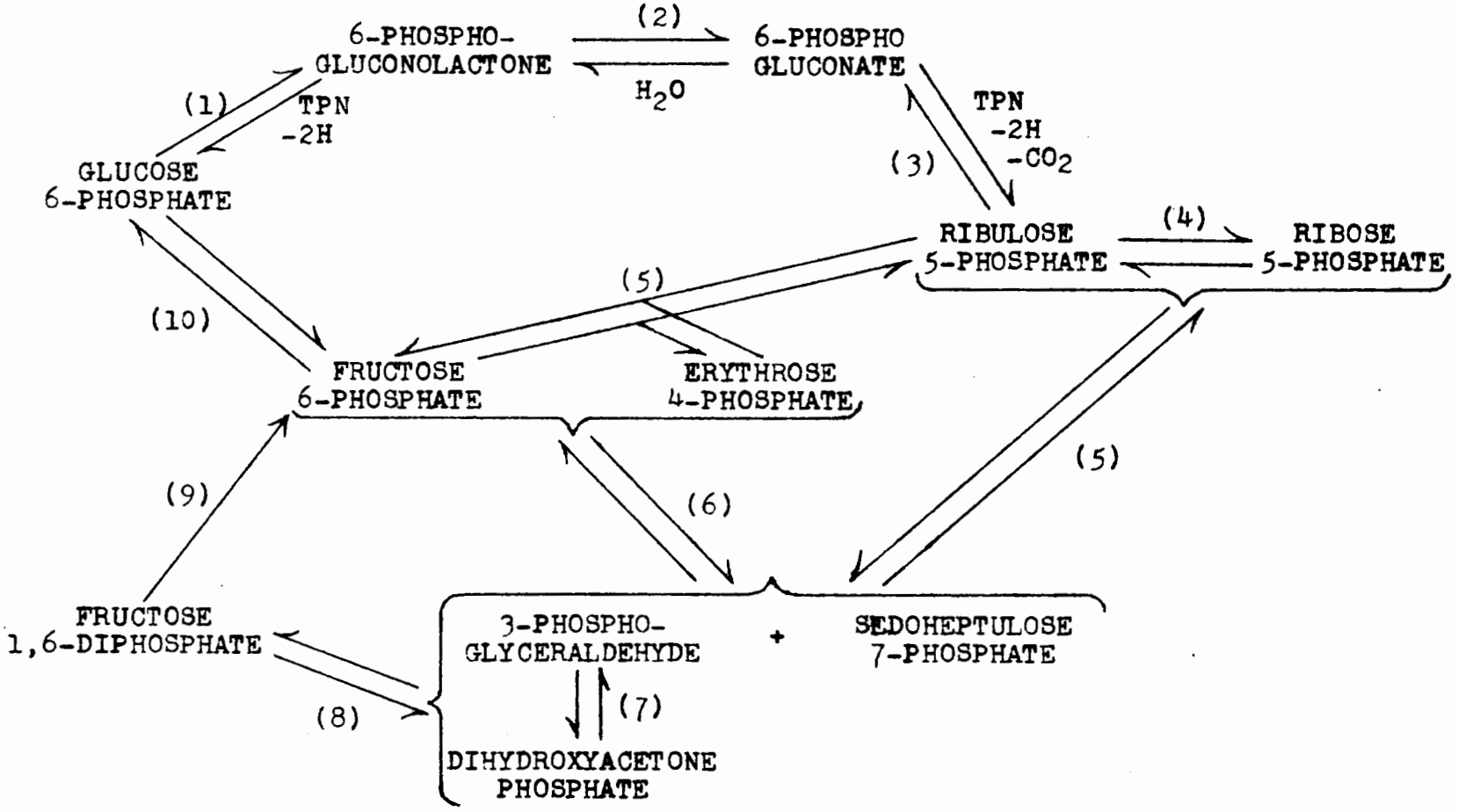
and the concentrations of the ATP and 2, 3-DPG occurs. If adenosine is then added to the medium, an increase in the uptake of P^{32} by the cells may be observed, accompanied by an increase in the concentration and relative specific activity of the ATP and 2, 3-DPG (56, 79). With hemolysates containing added adenosine, approximately one mole of inorganic phosphate is esterified in the form of organic phosphate per mole of adenosine utilised (76).

Kalckar (65) demonstrated that certain tissues contain an enzyme which catalyses the phosphorolysis of nucleosides. This enzyme is analogous to the phosphorylase which forms glucose-1-phosphate from glycogen and inorganic phosphate. The product of the phosphorolysis of the nucleosides is ribose-1-phosphate. Abrams and Klenow (68) found a mutase which catalyses the conversion of the ribose-1-phosphate to ribose-5-phosphate. A nucleoside phosphorylase has recently been isolated and purified from erythrocyte hemolysates (78), which has an absolute requirement for orthophosphate or arsenate for the phosphorolysis or arsenolysis respectively. In the presence of arsenate, however, there is no increase in the formation of intracellular phosphate esters, since arsenolysis yields the free sugar (56).

The scheme presented in Figure 3 (242), compiled from numerous sources, but based largely on evidence obtained by Horecker et al. (243, 244), summarises the present concept of the sequence of reactions comprising the hexose monophosphate shunt.

FIGURE 3

The Hexose Monophosphate Shunt.



Enzymes of the Hexose Monophosphate Shunt

1. Glucose-6-phosphate dehydrogenase
2. Lactonase
3. Phosphogluconic dehydrogenase
4. Ribose phosphate isomerase
5. Transketolase
6. Transaldolase
7. Triosephosphate isomerase
8. Aldolase
9. Fructose diphosphatase
10. Hexose phosphate isomerase

The possibility that an enzyme system exists in the erythrocyte which promotes the breakdown of 6-phosphogluconic acid directly to triosephosphate and pyruvate, as occurs in Pseudomonas saccharophila (64) has not been reported. The idea that the TPNH formed in the first two reactions of the shunt might be reoxidised by the lactic dehydrogenase during the reduction of pyruvate is attractive, but improbable since such a transformation would be wasteful of energy. That the oxidative portion of the shunt is functional in the erythrocyte is, for the time being, doubtful, since no mechanism has been found and shown conclusively, to reoxidise the TPN reduced by the dehydrogenases involved in this alternative pathway of glucose metabolism.

d) Respiration of the Erythrocyte

The respiratory activity of the mature mammalian erythrocyte is so slight, if it occurs at all, that most authorities doubt whether it can be of any significance in the energy metabolism of the cell. As already pointed out, many workers have claimed that the oxygen consumption of the red cells is attributable entirely to the reticulocytes which invariably are present in blood specimens (41, 42). Ramsey and Warren (70) on the other hand, examined the question very carefully and arrived at the conclusion that the mature erythrocyte has a significant respiratory activity. They carefully removed the white cells from the samples of rabbit blood, and measured the oxygen consumption of the remaining cells. After

correcting for the oxygen consumption of the reticulocytes in the sample, they concluded that the mature rabbit erythrocyte does have a small but nevertheless significant oxygen consumption. The QO_2 for mature rabbit erythrocytes as calculated from their data was approximately 0.02. That of the reticulocytes was about 30 times as great. In a previous paper (71), Ramsey and Warren had shown that on hemolysis of rabbit or human erythrocytes, there is a sudden increase in the oxygen uptake, accompanied by the evolution of CO_2. The calculated R. Q. was found to be within the physiological range, and the respiratory activity was accompanied by an evolution of heat. The same authors later provided evidence that the sudden increase in the oxygen uptake, in part at least, was attributable to autoxidation of lipid material in the plasma (245).

Harrop and Barron (72) had previously discovered that methylene blue, added to a suspension of mammalian red cells, greatly increases the oxygen consumption. This increased respiratory activity proved to be insensitive both to cyanide and to carbon monoxide. When avian cells, which are nucleated and have a considerable and genuine oxygen consumption (41) were tested, methylene blue produced only a small increase in the respiratory activity. Methylene blue caused little, if any, alteration in the rate of utilization of glucose by the cells (73), but caused a significant reduction in the amount of lactate formed. Ordinarily, the ratio:

$$\frac{\text{millimoles lactate formed}}{\text{2 x millimoles glucose utilised}}$$

in the mammalian cell is unity, but in the presence of methylene blue it may be reduced to 1 percent of the previous value obtained in the absence of the dye. These calculations cannot be applied to measurements on chicken erythrocytes, since these cells are incapable of aerobic glycolysis (73). Warburg and Christian (60, 61) showed that the respiration in the presence of methylene blue was attributable to the oxidation of glucose-6-phosphate to 6-phosphogluconic acid by glucose-6-phosphate dehydrogenase with TPN as coenzyme.

Michaelis and Salomon (74), observed that the addition of a saline extract of certain tissues, especially liver, to a suspension of mammalian erythrocytes, increased the oxygen uptake more than did methylene blue. Like methylene blue, the extracts had no effect on the respiration of avian erythrocutes. The effect of the extracts on mammalian red cells, as with methylene blue (72), is not sensitive to carbon monoxide. The metabolic significance of these findings is not yet known.

That the maintenance of hemoglobin in the reduced state is connected with the metabolism of the erythrocyte was known by Warburg and co-workers (82) as long ago as 1931. Kiese (83) noted that the reduction of methemoglobin by red cells, in the presence of glucose caused the accumulation of pyruvate, and a depression of the normal formation of lactate. Drabkin (84) found that there is a parallelism between the

disappearance of glucose and the in-vitro reduction of methemoglobin, in preparations which contained up to 50 percent of the hemoglobin, in the oxidised form as methemoglobin. Cox and Wendel (85) had previously shown that in the erythrocyte of the dog, both in vivo and in vitro, methemoglobin disappears by reduction, at a rapid rate, but they could not establish any relationship between the reduction and the concentration of glucose in blood.

Gibson (86) showed that the reduction of methemoglobin could be effected in the presence of lactate or glucose. The ratio of pyruvate accumulated:methemoglobin reduced was about one half.

3) Lactate + 2HbOH $\longrightarrow$ Pyruvate + 2Hb

4) Glucose + 4HbOH $\longrightarrow$ 2 Pyruvate + 4Hb

The action of lactate is straight-forward as indicated in equation 3: lactate is acted upon by lactic dehydrogenase, and the hydrogens removed can be utilised for the reduction of the methemoglobin. With glucose, on the other hand, there appear to be at least three possible mechanisms. Two possibilities may arise from glycolysis as indicated below:

5) (a) Glucose $\longrightarrow$ 2 Lactate

(b) 2 Lactate + 4HbOH $\longrightarrow$ 2 Pyruvate + 4Hb

6) (a) Glucose + 4HbOH $\longrightarrow$ 2(1, 3-DPG) + 4Hb

(b) 2(1, 3-DPG) $\longrightarrow$ 2 Pyruvate

The third possibility involves the hexose monophosphate shunt, coupled with TPN.

Studies with inhibitors have been carried out by Gibson (86). Blood containing added glucose and fluoride, the latter to inhibit enolase and thus block the glycolytic system by preventing the formation of hydrogen acceptors to couple with triosephosphate dehydrogenase, was found to utilise glucose provided methemoglobin were present to serve as acceptor. Similarly the reduction of methemoglobin using lactate to provide the hydrogen, was not inhibited. When iodoacetate was added to blood containing glucose, there was a 90 percent inhibition of methemoglobin reduction, since in this case the triosephosphate dehydrogenase was inhibited. However, reduction could still proceed in the presence of lactate. Glucose has also been found (88) to increase the rate of reduction of methemoglobin in the presence of an excess of lactate. These observations suggest that two mechanisms exist for the conversion of methemoglobin to hemoglobin, one for glucose and one for lactate. From these observations it appears unlikely that the oxidative steps of the hexose monophosphate shunt are directly involved in the process.

According to Gibson (86), TPNH will bring about the reduction of methemoglobin only in the presence of methylene blue, but Kiese (87) and Francoeur in our laboratory (59), found that TPNH can react with methemoglobin through an intermediate step mediated possibly by the flavoprotein methemoglobin reductase. Similarly, with DPNH as the hydrogen donor, a flavoprotein appears to be required as an intermediate, since

the reaction of DPNH and methemoglobin, directly, is very slow (59).

It appears that the system normally employed for the reduction of methemoglobin by the red cell involves DPN rather than TPN, and anything which interferes with the production of DPNH will also interfere with the reduction of methemoglobin. Such is the case with malonate and pyruvate, both of which inhibit lactic dehydrogenase. Fumarate and malate, which can promote the reduction of DPN, on the other hand, can act also as donors for the reduction of methemoglobin (88) according to the following reactions:

7) Fumarate + H_2O $\overset{\text{fumarase}}{\rightleftharpoons}$ Malate

8) Malate + DPN^+ $\overset{\text{malic dehydrogenase}}{\rightleftharpoons}$ Oxalacetate + DPNH + H^+

Hemoglobin is said (80) to undergo spontaneous oxidation to methemoglobin. This process involves the uptake of some oxygen according to equation 9:

9) 2 Hb + 1/2 O_2 + H_2O $\longrightarrow$ 2 HbOH

Hence, the erythrocyte is potentially capable of some oxygen consumption, but this process is not to be considered as "respiration".

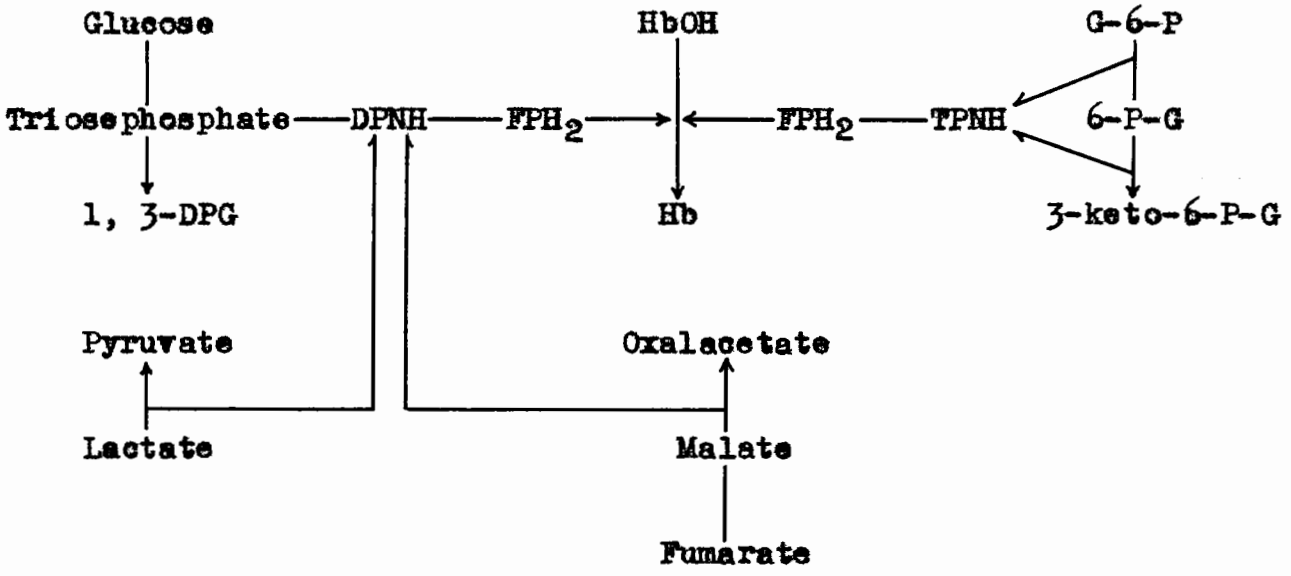
According to Lemberg and Legge (81) in reference to the hemoglobin-methemoglobin system, the presence of a complete oxidative cycle would lead to a ". . . wasteful and useless oxidation of large quantities of glucose . . . ." If, however,

methemoglobin is formed spontaneously, it is essential, if the cell is to fulfil its obligations as an oxygen carrier, that some system exist for the maintenance of the hemoglobin in its reduced state.

The reactions involved as postulated, or demonstrated, may be summarised, after Francoeur (59), as shown in Figure 4.

FIGURE 4

Mechanisms for the Reduction of Methemoglobin in the Erythrocyte



III

THE TRICARBOXYLIC ACID CYCLE

a) Introduction

This metabolic cycle is variously known as the "Krebs cycle", "citric acid cycle", or "tricarboxylic acid cycle". Numerous excellent reviews are available and continue to appear on the subject in annual issues of Recent Advances in Enzymology, Annual Reviews of Biochemistry, and other journals.

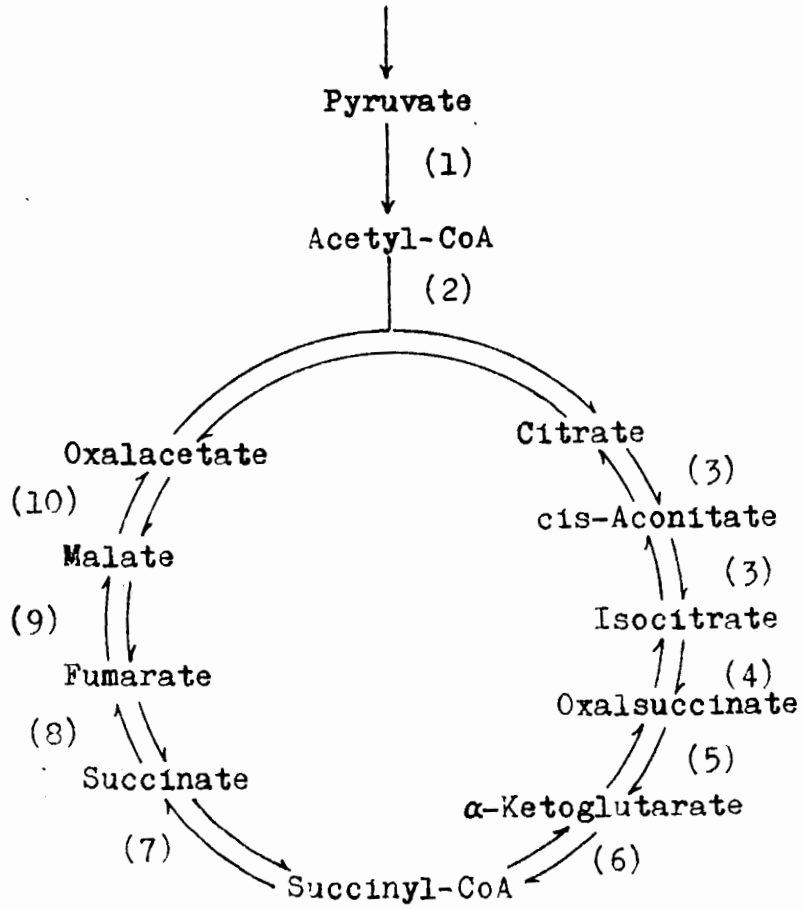
The tricarboxylic acid cycle is the major pathway of the oxidation of the metabolites of foodstuffs, the ultimate end products of which oxidations are CO_2 and H_2O. Notwithstanding the intensive investigations carried out in the past two decades, the original concepts proposed by Krebs and Johnson in 1937 (89), have remained almost unchanged, although many amplifications have been added. Schneider and Potter (90), and Kennedy and Lehninger (91), showed that the enzymes of the cycle act as a unit rather than as separate entities, and they are confined, in their cytological distribution, to the mitochondria. Later their results were confirmed in Green's laboratory, by Harman (92).

The enzymes of the cycle are indicated in Figure 5.

In discussing the various enzymes, it is convenient to group them according to their similarity of function, or close association.

FIGURE 5

The Tricarboxylic Acid Cycle.



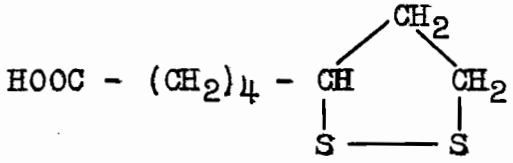
1. Pyruvic oxidase
2. Condensing enzyme
3. Aconitase
4. Isocitric dehydrogenase
5. Oxalsuccinic carboxylase
6. α-Ketoglutaric oxidase
7. Succinyl-CoA deacylase or P-enzyme
8. Succinic dehydrogenase
9. Fumarase
10. Malic dehydrogenase

b) Pyruvic and α-Ketoglutaric Oxidases

(i) Dehydrogenases

These enzymes are here considered together because, with only a few exceptions, the mechanism of action of the two systems is now believed to be the same (93-96). They catalyse the dehydrogenation and decarboxylation of their respective substrates, with the eventual formation of an acyl-CoA complex. The reactions require DPN, Mg^{++}, thiamine pyrophosphate, and CoA as cofactors.

In 1948, O'Kane and Gunsalus (97), found that another cofactor was needed for the oxidation of pyruvate by Strep. faecalis. This factor later was isolated from liver by Reed et al. (98), crystallised and identified as α-lipoic acid:



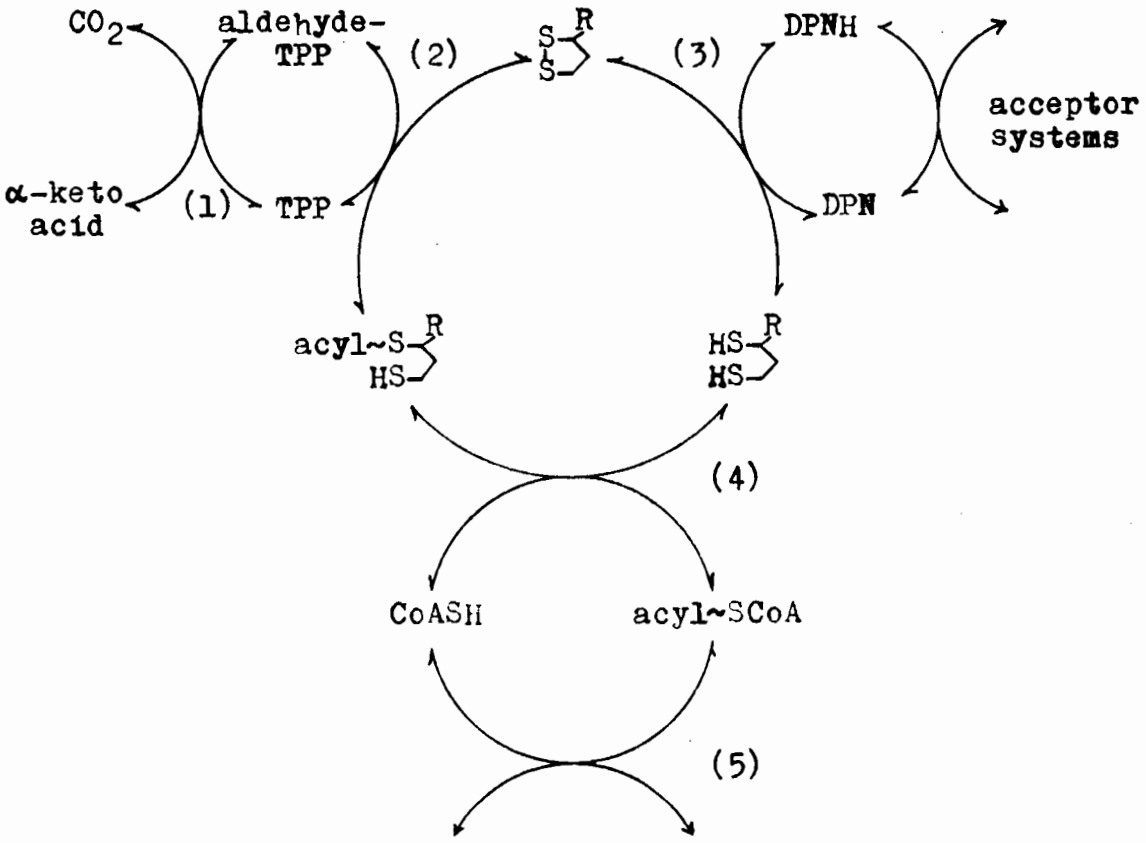
α-Lipoic acid has been found to be associated with the pyruvic and α-ketoglutaric oxidases of bacteria (97), and also has been prepared in purified form from animal tissues (99, 100).

A scheme indicating the role of TPP and of α-lipoic acid as cofactors in α-ketoglutaric oxidation is shown in Figure 6, from Gunsalus (101).

The initial reaction in the system is catalysed by a carboxylase and effects the removal of CO_2. All that is known

FIGURE 6

Transfer Reactions of Lipoic Acid. (101).



1. Carboxylase
2. TPP-lipoic transacylase
3. Lipoic dehydrogenase
4. Lipoic transacylase
5. Acyl transfer systems

about this step is that TPP is required along with a divalent cation of which Mg^{++} is the most effective. Seaman (102), carried out an anaerobic incubation of α-lipoic-acid-free preparations of pyruvic oxidase from Tetrahymena pyriformis S., with pyruvate, $C^{14}O_2$, and TPP. He found that C^{14} was incorporated into pyruvate to a greater extent in the absence of α-lipoic acid, thus demonstrating that the carboxylase catalysed reaction is reversible and independent of α-lipoic acid.

The next reaction is presumed (101) to be a transfer of an "aldehyde" group from an aldehyde-TPP complex (hypothetical) to α-lipoic acid, with the splitting of the S-S linkage and the reduction of one of the sulphur atoms to a thiol, and combination of the other one with an acyl group. This reaction is supposed to be catalysed by a TPP-lipoic transacylase system, the existence of which has not yet been proved.

Evidence for the involvement of lipoic transacetylase and lipoic dehydrogenase in the pyruvic oxidase system was obtained by separating the two enzymes and testing them with the aid of single-step assays, linked with suitable systems (101).

Lipoic transacetylase has been measured by linking it with phosphotransacetylase in the following manner:

10) $\text{Acetyl}\sim\text{P} + \text{CoASH} \rightleftharpoons \text{Acetyl}\sim\text{SCoA} + P_i$

11) $\text{Acetyl}\sim\text{SCoA} + \text{Lip(SH)}_2 \rightleftharpoons \text{Acetyl}\sim\text{SLipSH} + \text{CoASH}$

The first reaction was measured by estimating the hydroxamic acid formed in the presence of hydroxylamine (103):

12) $RSCOCH_3 + NH_2OH \longrightarrow RSH + CH_3C(=NOH)$

and the overall reaction was measured by following the disappearance of thiol groups.

The lipoic dehydrogenase can be followed by linking it to lactic dehydrogenase:

13) $Lip(SH)_2 + DPN^+ \rightleftharpoons LipS_2 + DPNH + H^+$

14) $DPNH + H^+ + Pyruvate \rightleftharpoons Lactate + DPN^+$

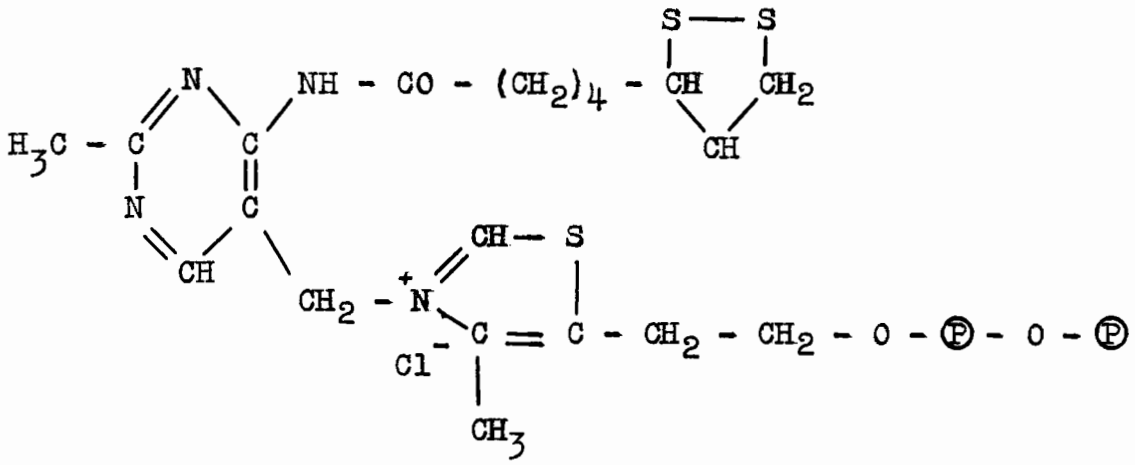
The reversibility of the reaction can be demonstrated by measuring either the oxidation or the reduction of the pyridine nucleotide, spectrophotometrically.

Little as yet is known about the α-ketoglutaric oxidase, but it is presumed to have the same mechanism of action as pyruvic oxidase. Evidence for this view has been presented by Hager and Gunsalus (104), who in the course of fractionation of α-ketoglutaric oxidase from E. coli, obtained a lipoic dehydrogenase identical with that of pyruvic oxidase.

The three reactions involving α-lipoic acid, namely the reduction and acylation of the disulphide group, the transacylation to CoASH, and the dehydrogenation of the thiols to reform the disulphide, are reversible (101). The carboxylase, for the pyruvic oxidase at least, also is reversible (102).

Reed and DeBusk (105), working with a mutant of E. coli grown in synthetic media, found that these organisms

could use neither TPP nor α-lipoic acid for growth, and that pyruvic oxidase and α-ketoglutaric oxidase could be reactivated by a compound formed from both TPP and α-lipoic acid (106). They called the substance, lipothiamide pyrophosphate (LTPP). The structure proposed for the compound is as follows:



The lack of response of the mutant to TPP and α-lipoic acid, has been ascribed to the absence of a lipoic acid conjugase, the enzyme presumably required to synthesise LTPP (107).

How these results can be reconciled with the postulated mechanisms of Gunsalus (101), Hager and Gunsalus (104), and Seaman (102), is difficult to tell at the present time. There may be a connection between the conjugase of Reed and Debusk (107) and the TPP-lipoic transacylase of Gunsalus (101).

The end product of this first series of reactions is an acyl-CoA complex. Acetyl-CoA is obtained from the pyruvate as succinyl-CoA from α-ketoglutarate. The fate of these compounds as far as the tricarboxylic acid cycle is concerned is discussed in the following section.

(ii) Deacylating Systems

(1) Condensing Enzyme

Among the many reactions involving acetyl - CoA, one of immediate interest is the condensation of the acetyl group from the breakdown of carbohydrate, with oxalacetate, to form citrate. Much of the early work in this field, including the isolation, purification, and partial determination of the structure of CoA was done by Lipmann and his group (108, 109), and by Snell et al. (110). The identity of the "active acetate" fragment became known with the isolation of acetyl - CoA from yeast, by Lynen et al. (111, 112).

The final proof as to the nature and function of the active acetate was provided by the work of Ochoa and his collaborators. Soluble enzyme preparations from animal tissues were found to be capable of forming citrate when provided with acetyl - phosphate, CoA and bacterial transacetylase (113), and oxalacetate, according to the following reactions:

15) $\text{Acetyl} \sim \text{P} + \text{CoASH} \rightleftharpoons \text{Acetyl} \sim \text{SCoA} + \text{P}_i$ (C.kluyveri transacetylase)

16) $\text{Acetyl} \sim \text{SCoA} + \text{Oxalacetate} \rightleftharpoons \text{Citrate} + \text{CoASH}$ (condensing enzyme)

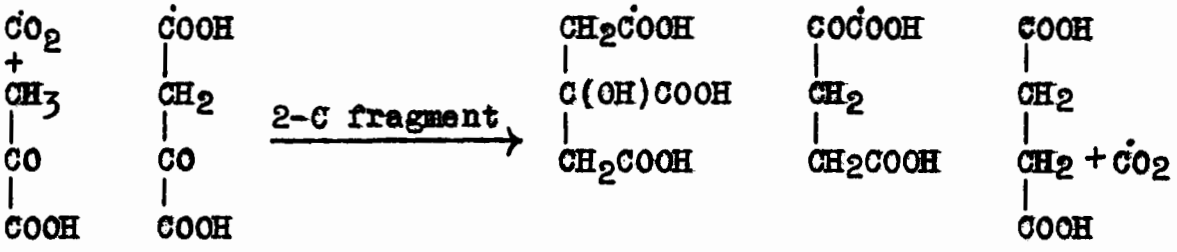
The crystallization of the condensing enzyme from pig heart (116) and the isolation of citrate confirmed the theory that this was in fact the product of the condensation (116). The reversibility of the reaction was demonstrated by coupling the condensation reaction with malic dehydrogenase,

as shown in reactions 17 and 18', and measuring either the reduction of DPN or the oxidation of DPNH, spectrophotometrically (117).

17) Malate+$DPN^+ \rightleftharpoons$ oxalacetate+DPNH + H^+ (malic dehydrogenase)

18') Oxalacetate+Acetyl $\sim$ SCoA $\rightleftharpoons$ Citrate + CoASH (condensing enzyme)

With reference to reaction 18', i.e. the condensation, the acetyl fragment from acetyl - CoA, condenses to form asymmetrically labelled citrate. Wood (118) showed that oxalacetate labelled with C^{13} by $C^{13}O_2$ fixation with pyruvate, gave rise to α-ketoglutarate labelled in the carboxyl next to the carbonyl group. These results were interpreted as being evidence against the view that citrate, which is a symmetrical molecule, is an intermediate in the cycle. Instead, it was thought that cis-aconitate was the first tricarboxylic acid to be formed, and that citrate was formed as a side reaction product. Ogston (119) proposed that an asymmetric enzyme might be able to distinguish between the identical groups of a symmetrical compound, by a three point attachment of enzyme and substrate. Potter and Heidelberger (120) confirmed Ogston's hypothesis by means of $C^{14}O_2$ fixation and production of succinate in the following reactions:



Later, the successful isolation of citrate from reactions with the purified preparations settled the dispute (116).

(2) Enzymes which Hydrolyse Succinyl - CoA

The observation that, in crude systems, the oxidation of α-ketoglutarate occurs without the intermediation of acceptors for the acyl part of the complex (121), led to the assumption that there was an enzyme catalysing the hydrolysis of succinyl - CoA to succinate and CoASH. Gergely et al. (122) succeeded in purifying such an enzyme, and they named it succinyl - CoA deacylase.

In the presence of a suitable acceptor, the hydrolysis of succinyl - CoA produces the esterification of orthophosphate, and the same enzyme produces the formation of inorganic phosphate from ATP, provided CoASH and succinate also are present. The reaction was believed to proceed as follows:

19) $\text{Succinyl} \sim \text{CoA} + \text{ADP} + P_i \rightleftharpoons \text{Succinate} + \text{CoASH} + \text{ATP}$

Kaufman et al. (95) have purified both the deacylase and the phosphorylating enzyme (designated "P"), which catalyses reaction 19.

The phosphate acceptor has been found to be guanosine diphosphate (124) and not ADP. The reaction with ADP would be explained by assuming the mediation of a nucleoside diphosphokinase (125) as the catalyst:

20) ATP + GDP $\rightleftharpoons$ ADP + GTP

Thus the presence in the preparation, of guanosine nucleotides in small amounts, would suffice to permit the P-enzyme to operate with the accumulation of ATP, from succinyl - CoA and ADP.

When purified systems containing the α-ketoglutaric "dehydrogenase" are used along with catalytic amounts of CoASH and α-ketoglutarate, no reaction occurs, since the cycle cannot operate owing to tying up of all the CoASH. However, on adding purified deacylase (122, 123), the reaction proceeds, and can be followed by the measurement of DPN reduction. The deacylase could be substituted by the P-enzyme, provided ADP, inorganic phosphate, and Mg^{++}, also were present (123). The formation of ATP was demonstrated to accompany the reaction, by coupling with hexokinase in the presence of glucose, and estimating the amount of glucose-6-phosphate formed.

The reversibility of the reaction catalysed by the P-enzyme was suggested by the experimental results (123) which showed that when CoASH is present in catalytic amounts along with the P-enzyme, Mg^{++}, ATP, and succinate, liberation of inorganic phosphate does not occur in significant amounts, unless the deacylase, or hydroxylamine is present. The reactions proceed as shown below:

21) Succinate + ATP + CoASH $\xrightarrow[\text{P-enzyme}]{Mg^{++}}$ Succinyl~SCoA + ADP + P_i

22) Succinyl~SCoA $\xrightarrow{\text{Deacylase}}$ Succinate + CoASH

Succinyl~SCoA $\xrightarrow{NH_2OH}$ $COOHCH_2CH_2C(=NHOH)=O$ + CoASH

In this manner the CoASH is set free and is available for recycling.

Because of the great difference between the free energy of the succinyl - CoA and of succinate, the deacylase, for thermodynamic reasons, is not freely reversible.

In a complete system, both the action of the P-enzyme and of the deacylase can occur. Hence the ratio of inorganic phosphate esterified, to CO_2 output, does not reach the theoretical value of unity, the maximum expected for the substrate-level phosphorylation during the oxidation of α-ketoglutarate to succinate. That the ratio is less than unity arises from the fact that some of the succinyl-CoA is split by the deacylase without the retention of any of the energy of the thio bond.

c) Dehydrogenases

(i) Succinic Dehydrogenase

Succinic dehydrogenase forms part of a closely integrated system of enzymes known as the succinoxidase system,

which requires no pyridine nucleotide carrier for hydrogen and electrons, but utilises the cytochrome system (126). The overall reaction brought about by this system is:

23) Succinate + 1/2 O_2 $\longrightarrow$ Fumarate + H_2O (127)

The reaction catalysed by the dehydrogenase itself is

24) Succinate $\rightleftharpoons$ fumarate + $2H^+$ + 2e

Quastel and Whetham (129) showed that this reaction is reversible.

The succinoxidase activity is associated with the mitochondria (129, 130), and Hogeboom (129) found that the dehydrogenase was very tightly bound in the particulate fraction, but that it could be solubilised. The yields, however, after solubilization, amounted to only 10 percent of the original activity.

Singer and Kearney (131), on the other hand, recently have solubilised succinic dehydrogenase from beef heart mitochondrial preparations with the aid of TRIS buffers. They obtained a recovery of activity of 96 percent. Furthermore, they partially purified the material and achieved a 75-fold increase in the specific activity.

The purest preparations of succinic dehydrogenase contained no iron-porphyrin component, although iron was found to be present in significant amounts and in very tightly bound form (132) α-Lipoic acid also was found to be present in the preparation, which on ultracentrifugal analysis (132), was found

to be almost pure. However, it is possible that the lipoic acid was present as a contaminant. On the other hand, if α-lipoic acid is an integral part of the succinic dehydrogenase system, its function may possibly be analogous to that in the pyruvic and α-ketoglutaric oxidases. A lipoic dehydrogenase linked to a flavoprotein rather than to a pyridine nucleotide has been found in Clostridium butyricum (101).

Cooper et al. (133) recently have reported the isolation of another factor required for succinate oxidation. It is active only in the thiol (reduced) form, and has tentatively been identified as desamino-CoA.

The inhibitability of the succinate oxidation by oxalacetate has been known for several years (134). The reaction is inhibited also by DPN, due, according to Mann and Quastel (136), to the formation of oxalacetate, presumably by way of the reactions catalysed by fumarase and malic dehydrogenase. The strong activation of succinoxidase activity by Ca^{++}, as demonstrated by Axelrod et al. (137), was later shown to be due to the influence of the Ca^{++} in promoting the breakdown of DPN, thus preventing the formation of oxalacetate. This is further indicated by the inability of Ca^{++} to increase succinate oxidation in the presence of nicotinamide, which protects DPN, and also in the presence of oxalacetate (137).

The inhibition by oxalacetate can be nullified by the addition of ATP (138). Pardee and Potter (138) suggested that if the ATP could form a compound such as phosphoenoloxal-

acetate from oxalacetate, the configuration of the inhibitor would be sufficiently changed as to make it lose its affinity for the succinoxidase system. Utter and Kurahashi (139) have demonstrated that partially purified oxalacetic carboxylase will catalyse the following reaction:

25) CO_2 + phosphoenolpyruvate + IDP $\rightleftharpoons$ Oxalacetate + ITP

ADP and ATP also were found to be used, although they proved to be less active than the inosine nucleotides (139). The action of the adenine nucleotides may be due to the presence of small amounts of inosine nucleotide contaminants, which, with the mediation of the nucleoside diphosphokinase (125) could maintain a supply of the inosine nucleotides for reaction 25, by the following reaction:

26) ATP + IDP $\rightleftharpoons$ ADP + ITP

The inhibition of succinic dehydrogenase by malonate, was described by Quastel and Wheatley (177), and has been of great importance, since it provided a basis for the theories of competitive inhibition.

(ii) Malic Dehydrogenase

This enzyme, discovered by Batelli and Stern (140), catalyses the reaction:

27) Malate + DPN^+ $\rightleftharpoons$ Oxalacetate + DPNH + H^+

The enzyme, which is DPN-linked, can also function with TPN, but the rate in the latter case is less than 5 percent of that with DPN (141).

The only substrates used by this dehydrogenase are malate and oxalacetate, and the equilibrium is far towards the formation of malate:

$$\frac{[\text{oxalacetate}]\ [\text{DPNH}]\ [H^+]}{[\text{malate}]\ \ [\text{DPN}^+]} = 7.5 \times 10^{-13} \quad (142).$$

(iii) Isocitric Dehydrogenase and Oxalsuccinic Carboxylase

These two enzymes may logically be considered together because they are always found together, and as yet have not been separated.

They catalyse the overall reaction:

28) isocitrate + $TPN^+ \xrightleftharpoons{Mn^{++}}$ α-ketoglutarate + TPNH + H^+ + CO_2

Ochoa (143, 144) demonstrated that the above reaction actually involves two independent steps:

29) isocitrate + $TPN^+ \rightleftharpoons$ oxalsuccinate + TPNH + H^+ (dehydrogenase)

30) oxalsuccinate $\xrightleftharpoons{Mn^{++}}$ α-ketoglutarate + CO_2 (carboxylase)

Both reactions are reversible. The dehydrogenase can be studied alone if Mn^{++} is absent from the system, while the decarboxylase may be studied with Mn^{++} present, since this ion (or Mg^{++}) is essential for the carboxylase (147). Ochoa (145) pointed out

that oxalsuccinate is spontaneously decarboxylated at a considerable rate, in aqueous solutions, and also that the rate of decarboxylation is increased by the addition of polyvalent cations exclusive of Mg^{++} and Mn^{++} which, in the absence of the enzyme, are without effect.

Grafflin and Ochoa (146) have obtained a partial purification of the two enzymes from pig heart. The degree of purification was the same for both enzymes, a circumstance which gave rise to doubt as to whether two enzymes are involved. However, the inhibition of the decarboxylation of oxalsuccinate by isocitrate was demonstrated both manometrically (147), and spectrophotometrically (146). In the latter case the inhibition was complete with 3.5×10^{-5} M dl-isocitrate (146). The inference drawn from these results contrary to the results of attempts at purification of the enzymes was that two enzymes were involved. It is possible that the two enzymes act as one structural unit (148).

Both DPN and TPN specific isocitric dehydrogenases have been isolated from yeast (149), and bacteria (150). A DPN specific dehydrogenase also has been demonstrated in animal tissues (151). With bacteria (150) and yeast (149), AMP and Mg^{++} are required for activity, but AMP apparently, is not essential in the case of the enzyme in animal tissues.

The DPN specific dehydrogenase of yeast (149) does not react with oxalsuccinate to produce α-ketoglutarate and

CO_2, nor does it cause the reduction of oxalsuccinate by DPNH to yield isocitrate. The reversal of the following reaction:

31) isocitrate + DPN^+ $\rightleftharpoons$ α-ketoglutarate + DPNH + H^+

could not be demonstrated, although from left to right, the reaction proceeds with the stoichiometric formation of α-ketoglutarate and DPNH.

Isocitric dehydrogenase is an exception among the various enzymes of the citric acid cycle, in that more than 80 percent of its activity is found in the cytoplasm, while only 12 percent of the activity could be recovered in the mitochondria (152).

d) Hydrases

(i) Aconitase

The conversion of citrate to isocitrate by way of cis-aconitate was first described by Martius (153). The reactions involved are as follows:

32) citrate $\overset{\text{citrase}}{\rightleftharpoons}$ cis-aconitate $\overset{\text{isocitrase}}{\rightleftharpoons}$ isocitrate

In other words, aconitase catalyses the addition of the elements of water to cis-aconitate to form either citrate or isocitrate. Whether the reactions involved are catalysed by one or two enzymes is not known.

Jacobsohn and Tapadinhas (154) suggested that two enzymes are involved, while Buchan and Anfinsen (155) obtained partial purification of aconitase without any change in the ratio of citrase to isocitrase activity. Similarly, Morrison (156) prepared electrophoretically homogeneous preparations which were estimated to be 75-80 percent pure, and had full aconitase activity. Racker, on the other hand, although obtaining no separation of the two type of enzymatic activity, obtained changes in the ratio of isocitrase to citrase, with various degrees of purification of aconitase (157). The above ratio in heart muscle, increased from 2.1 to 7.5, with increasing purification. Yeast aconitase exhibited a similar behaviour.

Dickman and Cloutier (158, 159) reported that ferrous ions along with cysteine, ascorbate, or glutathione, stabilise the aconitase in crude preparations, and restore the activity lost during dialysis. Addition of Fe^{++} restores the activity lost on treatment with o-phenanthroline, or α, α - bipyridyl, which are strong Fe^{++} complexing agents (159). No other cation tested produced reactivation. From polarographic, spectrophotometric, and titrometric data, Dickman and Cloutier (159) inferred that a complex forms, both with the substrate and with the enzyme. They suggest that the complexing agent is the ferrous ion. One purpose of the reducing agent could be to maintain the iron in the ferrous form, but the reducing agent seems also to have an effect on the enzyme (159, 160)

and there is evidence that both one Fe^{++} ion and one molecule of reducing agent react with each active site on the enzyme (160).

The distribution of aconitase in cells has been studied by Dickman and Speyer (161) who found the aconitase in the cytoplasm to have a pH optimum of 7.3, while that of mitochondria has optima at 5.7 and 7.3. The intensity of activity at the two pH values varied; thus at pH 7.3, 85 percent of the activity was found in the soluble fraction, while at pH 5.8 only 40 percent of the activity was in the cytoplasm.

Krebs, studying the kinetics of the aconitase reaction, found that at pH 7.4 and 25°C., the ratio of isocitrate: cis-aconitate:citrate is 6.2:2.9:90.9 (162).

(ii) Fumarase

This enzyme was first described by Batelli and Stern (163). It catalyses the reversible hydration of fumarate to l-malate, and occurs in many plant and animal tissues, and in bacteria. The enzyme has been purified and crystallised by Massey (164) from pig-heart muscle. The crystalline protein was found to be electrophoretically homogeneous, and these observations were corroborated by sedimentation and diffusion measurements in the ultracentrifuge, in analyses performed by Cecil and Ogston (165) who found the purity to be 96 percent and the molecular weight approximately 200,000.

The enzyme acts only on fumarate and malate among the compounds tried (164); the turnover number for the reaction from fumarate to malate at pH 7.3 and 20°C. is more than 100,000 (164). At equilibrium, at pH 7.4 and 25°C., the ratio of malate to fumarate is 4.42 (162). At pH levels below 5.5, the ratio increases rapidly, reaching a maximum of about 65 at pH 1.

e) Electron Transport

The transport of electrons as it is believed to be involved in the tricarboxylic acid cycle is shown in Figure 7.

The energy released during the breakdown of the substrates from carbohydrate metabolism, to CO_2 and H_2O, is conserved in the form of high-energy phosphate compounds, produced by the esterification of orthophosphate into nucleotide pyrophosphates. The most prominent of these, to our present knowledge is ATP.

The esterification of inorganic phosphate can take place at the substrate level as occurs with the P-enzyme system, or it can take place at the electron-to-oxygen transfer level. A scheme depicting the phosphorylations that occur in the course of carbohydrate metabolism is shown in Figure 8. The theoretical values for the number of phosphorylations taking place during the glycolysis of glucose, and in the tricarboxylic acid

FIGURE 7

Electron Transport Mechanisms Associated with the Tricarboxylic Acid Cycle (166)

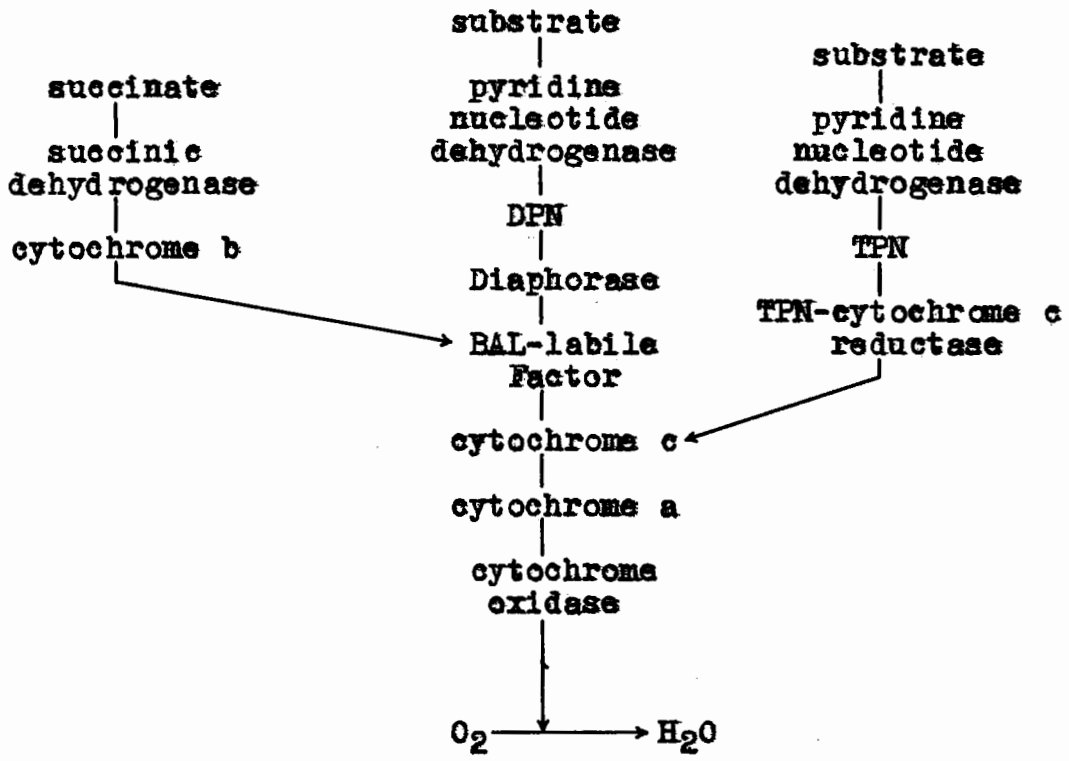
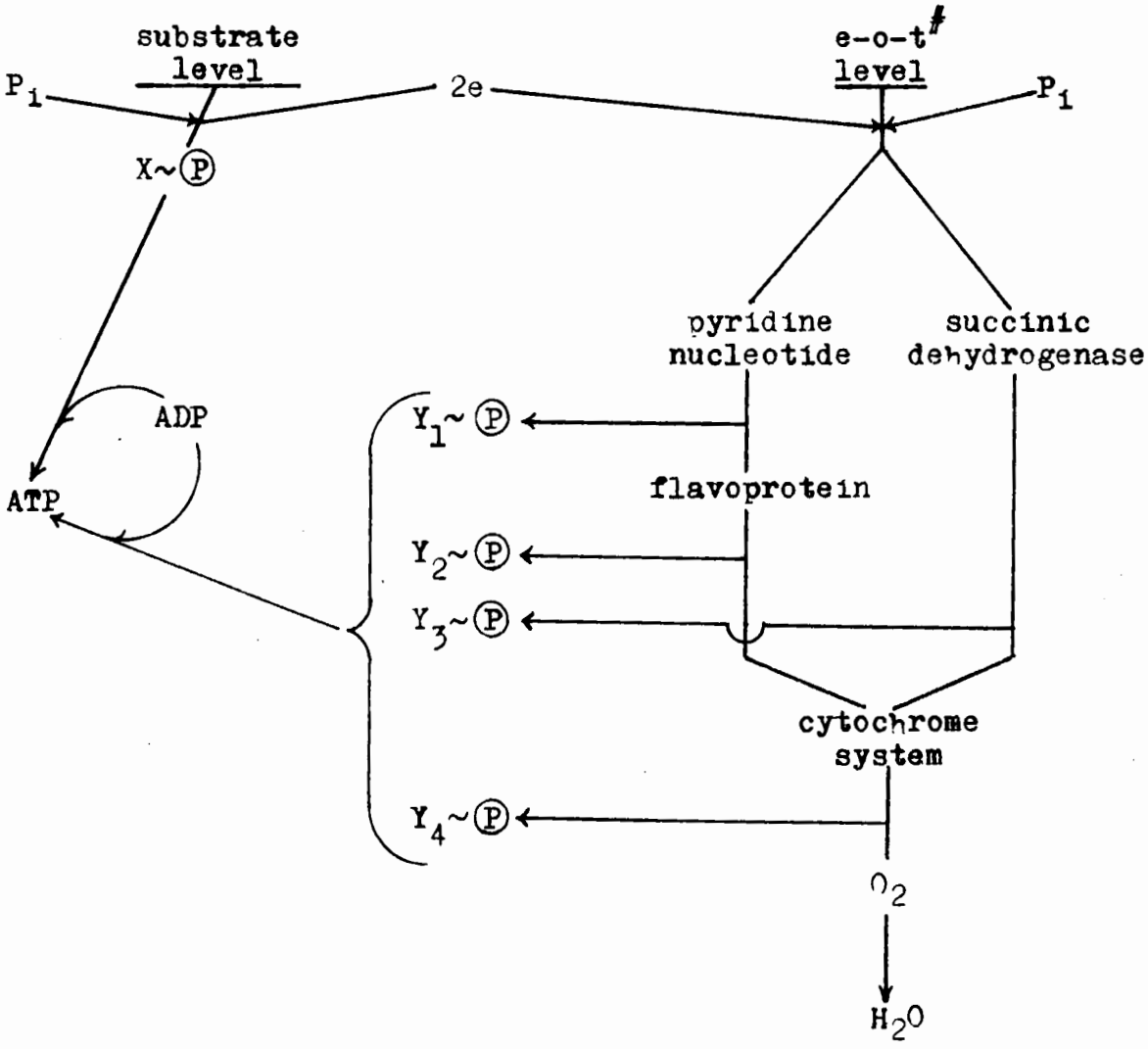


FIGURE 8

Hypothetical Scheme for the Phosphorylations Associated with Carbohydrate Metabolism. (69).



X, Y — hypothetical phosphorylated intermediates

electron to oxygen transfer

cycle as shown in Table I.

The total number of molecules of inorganic phosphate esterified per molecule of glucose completely oxidised to CO_2 and H_2O, is 40, and since two molecules at ATP are used in the initial steps of glycolysis, when glucose is the substrate, the net gain is of 38 molecules of high energy phosphate.

To gain an understanding of the metabolic changes that occur during the maturation of the red cell, the author in the present investigation studied the progressive changes in activity of numerous enzymes in blood specimens containing a high proportion of reticulocytes. In the study, attention was given mainly to the activity of enzymes involved in the energy of the cell.

TABLE I

Phosphorylations per Molecule of Glucose Oxidised (69)

	Level	Enzyme System	P esterified
1a	substrate	3-phosphoglyceraldehyde + DPN^+ ⟶ 3-PG + DPNH + H^+	1
b	e-o-t	DPNH + H^+ + 1/2 O_2 ⟶ DPN^+ + H_2O	3
2	substrate	phosphoenolpyruvate + H_2O ⟶ pyruvate	1
3a	substrate	pyruvate + CoA + DPN^+ ⟶ acetyl-CoA + DPNH + H^+ + CO_2	0
b	substrate	acetyl-CoA + oxalacetate ⟶ citrate + CoA	0
c	e-o-t	DPNH + H^+ + 1/2 O_2 ⟶ DPN^+ + H_2O	3
4a	substrate	isocitrate + TPN^+ ⟶ oxalsuccinate + TPNH + H^+	0
b	e-o-t	TPNH + H^+ + 1/2 O_2 ⟶ TPN^+ + H_2O	3
5a	substrate	α-ketoglutarate + CoA + DPN^+ ⟶ succinyl-CoA + DPNH + H^+ + CO_2	0
b	substrate	succinyl-CoA ⟶ succinate + CoA	1
c	e-o-t	DPNH + H^+ + 1/2 O_2 ⟶ DPN^+ + H_2O	3
6	e-o-t	succinate + 1/2 O_2 ⟶ fumarate + H_2O	2
7a	substrate	malate + DPN^+ ⟶ oxalacetate + DPNH + H^+	0
b	e-o-t	DPNH + H^+ + 1/2 O_2 ⟶ DPN^+ + H_2O	3

EXPERIMENTAL

IV

METHODS

a) Method of Obtaining Reticulocytes

The injection of phenylhydrazine is a commonly used method for causing intravascular hemolysis and increasing the proportion of reticulocytes in the circulation. The degree of stimulation of the bone marrow to increase the production of red cells varies with the severity of the anemia caused by the drug. The output of new cells appears to be so greatly accelerated that a high proportion, if not all, of the cells enter the circulation as reticulocytes (see Figure 2, page 20). Acetylphenylhydrazine is less toxic, and just as effective as phenylhydrazine, and is now more extensively used.

These drugs probably cause the initial formation of highly refractile bodies, known as Heinz bodies, in all the mature erythrocytes in the circulation (30). Warburg et al. (228) showed that treatment of rabbit erythrocytes, in vitro, with phenylhydrazine, gives rise to the formation of a brown colour which is stable in the presence of air. The brown substance apparently is not methemoglobin. On hemolysis of cells treated with phenylhydrazine, denatured globin precipitates out. Warburg and his associates (82) later demonstrated that the Heinz bodies which form in erythrocytes treated with

phenylhydrazine, agree closely in chemical properties with denatured globin. When a large proportion of the globin in the hemoglobin becomes denatured the oxygen carrying properties of the pigment is lost and the cell usually is promptly destroyed and removed from the circulation. The high percentage of reticulocytes in the circulation may be attributable to the accelerated rate of erythrocyte formation, and entrance of the reticulocytes into the circulation before maturation of the cells is complete. An alternate explanation has been offered (30), namely, that acetylphenylhydrazine interferes with the maturation of the reticulocyte.

With repeated doses of the drug, the proportion of reticulocytes in the circulation can be increased to almost 100 percent since these immature cells apparently are not destroyed by the drug (30, 229). Heinz bodies appear in the mature cells after treatment of the animal with acetylphenylhydrazine, in vivo, or the cells, in vitro; it is only very rarely that these bodies appear in the reticulocytes.

This behaviour of the hemoglobin of the mature and immature cells suggests the possibility that different hemoglobins may exist in the two cells. (See Appendix 1).

The reticulocytes used for the experimental work of this thesis were produced in healthy, adult rabbits of New Zealand or American strains, by the subcutaneous administration of six daily doses of one ml. containing 25 mgm acetylphenylhydrazine in 50 percent ethanol. No injection

was given on the 7th. day, and the animal was bled on the 8th. day.

The degree of reticulocytosis produced by this method ranged from 50 percent to over 90 percent of the total cell count. The animals did not appear to suffer from the anemia, having recovered their usual state of well-being within two or three days after being bled.

Sherwood Jones et al. (181) showed that reticulocytes induced by phenylhydrazine and those produced during recovery from Plasmodium berghei malaria, behaved similarly with respect to their metabolic activity. In view of these findings, the assumption that the reticulocytes produced with acetylphenylhydrazine, and used in our experiments, are normal products of erythropoiesis, and are normal in metabolic and other activity, is fully justified.

b) Staining and Counting Reticulocytes

The staining of reticulocytes was accomplished by "the dry method" (233) using brilliant cresyl blue as the dye for the staining of the reticulum. The cells were counterstained with Wright's stain, and about 1000 cells from duplicate preparations were counted in the estimation of the proportion of reticulocytes in the sample.

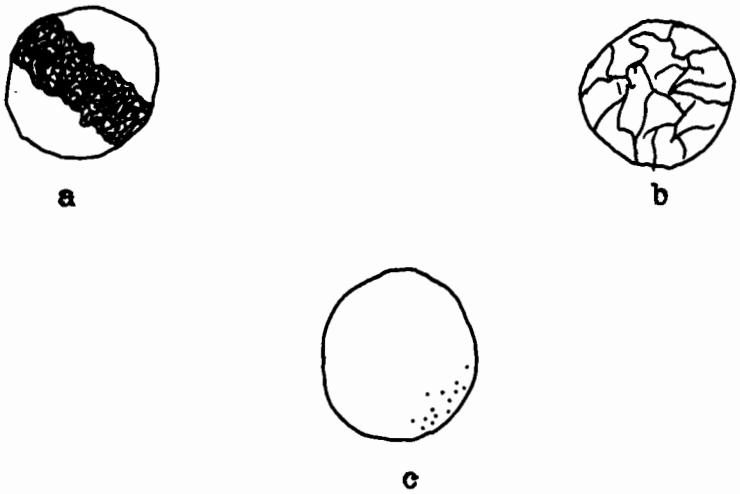
Every cell containing any material stainable by the brilliant cresyl blue was considered to be a reticulocyte.

According to Bessis (21) the value when determined in this manner tends to be low. Bessis compared the values obtained by counting the cells in preparations stained with brilliant cresyl blue, with the aid of the ordinary microscope and with phase-contrast. The values by the latter method were consistently higher. Apparently, with ordinary microscopy some of the reticulocytes may escape detection.

The appearance of the stained reticulocytes may differ greatly from cell to cell. The stain may be distributed in a thick band across the cell as illustrated in Figure 9a, or it may be dispersed throughout the cell with the appearance of filaments as in Figure 9b. In some of the cells only a few "dots" of stained material, usually grouped together in one area of the cell, are observed, as shown in Figure 9c.

FIGURE 9

Reticulocytes Stained with Brilliant Cresyl Blue



In our studies, no effort was made to differentiate between different stages of reticulocyte development, as would be indicated by the density of the stain.

c) Preparation of Enzymes

Blood was obtained by making an incision in the marginal ear vein of the rabbit, and collecting it into a test tube containing heparin as the anticoagulant. The volume of blood collected seldom exceeded 40 ml. at any one time.

The volume of the collected blood was noted, and the sample was centrifuged in a refrigerated centrifuge, at 2500 r.p.m., for about five minutes. The supernatant plasma was removed by aspiration, and discarded. The "buffy layer", containing the white cells, was also carefully removed. The red cells were then washed four times by repeated suspension in fresh, cold, isotonic (0.154 M) KCl, followed each time by centrifugation and removal of the supernatant fluid which was discarded. After the last washing the volume of the sample was made up to the original value with isotonic KCl, and this preparation was either hemolysed or used as whole cells, depending on the requirement.

Hemolysis of the erythrocytes was produced by freezing at -79°C. in an alcohol-dry ice mixture bath, followed by thawing. This procedure being repeated three times, to ensure complete breakdown of the cells. The preparation obtained in this manner is designated "whole hemolysate".

If necessary, the whole hemolysate was centrifuged at 3000 r.p.m. for ten minutes, to precipitate the solid residues from the cell membrane or "stroma". Thus a fractionation into a soluble, stroma-free hemolysate (SFH) and a "stroma" or particulate fraction[#] was obtained. The perfectly clear stroma-free hemolysate was removed by aspiration and was made up to the same concentration at which it was present in the whole hemolysate, by addition of 0.154 M KCl. The stroma was washed repeatedly, with isotonic KCl, until the supernatant was visibly free from hemoglobin (4 or 5 times were generally sufficient). The volume of this fraction also was made up to the original concentration by the addition of isotonic KCl.

d) Materials

All substrates, coenzymes and inhibitors were obtained commercially, with the exception of isocitrate (barium salt) which was prepared in these laboratories by Dr. S. G.A. Alivisatos, according to the method of Fittig et al. (169). A preparation of crude nucleotides from hog liver, prepared by Dr. Rubinstein, in our laboratory, according to the method of LePage and Mueller (170) was used as a source of TPN.

[#]The assumption was made that the material which settles out on centrifugation of the whole hemolysate of reticulocytes consisted only of stroma. This is based on the findings that the last erythroblastic stages have already lost all microscopically visible particles from the cytoplasm (21). Even the reticulum has been shown to be firmly attached, if not actually a part of the stroma (12-16).

All the pure chemicals referred to above, were used in the form of sodium or potassium salts, their solutions being neutralised to a pH of about 7.4 before being used.

The ordinary chemicals used, were all C.P. or Reagent Grade. Cyanide solutions used for the fixation of keto-acids as the cyanhydrins, were prepared by neutralising a solution of sodium cyanide to a pH of about 7.4 with concentrated HCl[#]. The neutralised solution contains cyanide mostly in the form of HCN. The final cyanide concentration was about 0.64 M. A relatively large concentration of cyanide must be added in the manometric experiments, because of the extensive volatilization and loss of the HCN during the gassing of the vessels.

e) Assay of Enzymatic Activity

(i) Introduction

The methods employed in the enzymatic studies, included manometric, spectrophotometric, and chemical or enzymatic analytical procedures.

The manometric determinations were carried out in a conventional Warburg apparatus at constant temperature of 37.5°C. ± 0.01, the manometers being agitated at 120 oscillations per minute. The spectrophotometric studies were done

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[#]636 mgm NaCN were dissolved in 19 ml. of distilled water, and 1 ml. of concentrated HCl (S.G.:1.18) was added with mixing. The resultant pH was close to 7.5 (190).

with a Beckman Model BU spectrophotometer, equipped with a photomultiplier unit.

(ii) Respiration

The respiratory activity was measured manometrically as uptake or oxygen, the CO_2 produced having been absorbed by KOH solution placed in the center well of the flasks. The exact reaction mixtures for this and other procedures, will be given as footnotes to the Tables of results.

(iii) Cytochrome Oxidase

The activity of this system also was measured in terms of the oxygen consumption in the presence of added cytochrome c. p-Phenylenediamine was used as the reducing agent for cytochrome c (173).

The assay consisted of determining the activity of cytochrome oxidase at three concentrations of the enzyme. The straight line obtained on plotting the activity against the enzyme concentration was extrapolated to zero concentration to obtain the "blank" value which must be subtracted from that of the experimental runs.

(iv) Dehydrogenases

Two simple methods are available for the determination of the dehydrogenases which require a pyridine nucleotide

(DPN or TPN) as coenzyme. One procedure involves the spectrophotometric estimation of the change in concentration of the reduced form of the coenzyme at a wave length of 340 mμ where the absorption of DPNH and TPNH is a maximum. The alternative method is the well known procedure of Quastel and Wheatley (171) in which ferricyanide is used as terminal electron acceptor according to the following reactions:

32) $\text{Substrate.2H} + \begin{matrix}\text{DPN}^+\\ \text{or}\\ \text{TPN}^+\end{matrix} \xrightarrow{\text{dehydrogenase}} \text{substrate} + \begin{matrix}\text{DPNH}\\ \text{or}\\ \text{TPNH}\end{matrix} + \text{H}^+$

33) $\begin{matrix}\text{DPNH}\\ \text{or}\\ \text{TPNH}\end{matrix} + 2\ \text{Fe(CN)}_6^{\equiv} \longrightarrow \begin{matrix}\text{DPN}^+\\ \text{or}\\ \text{TPN}^+\end{matrix} + 2\ \text{Fe(CN)}_6^{\equiv\equiv} + \text{H}^+$

34) $2\ \text{H}^+ + 2\text{HCO}_3^- \longrightarrow 2\text{H}_2\text{CO}_3 \longrightarrow 2\text{H}_2\text{O} + 2\text{CO}_3$

The 2 H^+ ions liberated, react with the bicarbonate buffer with the liberation of 2 molecules of CO_2 gas. The gas output is measured manometrically.

Succinic dehydrogenase also can be measured by the ferricyanide method (171) and can therefore be linked with ferricyanide reduction even though oxidation of succinate does not involve a pyridine nucleotide. The ferricyanide, in this case, may accept the electrons from cytochrome b(226) rather than directly from the dehydrogenase as postulated by Quastel and Wheatley. Singer and Kearney (131), who worked with the purified dehydrogenase, have reported that ferricyanide acts very inefficiently as an electron acceptor at this step.

The lactic, malic, isocitric and succinic dehydrogenases were routinely assayed by the ferricyanide technique. The experiments were carried out at pH 7.4, the pH of the medium being adjusted by means of the bicarbonate concentration as calculated from the chart devised by Umbreit et al. (172). For the first three dehydrogenases, cyanide was added to the flasks to fix the keto acids produced by the dehydrogenation of the respective substrates. This precaution is essential, especially in the case of the lactic and malic dehydrogenases which are very strongly inhibited by the keto acids formed.

The flasks used as "controls" contained the complete reaction mixture used in the experimental flasks except that the substrate was omitted.

Glucose-6-phosphate dehydrogenase, because of its strong activity, could be measured routinely in the spectrophotometer. The spectrophotometric method has an obvious limitation, namely, that in cases when the tissue containing the enzyme is highly coloured, e.g. blood, only very small amounts of the tissue can be used. Unless the enzyme is present in very active form, assays by this method become difficult. The activity of the enzyme was estimated by following the reduction of TPN in the spectrophotometer at a wavelength of 340 mμ, at pH 7.4, in the presence of glucose-6-phosphate. The reaction was generally followed for at least ten minutes with readings every one or two minutes. During this period the rate of the dehydrogenation remained practically constant.

When the above method was applied to lactic dehydrogenase it was not very successful, since the formation of pyruvate inhibited the reaction. Cyanide could not be used to fix the pyruvate in this case, since DPN forms a complex with cyanide which would interfere with the measurement of DPNH concentration (202).

If the pH of the medium was raised to the vicinity of 9 by addition of sodium pyrophosphate, the reaction rate remained constant with time, and the readings were very substantial. Thus in the presence of 0.03 ml. of an SFH preparation from normal erythrocytes, lactate, DPN, and phosphate buffer at pH 7.4, all in a final volume of 3.0 ml., the reduction of DPN reached a standstill within twenty minutes, with a total change in the optical density of less than 0.200. When the reaction was carried out in the presence of 0.03 M $Na_4P_2O_7$ instead of phosphate buffer at pH 7.4, the reduction of DPN was still in progress after thirty minutes, with an optical density change, at this time, of more than 1.000. The reason for the great difference in the optical density in the two systems, is that in an alkaline medium the equilibrium of the reaction is driven towards the reduction of DPN by the removal of the liberated H^+ ions, by means of the alkali.

Malic dehydrogenase, which is less active than lactic dehydrogenase, behaved in the same manner as the lactic dehydrogenase. Typical results of the spectrophotometric measurement

of the activity of lactic and malic dehydrogenases are illustrated in Figure 10, page 82.

Isocitric dehydrogenase was not active enough to be measured on incubation at room temperature, and had to be incubated at 37°C. for significant readings to be obtained.

The use of the spectrophotometric method for assay of dehydrogenases other than glucose-6-phosphate dehydrogenase, was only used in preliminary experiments, and was not very extensively employed.

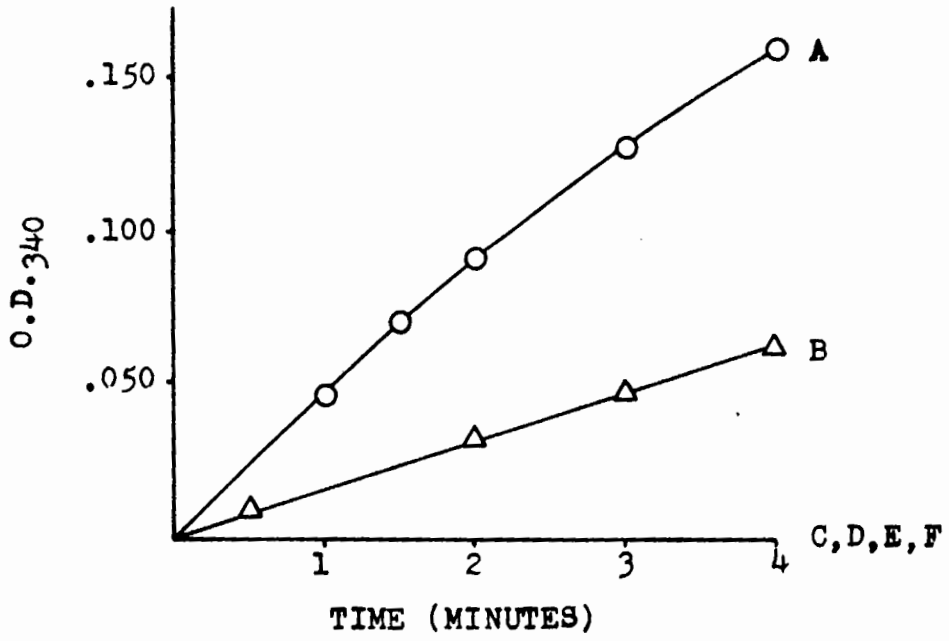
(v) Aconitase and Fumarase

These enzymes were assayed spectrophotometrically by following the increase in the optical density at 240 mμ. The method was that introduced by Racker (157) and depends on the formation of fumarate from malate, or cis-aconitate from citrate (or isocitrate). Both fumarate and cis-aconitate contain a carbon-to-carbon double bond in their structure; the bond gives a maximum absorption at 240 mμ.

Fumarase, in the erythrocyte, is sufficiently active to be measurable by Racker's method. The course of the reaction in our experiments was followed with the spectrophotometer over the period of one hour. Aconitase on the other hand, is much less active and was estimated by Rubinstein's (174) modification of Racker's method. This method consists

FIGURE 10

Spectrophotometric Measurement of Lactic and Malic Dehydrogenases.



Cuvette contents:
A. Lactic dehydrogenase: 0.01 ml. SFH (normal erythrocytes); 0.03M $Na_4P_2O_7$; 0.033M dl-lactate; 0.5 mgm. DPN; volume made up to 3.0 ml. with 0.154M KCl. Blank contained no lactate.
B. Malic dehydrogenase: same as A except that 0.02 ml. enzyme was used and 0.06M l-malate was substituted for the lactate. Blank contained no malate.
C. As in A but omitting the enzyme.
D. As in A but omitting the DPN.
E. As in B but omitting the enzyme.
F. As in B but omitting the DPN.

in incubating relatively large quantities of the enzyme preparation at 37°C., taking aliquots periodically during one hour, deproteinising them by acidification and heating for five minutes in a boiling water bath, and finally, determining the absorption of the filtrate at 240 mμ.

Attempts were made to measure the formation of cis-aconitate by incubating the enzyme and citrate at room temperature in the presence of Fe^{++} and cysteine, as recommended by Morrison and Peters (199), but the effort was unsuccessful. Within a few minutes, in our case, the specimens gave an optical density of infinity, both in the presence and absence of enzyme, presumably due to the formation of a complex of citrate with the Fe^{++} ions (199).

(vi) Hexokinase

This enzyme was assayed by measuring the rate of disappearance of added glucose, from the medium, using the method of Nelson (175).

The enzyme preparation was incubated with phosphate, Mg^{++}, ATP, F^-, glucose and glycyl-glycine buffer as described in the section on results. The F^- was added to inhibit the action of ATPase contained in the cell stroma. A reaction mixture containing the ingredients mentioned, except glucose and ATP, was preincubated at 37°C., since this treatment was

found to favour more consistent results. After the preincubation period, the ATP and glucose were added and an additional incubation treatment of one hour, at 37°C., was permitted. The samples were then cooled rapidly, diluted from an original volume of 1.5 ml., to 11.5 ml., and were then deproteinised by the addition of 2 ml. of 0.3N $Ba(OH)_2$ and 2 ml. of 5 percent $ZnSO_4$. After centrifugation, 2 ml. samples from the supernatant fluid were used for glucose analysis.

(vii) DPNase

This assay depends on the measurement of the amount of DPN remaining in the reaction medium after a specified (15 minutes) period of incubation of the DPN and the enzyme at 37°C. After the incubation, the preparations were deproteinised, either by heating, or by means of 5 percent trichloracetic acid, and the DPN in the supernatant was then determined, by either of two methods. One involved the formation of a DPN-cyanide complex which absorbs maximally at 325 mμ (202); the other, by means of a purified alcohol dehydrogenase and ethanol, in an alkaline medium. The DPN is reduced and measured at 340 mμ (213). The molar extinction coefficient used was 0.63×10^{-7} for both the reduced DPN (213) at 340 mμ, and for the DPN-cyanide complex at 325 mμ (214).

(viii) Pyrophosphatase

This enzyme was measured by means of the Fiske-

Subbarow method for orthophosphate (182). The enzyme was incubated with Mg^{++} and inorganic pyrophosphate, at 37°C., for fifteen minutes, at the end of which time the inorganic orthophosphate liberated was determined.

f) Analytical Determinations

(i) Pyruvate, Lactate, and RNA

These were measured by the methods of Friedemann and Haugen (216), of Barker and Summerson (215), and of Schneider (192) respectively.

(ii) ATP

ATP was measured by making use of its reaction with glucose, in the presence of hexokinase, to form glucose-6-phosphate, which in turn is converted to 6-phosphogluconic acid by the action of glucose-6-phosphate dehydrogenase in the presence of added TPN. By the last reaction the TPN, which is added in excess, is reduced, and this form of the nucleotide can be measured spectrophotometrically at 340 mμ.

The use of erythrocyte SFH as a source of hexokinase and glucose-6-phosphate dehydrogenase proved to be unsatisfactory as the values for the reduced TPN increased to a maximum and then fell to the initial value. Because of this, the dehydrogenase was prepared by the method of Kornberg (217).

In this procedure Brewer's yeast[#] is allowed to autolyse for five hours at 40°C. In our experience it was found that autolysis for at least seventy-two hours was necessary to yield sufficient activity to make the purification of the enzyme worthwhile. Even with this modification, the yield of dehydrogenase was never more than 15 percent as much as Kornberg (217) obtained.

For the ATP assay, crystalline hexokinase (Nutritional Biochemicals) and an excess of 90 percent pure TPN (Sigma Chemicals) were employed. Blood specimens for the assay of ATP were deproteinised by heating in a boiling water bath for five minutes. After centrifugation the supernatant was assayed by Kornberg's method (217).

g) A Spectrophotometric Adaptation of the Ferricyanide Method for the Assay of Dehydrogenases

In 1938 Quastel and Wheatley (171), described their method in which ferricyanide is used as an electron acceptor (reaction 32, et seq., page 78). In the previous year Haas (218) had shown that ferricyanide can oxidise reduced DPN without the mediation of any enzyme or cofactor. Hence the DPN- and presumably also TPN-linked dehydrogenases can be

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[#]The yeast used in our experiments was donated by Dow Brewery, Montreal, and was a bottom yeast used in the manufacture of lager.

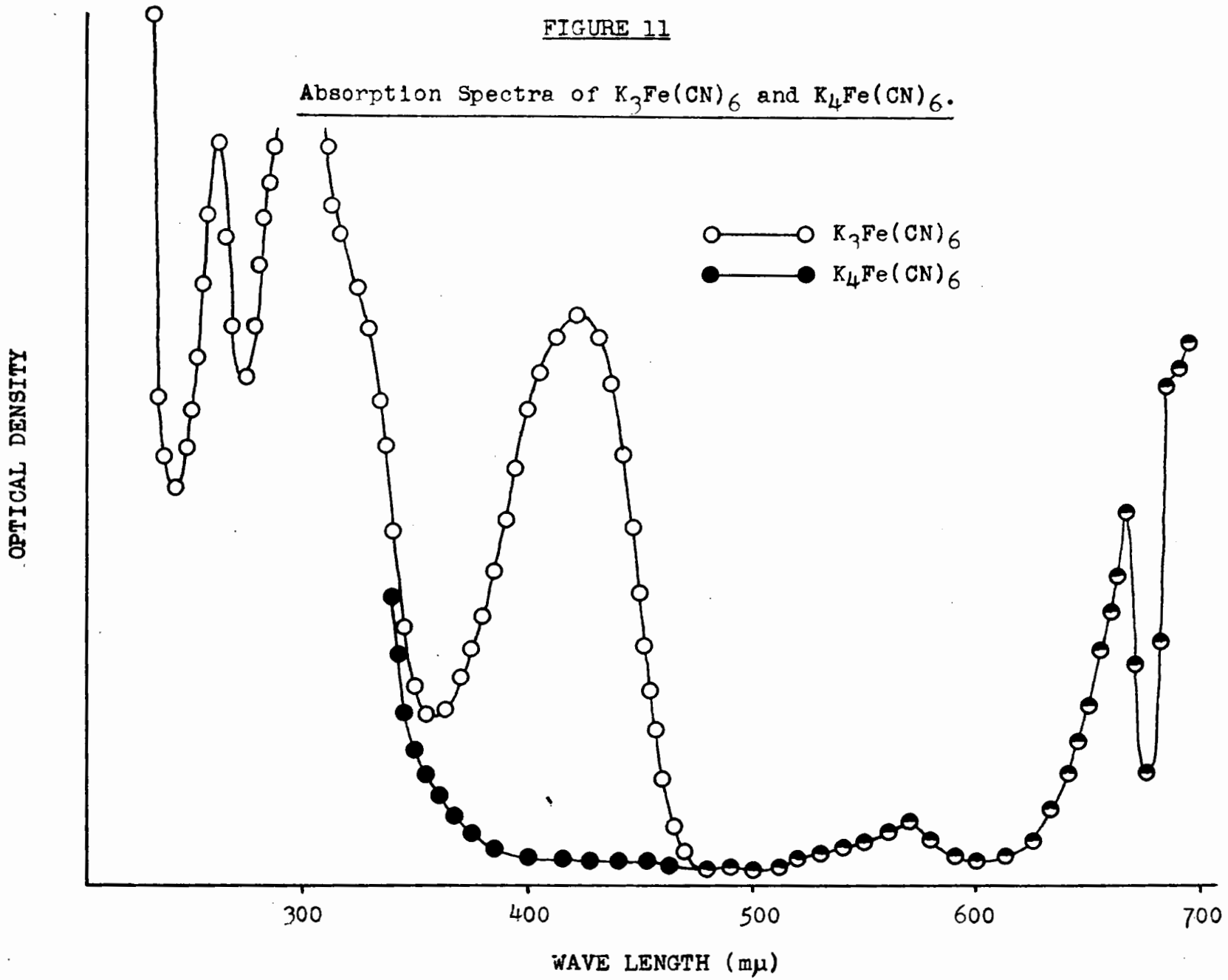
studied by this method. In the case of succinic dehydrogenase it is more difficult to say whether the dehydrogenase is directly responsible for the reduction of ferricyanide, since this enzyme is not linked with a pyridine nucleotide cofactor. It has recently been shown that the purified dehydrogenase can reduce ferricyanide only very slowly (131), so that it appears that in this case some other factor is necessary for the transfer of electrons to the ferricyanide.

In certain experiments to be described later (Results, page 116) it was necessary to estimate the DPN remaining in the flasks after periods of incubation during which the ferricyanide technique had been employed to measure the activity of dehydrogenases. The assay of DPN by the spectrophotometric methods was found to be impossible due to interference by ferricyanide. It was observed, however, that the interference was least in samples in which the dehydrogenase activity had been strongest.

The observations just cited warranted further investigation. As a first step, the spectra of both ferricyanide and ferrocyanide (as the potassium salts) were investigated in both the visible and ultraviolet regions. The absorption spectra are illustrated in Figure 11. Thus, ferricyanide has an absorption peak at 420 mμ which is not given by ferrocyanide. However, after oxidation of the ferrocyanide with H_2O_2, the peak at 420 mμ reappeared. Plotting the concentration of ferricyanide against the intensity of absorption at 420 mμ the relationship

FIGURE 11

Absorption Spectra of $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$.



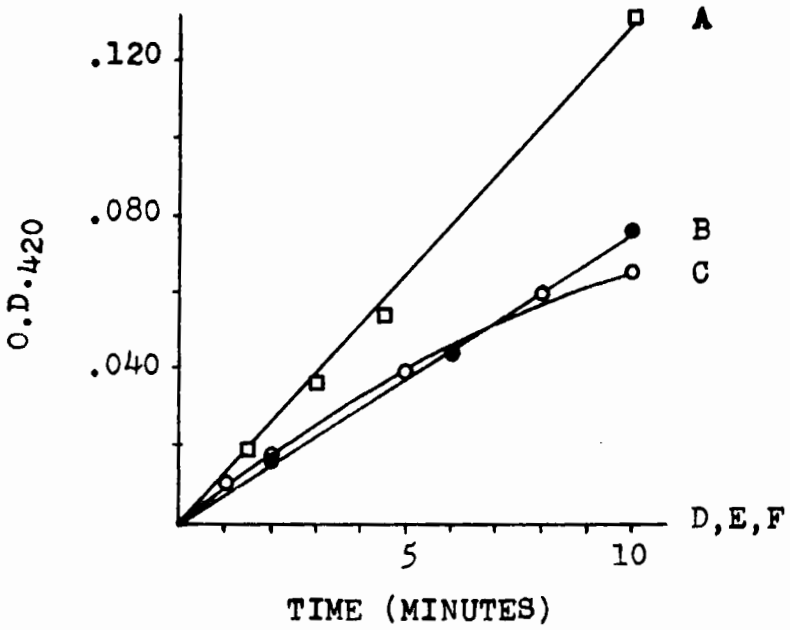
behaves according to Bear's Law.

The assay of the dehydrogenase activity of a liver preparation, by following the reduction of ferricyanide spectrophotometrically was attempted. A male albino rat was decapitated and the liver was immediately removed. A 10 percent homogenate in water was prepared in the cold, and the preparation was centrifuged at 1000 r.p.m. for ten minutes. The supernatant was dialysed for three hours against distilled water at a temperature below 5°C. This preparation was used as the source of the enzymes to be assayed.

As these were merely exploratory experiments, various systems were studied, including lactic dehydrogenase (DPN-linked), glucose-6-phosphate dehydrogenase (TPN-linked), and succinic dehydrogenase (not pyridine-nucleotide-linked). The oxidation of tyramine by liver homogenate, and the activity of a commercial preparation (Sigma Chemicals) of purified alcohol dehydrogenase (DPN-linked), also were tested. The results of these assays are indicated in Figures 12 and 13. The "blanks" used, comprised the complete system, less the substrate. The blank was designed to correct for any endogenous activity of the enzyme preparation, or for possible interactions of the components of the reaction mixture. During the experiment the blanks were kept at an optical density of 0.300. The changes that occurred were periodically recorded as the decrease in the optical density.

FIGURE 12

Oxidation of Glucose-6-phosphate, Lactate, and Tyramine by Liver Homogenates, with Ferricyanide as the Terminal Electron Acceptor.



Cuvette contents:

A. Glucose-6-phosphate dehydrogenase: 0.1 ml. 10% liver homogenate; 0.0011M glucose-6-phosphate; 0.2 mgm. 90% TPN; 0.0066M phosphate buffer at pH 7.2; 0.0005M $K_3Fe(CN)_6$; volume made up to 3.0 ml. with water.

B. Lactic dehydrogenase: 0.1 ml. 10% liver homogenate; 0.016M dl-lactate; 0.5 mgm. DPN; 0.03M $Na_4P_2O_7$; 0.0005M $K_3Fe(CN)_6$; volume made up to 3.0 ml. with water.

C. Amine oxidase#: 0.03 ml. 20% liver homogenate; 0.013M tyramine hydrochloride; 0.007M TRIS buffer at pH 7.2; 0.0005M $K_3Fe(CN)_6$; volume made up to 3.0 ml. with water.

D. Same as A but without enzyme.

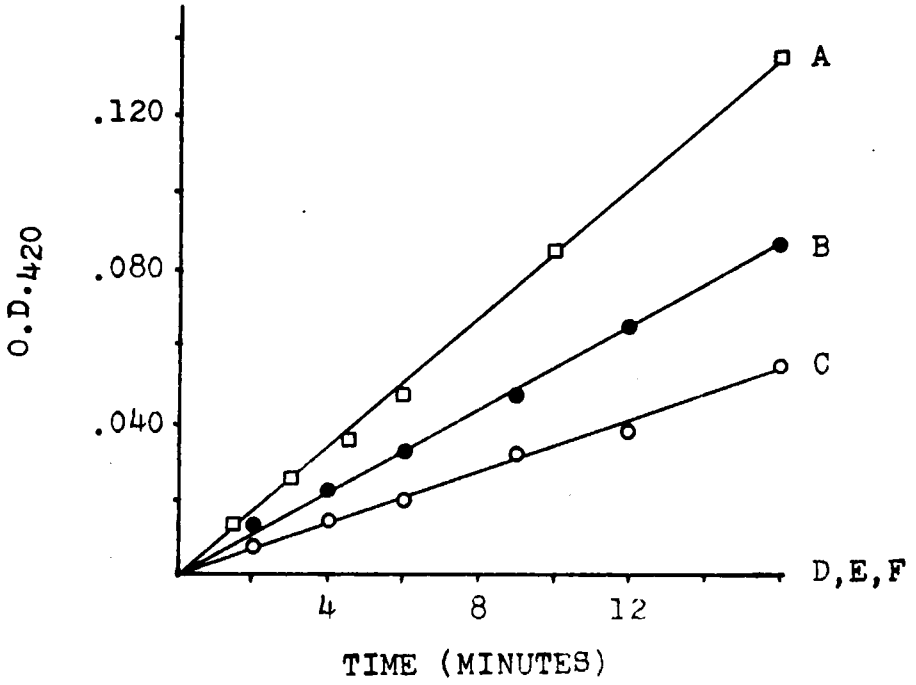
E. Same as B but without enzyme.

F. Same as C but without enzyme.

#This determination was done with the collaboration of Mr. J.R. Lagnado, of the Department of Biochemistry, Allan Memorial Institute of Psychiatry, McGill University. The enzyme preparation used was dialysed against cold, distilled water, for 25 hours.

FIGURE 13

Oxidation of Succinate by Liver Homogenate, using Ferricyanide as the Terminal Electron Acceptor.



Cuvette contents:
A. 0.1 ml. enzyme preparation; 0.016M succinate; 0.0066M phosphate buffer at pH 7.2; 0.0005M $K_3Fe(CN)_6$; volume made up to 3.0 ml. with water.
B. As in A, but 0.0035M TRIS buffer at pH 7.2 substituted for the phosphate buffer.
C. As in B, but 0.06 ml. enzyme instead of 0.1 ml.
D. As in A, but without enzyme.
E. As in B, but without enzyme.
F. As in B, but with 0.007M TRIS buffer instead of 0.0035M TRIS buffer.

To take account of the possibility that a reduction of ferricyanide might take place even in the absence of enzyme, a second set of cuvettes was employed, in which the conditions in the experimental and blanks were the same as mentioned above, with the exception that the enzyme was omitted. No decrease in the optical density was observed in the presence of any of the substrates tested.

The lactic dehydrogenase activity was assayed at an alkaline pH so as to drive the reaction towards pyruvate formation by removing the H^+ formed in the reduction of lactate. The assays of the oxidations of tyramine and of glucose-6-phosphate, were carried out at about pH 7.

The assay of succinic dehydrogenase was first tried in a medium buffered with 7×10^{-3} M TRIS, at pH 7.2. Under these conditions, no reduction of ferricyanide occurred. When the TRIS concentration was reduced to one half, i.e. to 3.5×10^{-3} M, there was some enzymatic activity, but with phosphate buffer at the same pH, the activity was more than doubled.

It is possible, therefore, as has been found elsewhere (219), that the succinoxidase system is dependent upon the presence of phosphate. According to Bonner (226) on the other hand, the reactions involved in the reduction of ferricyanide do not require the presence of phosphate. It is possible that TRIS acts as a true inhibitor of some reaction involved in the system. Singer and Kearney (131) using mildly alkaline TRIS buffers (0.06 M, pH 8.9) succeeded in completely solubilising the succinic dehydrogenase, with the result that

ferricyanide became a very poor electron acceptor for the dehydrogenation of succinate. Even though in our case the pH of the buffer was neutral, it is possible that the same effect may have taken place.

Using purified alcohol dehydrogenase with ethanol and DPN over a range of pH from 1.5 to 9.5, and with several different types of buffer, no reduction of ferricyanide occurred, even when the DPN was completely reduced, as indicated by the pronounced absorption at 340 mμ. From this experiment it appears that ferricyanide, in low concentrations, will not oxidise DPNH.

Hemolysates of mature erythrocytes produced no reduction of ferricyanide in the presence of a variety of substrates, but it appears that the reduction occurs with reticulocytes, at least with malate as the substrate.

One important conclusion from the above results is that the mode of action of ferricyanide depends on the concentration at which it is used. In the manometric method (171) the ferricyanide concentration used is about 2×10^{-2} M, and at this concentration it is inhibitory towards the activity of dehydrogenases. This inhibitory action is prevented in the presence of substrate. On the other hand, when ferricyanide is used in concentrations of 5×10^{-4} M, as in the experiments described above, it will not act as an electron acceptor for reduced DPN as was the case at the higher concentrations of ferricyanide. This may be inferred from the negative results with alcohol dehydrogenase, and with mature erythrocyte

hemolysates. From the experiments with mature erythrocyte hemolysates a second point arises. Methylene blue which acts as an electron acceptor in erythrocyte preparations, is known to accept the hydrogen and electrons at the flavoprotein stage. The failure of ferricyanide to be reduced by erythrocyte hemolysates, indicates that another factor, past the flavoprotein stage in the respiratory chain, is essential for the reduction of this reagent.

From studies on the mechanism of phosphorylations using the DPN-linked β-hydroxybutyric dehydrogenase, Lehninger (221) found that no phosphorylation occurred if ferricyanide was used as the electron acceptor. Phosphorylation did take place, however, in the presence of cytochrome c. Subsequently Copenhaver and Lardy (222) using 1.6×10^{-2} M ferricyanide, showed that one molecule of inorganic phosphate was esterified into organic phosphate for every two molecules of ferricyanide reduced to ferrocyanide, when β-hydroxybutyrate was oxidised to acetoacetate. These authors showed also that Antimycin A, which blocks oxidations at the Slater-factor stage in the respiratory sequence (223), completely prevented the oxidation of β-hydroxybutyrate when oxygen served as the terminal electron acceptor. With ferricyanide, on the contrary, neither the oxidation nor the phosphorylation accompanying it were in any way affected. Cross et al. (225) obtained values of 0.19 and 0.63 for the ratio of P: 2 $Fe(CN)_6$ in the oxidation of succinate and malate, respectively, in the presence of 3×10^{-2} M

ferricyanide. The mechanism of action of the above mentioned phosphorylations is not understood, but it does not appear that ferricyanide is acting as acceptor for electrons from DPNH.

Pressman (220), has only very recently shown that with much lower concentrations of ferricyanide, the stage at which this reagent taps the electron transport mechanism is shifted nearer to oxygen. With 3×10^{-4} M ferricyanide, the ratios P:2 $Fe(CN)_6$ for several substrates were as follows: β-hydroxybutyrate, 1.95; fumarate, 1.7; l-malate, 1.8; l-glutamate, 1.92; and succinate, 0.75. In these cases, Antimycin A inhibited both ferricyanide reduction and phosphorylation, while DNP acted in the usual manner (179, 180), by increasing the substrate oxidation and abolishing the phosphate uptake. On the basis of these results, Pressman has postulated that ferricyanide, in the low concentrations used, probably acts as an acceptor in the vicinity of, or actually at the stage where cytochrome c is involved, and possibly accepting electrons from free cytochrome c.

This postulate would be in agreement with the writer's results with erythrocyte hemolysates. The mature erythrocyte has no cytochrome oxidase, and thus it is possible that when this enzyme was lost, the rest of the cytochrome system also disappeared.

NOTE: During the preparation of this thesis, it has come to the notice of the writer that Slater and Bonner (227), in 1952, had used the spectrophotometric method employing ferricyanide as the electron acceptor for the study of the succinoxidase system.

V

RESPIRATION OF RETICULOCYTES

a) Influence of Substrates

It has long been known that the reticulocyte is capable of consuming oxygen (41-43), having a $-Q_{O_2}$ of about unity (47, 70). In studies of the respiration of reticulocytes, the influence of some of the substrates of the glycolytic and tricarboxylic acid cycles on the oxygen uptake of these cells was studied. The results are shown in Table II.

TABLE II

Influence of added substrates on the respiration of reticulocytes

Substrate added	$-Q_{O_2}$	% increase
None	1.08	
Glucose	1.24	15
Lactate	1.35	25
Succinate	2.12	97
Malate	1.19	10

Flask contents: 1.0 ml. cell suspension (93% reticulocytes); 0.1 M substrate (in side arm); 0.2 ml. 20% KOH in center well; volume made up to 2.5 ml. with Krebs-Ringer Phosphate buffer an pH 7.4. Gas phase: air; temperature: 37.5°C.

The rate of oxygen consumption remained relatively constant with time, and from the above results it is evident that succinate was the most effective stimulant of respiration of the substrates. This was observed also by Rubinstein for avian erythrocytes (174). This influence of succinate may be attributable to the more direct route to oxygen of the succinoxidase system, as compared to the pyridine-nucleotide-linked systems, or possibly also to the relatively greater rate of diffusion through the cell membrane which may exist for succinate compared with that of the other substrates tested. The relatively weak stimulation produced by glucose is in agreement with the results obtained by Sherwood Jones et al. (181), and Wright (43), and was further corroborated in the results obtained from experiments designed to measure the disappearance of added glucose, during incubations for periods of up to two hours, at 37.5°C. In these experiments, frequently there was no glucose utilization.

b) Influence of Inhibitors

(i) Effects on the Respiratory Activity of Reticulocytes

To obtain information on the mechanism by which the reticulocytes respire, the effect of malonate, fluoride and 2, 4-dinitrophenol (DNP) on the respiratory activity of these cells, both in the presence and absence of added substrate, was investigated. The results are indicated in Table III.

TABLE III

Influence of Metabolic Inhibitors on the Respiratory Activity of Reticulocytes

Inhibitor	Concentration of Inhibitor	Percentage Inhibition				
		Without Substrate	With Glucose	Concentration of glucose	With Succinate	Concentration of Succinate
Malonate	2.5×10^{-2}	52	42	2.5×10^{-2}	49	2.5×10^{-2}
	1×10^{-1}	77#			75	1×10^{-1}#
Fluoride	3×10^{-2}	64#	64	1×10^{-1}#		
DNP	1×10^{-4}	10			-31	2.5×10^{-2}

Flask contents: 1.0 ml. cell suspension (93-95% reticulocytes); substrate in side arm; 0.2 ml. 20% KOH in center well; volume made up with Krebs-Ringer. Phosphate buffer at pH 7.4.
Gas phase: air. Temperature: 37.5°C.

#Final volume: 2.5 ml. In others final volume was 3.0 ml.

The effect of added malonate on cell respiration is caused primarily by inhibition of succinic dehydrogenase (177). The degree of inhibition of the uptake of oxygen by malonate, therefore, is an indication of the presence of a respiratory system involving succinoxidase, and points to its being active in the reticulocyte.

Fluoride inhibits glycolysis at the enolase step, and the tricarboxylic acid cycle at succinic dehydrogenase (227). The inhibition shown here, was unaffected by glucose addition.

The metabolic effects of DNP have been postulated as being an uncoupling of the phosphorylating mechanisms at some stage of electron transport (179, 180); in the experiments shown in Table III, the effect of DNP is stimulatory, this result being expected for the concentrations of the inhibitor being used. In the presence of added succinate and DNP in the same concentration used here, we have obtained an increase in the oxygen consumption of up to 200 percent relative to the control which contained no added inhibitor.

(ii) Effects on Phosphorylation

The level of ATP in the cell was chosen as an index of the phosphorylations which take place in the cell. ATP was assayed by the use of the enzymes:hexokinase and glucose-6-phosphate dehydrogenase (see page 85).

The influence of malonate and DNP are indicated in Table IV. DNP, which was found (Table III) to increase the

TABLE IV

Influence of Inhibitors on Phosphorylation

Number	Inhibitor	Concentration	Substrate	Concentration	µMoles ATP / ml. blood	% Decrease in ATP concentration
I a	none		none		0.13 #	
	DNP	$1x10^{-4}$	none		0	100
b	none		Succinate	$2.5x10^{-2}$	0.22 #	
	DNP	$1x10^{-4}$	Succinate	$2.5x10^{-2}$	0	100
IIa	none		none		0.13 #	
	Malonate	$2.5x10^{-2}$	none		0	100
b	none		Succinate	$2.5x10^{-2}$	0.18 #	
	Malonate	$2.5x10^{-2}$	Succinate	$2.5x10^{-2}$	0.04	78
c	none		Glucose	$2.5x10^{-2}$	0.12 #	
	Malonate	$2.5x10^{-2}$	Glucose	$2.5x10^{-2}$	0.09	25

Flask contents: 1.0 ml. cell suspension (95% reticulocytes); substrates in side arm; 0.2 ml. 20% KOH in center well; volume made up to 2.0 ml. with Krebs-Ringer phosphate buffer at pH 7.4. Incubated for 2 hrs. under air, at 37.5°C. before assying for ATP.

#Control.

oxygen uptake, by uncoupling oxidative phosphorylation did not permit sufficient resynthesis of ATP to maintain the normal level in the cell. The equilibrium is thus shifted towards the breakdown of ATP and at the end of the experiment no measurable amount of ATP remained. The addition of succinate to the preparation did not improve the situation as it existed in the absence of added substrate.

The effect of malonate, which when alone did not permit the maintenance of the normal level of ATP of the cell, was somewhat reduced by the addition of succinate. Glucose on the other hand, in the presence of malonate, was capable of maintaining the level of ATP near to the normal value, thus showing that the glycolytic system and the first part of the citric acid cycle, are quite efficient in supplying the high energy phosphate needed by the cell, at least for a limited period of time.

The effect of fluoride could not be estimated, since this ion was found to interfere with the reaction catalysed by the glucose-6-phosphate dehydrogenase as had been shown by Glock and McLean (212). Attempts to remove fluoride by precipitation with magnesium and phosphate ions in an alkaline medium, by forming the insoluble magnesium fluorophosphate, were unsuccessful. Hence it was impossible to measure the ATP after incubation with fluoride. Fluoroacetate in the form of a commercial preparation of rat poison (Monsanto Chemicals), contained a high concentration of fluoride and thus could not successfully be used in these studies.

VI

ENZYMES OF THE RETICULOCYTE

a) Cytochrome Oxidase

This enzyme is the final oxidase in the respiratory chain. It catalyses the activation of oxygen and the terminal reaction in which the oxygen reacts with hydrogen to form water, and is essential for respiratory activity. Although it has never been shown to exist in the mature erythrocyte of mammals, it is present and active in the reticulocyte.

The results from an experiment showing the distribution of this enzyme and the relative activity in the various cell fractions, are presented in Table V.

TABLE V

Distribution and Activity of Cytochrome Oxidase in the Reticulocyte

Fraction	$-Q_{O_2}$	% of Activity in Fraction
Whole hemolysate	3.1	100
SFH	0.2	6
Stroma	30.1	89

Flask contents: 0.4, 0.8, 1.2 ml. enzyme preparation (60% reticulocytes); 0.08 M phosphate buffer at pH 7.4; 2 mgm. cytochrome c; 0.6 ml. of a 1.5 M solution of p-phenylenediamine, in side arm; 0.2 ml. 20% KOH in center well; volume made up to 3.0 ml. with 0.154 M KCl.
Gas phase: air. Temperature: 37.5°C.

The activity which in this case was recovered in the soluble stroma-free hemolysate, can be attributed to contamination with the enzyme, since the percentage usually recovered in this fraction is nil, or very small. Thus the cytochrome oxidase activity in the rabbit reticulocyte seems to be confined exclusively to the stroma. The activity of this fraction, per mgm. dry weight, is considerably greater than that of the whole hemolysate and is about ten times higher than that of the particulate fraction of the chicken erythrocyte (174). In the case of the avian cell, however, the particulate fraction comprises, not only the stroma material, but also a nucleus.

The total accountable activity after fractionation, i.e. the sum of the activity of the individual fractions, in the above case, (Table V) was 95 percent of the activity of the whole hemolysate. Values as high as this were not usually encountered. Often only about 50 percent of the original activity could be accounted for in the fractions.

b) Dehydrogenases

(i) Succinic Dehydrogenase

Succinate, when added to reticulocytes, increases the respiratory activity, approximately doubling the oxygen consumption. This circumstance along with the results of inhibition studies with malonate, afford a strong indication of the presence

in these cells, of a system capable of oxidising succinate.

Succinic dehydrogenase has not been demonstrated in the mature erythrocyte. We have confirmed the absence of succinic dehydrogenase activity as measured by the ferricyanide technique of Quastel and Wheatley (171). The enzyme is present in the reticulocyte. The manner of its distribution and the activity of this enzyme are indicated in Table VI.

TABLE VI

Distribution and Activity of Succinic Dehydrogenase in Reticulocytes

Fraction	Q_{CO_2}	% of Activity in Fraction
Whole hemolysate	1.7	100
SFH	0.2	10
Stroma	23.5	98

Flask contents: 1.0 ml. enzyme preparation (72% reticulocytes); 0.08 M sodium succinate; 0.015 M $KHCO_3$; 0.2 ml. neutralised 11% $K_3Fe(CN)_6$; in side arm; volume made up to 2.5 ml. with 0.154 M KCl.
Gas phase: 95% N_2 + 5% CO_2. Temperature 37.5°C.

As with the cytochrome oxidase, succinic dehydrogenase apparently is confined to the stroma, since most of the activity was found in this fraction. The activity of the cytoplasmic fraction was very low, and most probably was attributable to contamination of the fraction with stroma in finely divided form.

In liver, 70 percent of the succinic dehydrogenase activity of the cell is in the mitochondria, and only about 10 percent can be accounted for in the nuclei and other solid cell residues (183). In the chicken erythrocyte, also, the activity appears in the particulate fraction, which gives a Q_{CO_2} of the order of 4.5 (174), with succinate.

Comparing the activity of the stroma of the reticulocyte with that of the debris of the chicken red cell preparation referred to in the previous paragraph, it becomes apparent that the reticulocyte preparation, on the basis of dry weights, is relatively very active (Q_{CO_2} of 23.5 for the reticulocyte, compared with 4.5 for the chicken erythrocyte). The comparatively low activity of the whole hemolysate is attributable to the large proportion of this fraction which is taken up by inert material, i.e. hemoglobin. Even so, the whole hemolysate from rabbit reticulocytes is approximately twice as active as that from chicken erythrocytes (174).

One should not overlook the possibility that the absence of succinic dehydrogenase from the mature mammalian erythrocyte may be caused by the absence of an essential co-factor or activator which may be necessary for the measurement of the activity by the ferricyanide method. Evidence in support of this possibility has recently been provided by Singer and Kearney (131), who have demonstrated that succinic dehydrogenase, once purified, will not use any of the common dyes as

acceptors, and will act with ferricyanide very inefficiently.

The activity accounted for in the SFH and Stroma was 108 percent of the activity of the whole hemolysate.

(ii) Isocitric dehydrogenase

This enzyme catalyses the reversible reaction between isocitrate and oxalsuccinate, with TPN as the pyridine nucleotide carrier. The enzyme was studied by the ferricyanide technique, using a preparation of crude nucleotides prepared from hog liver, as a source of TPN.

Isocitric dehydrogenase was shown by Rubinstein (174) to be present in the mature erythrocyte of the rabbit, and to be TPN-linked. In the reticulocyte the activity and distribution of the enzyme are shown in Table VII, next page.

The enzyme is distributed between the two fractions, the stroma having a reasonably high specific activity, and the SFH containing a sufficient activity to indicate that its activity is not attributable merely to contamination with stroma material.

The results of a preliminary study of the isocitric dehydrogenase by means of a spectrophotometric method are indicated in Table VIII, next page. The reduction of pyridine nucleotide was followed by the measurement of the increase in optical density at 340 mμ.

From these results it appears that the isocitric dehydrogenase of the reticulocyte requires a cofactor in addition

TABLE VII

Distribution and Activity of Isocitric Dehydrogenase in the Reticulocyte

Fraction	Q_{CO_2}	% of Activity in Fraction
Whole hemolysate	2.7	100
SFH	1.8	60
Stroma	12.3	33

Flask contents: 1.0 ml. enzyme preparation (72% reticulocytes); 0.0154 M $KHCO_3$; 0.2 ml. neutralised 11% $K_3Fe(CN)_6$ in the side arm; 0.3 ml. of a solution of crude nucleotides containing 40 mgm. crude nucleotides per ml.; 0.06 M neutralised NaCN; 0.04M nicotinamide; 0.05 M dl-isocitrate; volume made up to 2.5 ml. with 0.154 M KCl.
Gas phase: 95% N_2 + 5% CO_2. Temperature: 37.5°C.

TABLE VIII

Spectrophotometric Assay of Isocitric Dehydrogenase Activity in the Reticulocyte

Fraction	Change in Optical Density at 340 mμ		
	a) DPN	b) TPN	c) Crude Nucleotides
Stroma	nil	nil	0.010
SFH	nil	nil	0.080

Reaction mixture: 0.03 ml. stroma or 0.02 ml. SFH (50% reticulocytes); 0.04M nicotinamide; 0.03 M dl-isocitrate; 0.04M $Na_4P_2O_7$; volume made up to 2.5 ml. with water. Incubated at 37°C. for 20 minutes.

Expt. a) 1 mgm. DPN
b) 0.2 mgm. 90% TPN
c) 0.3 ml. crude nucleotide solution.

to DPN or TPN. The preparation of the crude nucleotides contained both DPN and TPN, along with numerous other substances. The probability that one or more of the other substances is required by the dehydrogenase, seems to be indicated by the findings in this experiment. That there was reduction of a pyridine nucleotide is shown by the increase in the optical density at 340 mμ.

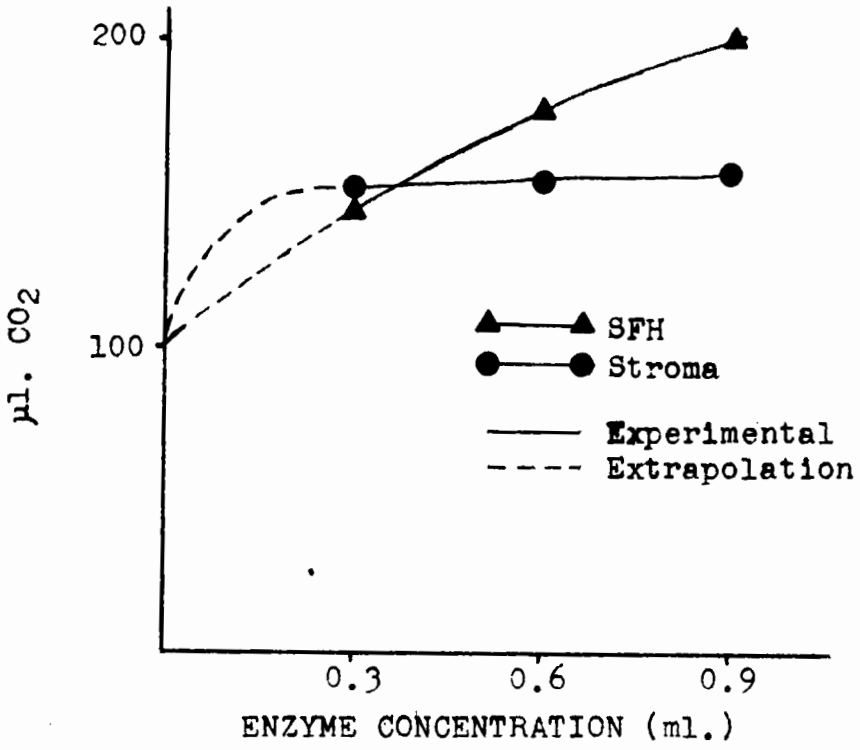
A second observation of interest is the apparent lack of activity in the stroma. The small increase in activity shown by the stroma preparations in the presence of crude nucleotides, cannot be taken as significant unless the result can be statistically established in repeated experiments.

To ascertain whether the stroma contains isocitric dehydrogenase activity, the activity of both the stroma and the SFH was estimated in the Warburg apparatus. The results which were obtained on varying the quantities of the enzyme preparation are indicated in Figure 14.

The results obtained were unexpected and anomalous. The activity of the SFH with varied enzyme concentration gives a straight line which, extrapolated, intercepts the axis at a value of about 100 μl CO_2. A similar treatment of the values of the activity of the stroma, would yield and intercept of 150 μl CO_2. It is inconceivable that the extrapolation of the two curves to zero enzyme concentration should give different intercepts; from the available evidence, the most likely

FIGURE 14

Isocitric Dehydrogenase Activity of Stroma and SFH.



Flask contents: enzyme preparation (50% reticulocytes); final volume 3.0 ml. Otherwise as for Table VII.

extrapolation is the one shown in Figure 14. For the present time, as no further work has been done on this problem, no explanation of the above effects can be attempted.

(iii) Lactic Dehydrogenase

The reversible reaction:

35) $$\text{pyruvate} + \text{DPNH} + H^+ \rightleftharpoons \text{lactate} + \text{DPN}^+$$

is catalysed by the enzyme lactic dehydrogenase#. The presence of this enzyme in the mammalian erythrocyte was discovered by Quastel and Wheatley in 1938 (171). The influence of the DPNases of the erythrocyte on the lactic dehydrogenase activity of the cell was studied by Alivisatos and Danstedt (184).

The lactic dehydrogenase activity of the reticulocyte was assayed in the whole hemolysate and the cell fractions using the ferricyanide method. The results obtained were found to have a peculiarity which became apparent only after fractionation of the hemolysate into the particulate fraction and the cytoplasm. As indicated in Table IX, next page, and in Figure 15, the sum of the activities of the stroma and SPH amounted to 230 percent of the activity of the original hemolysate, and furthermore, the activity of the recombined fractions was 123 percent of the activity of the whole hemolysate. Considering the errors inherent in the assay, however, the 23 percent

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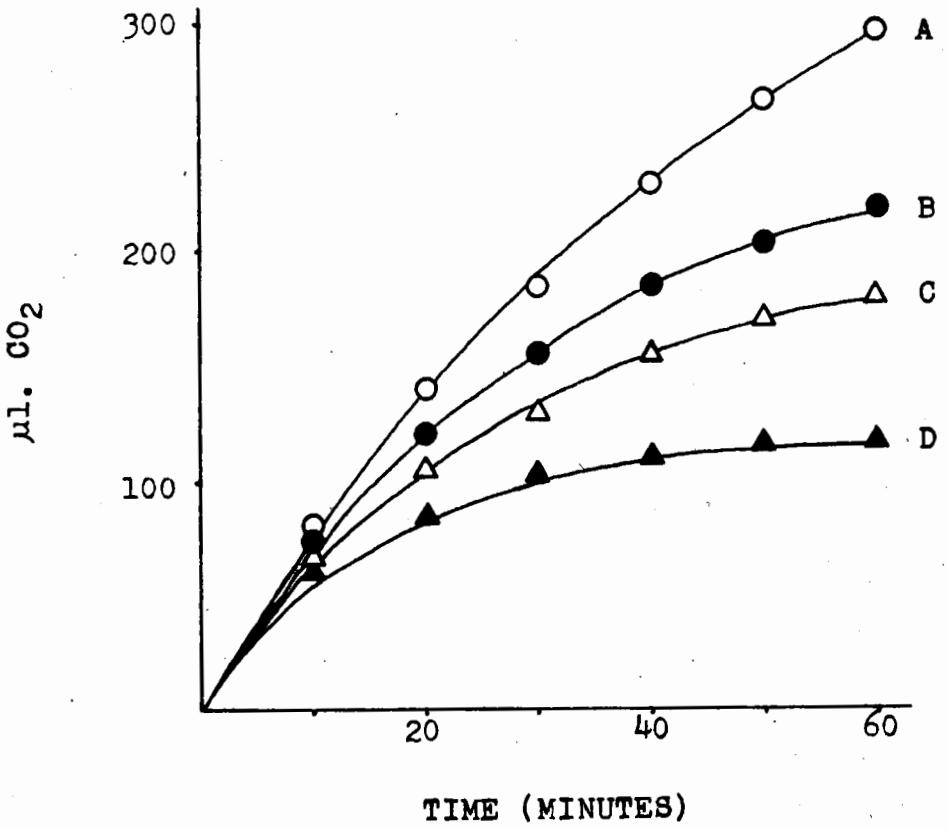
#Lactic dehydrogenase, like malic dehydrogenase, will function not only with DPN, but also with TPN as hydrogen acceptor. The rate with TPN is, however, much lower (for the lactic dehydrogenase, DPN/TPN = 170) (185).

FIGURE 15

Distribution and Activity of Lactic Dehydrogenase in the Reticulocyte.



A SFH
B SFH + Stroma
C Whole hemolysate
D Stroma

Flask contents: as in Table IX.

TABLE IX

Distribution and Activity of Lactic Dehydrogenase in the Reticulocyte

Fraction	Q_{CO_2}	% of Activity in Fraction
Whole hemolysate	10.1	100
SFH	16.4	166
Stroma	88.5	64
SFH + Stroma	12.6	123

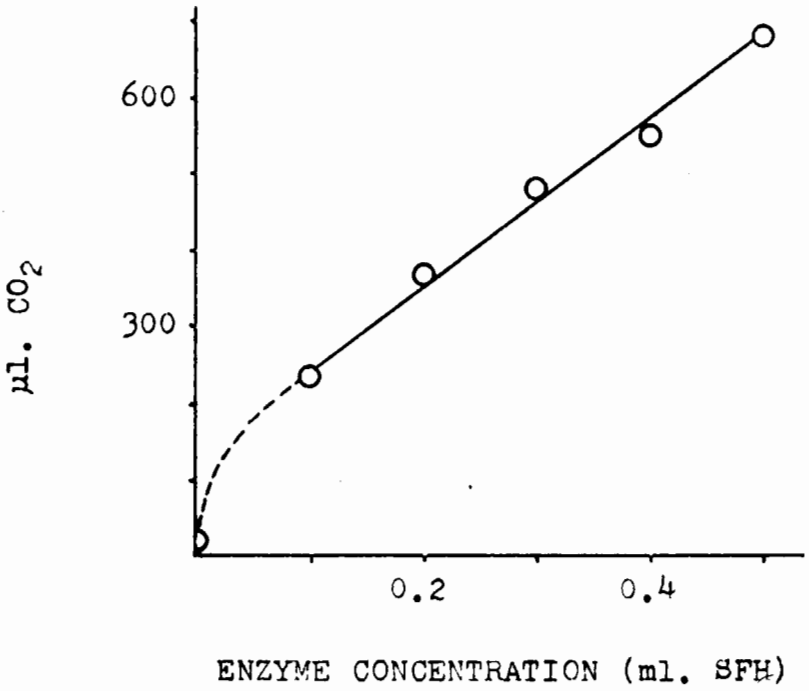
Flask contents: 0.5 ml. enzyme fraction (72% reticulocytes); 0.154 M $KHCO_3$; 0.2 ml. of neutralised 11% $K_3Fe(CN)_6$ in side arm; 0.04 M nicotinamide; 0.1 M dl-lactate; 1 mgm. DPN; volume made up to 2.5 ml. with 0.154 M KCl; 0.2 ml. 0.64 M, neutralised NaCN solution.
Gas phase: 95% N_2 + 5% CO_2. Temperature: 37.5°C.

increase in activity in the latter case is of doubtful significance. From the results in Table IX, and others to be presented later, it is apparent that the decrease in activity obtained with the recombined cell fractions represents an inhibitory effect of the stroma on the activity of the SFH.

Experiments preliminary to the investigation of the inhibition of lactic dehydrogenase activity by the stroma fraction, showed that the activity of this enzyme, when measured manometrically, did not give a straight-line relationship passing through the origin, when plotted against the enzyme concentration. The results from an experiment using the SFH are given in Figure 16. In this case the enzyme concentration

FIGURE 16

Enzyme Concentration Curve for Lactic Dehydrogenase.



Flask contents: as in Table IX except for amount of enzyme and lactate concentration which here was 0.06M.

was varied between 0.1 and 0.5 ml., but even when the enzyme concentration was lowered to 0.03 ml., the results obtained were essentially the same as before.

On trying to obtain a method which would give the usual relationship between the activity and the enzyme concentration, the spectrophotometric assay, measuring the reduction of DPN at a pH near 9, was used. The curve obtained in this case passed through the origin, but it was not a straight line, even though the enzyme concentration range, in this case, was very low (0.002 ml. to 0.015 ml.).

With the spectrophotometric assay, however, another difficulty arose; namely, that no inhibition of lactic dehydrogenase activity by stroma could be produced when using up to five times as much stroma as SFH (Table X, next page).

Since the spectrophotometric assay method was not found to be adaptable for these inhibition studies, the writer was obliged to use the manometric method.

That the inhibitory action manifested by the stroma was abolished upon heating the stroma for five minutes in a boiling water bath (Table XI, next page) could be due to the stroma having some enzymatic action which counteracts the activity of the lactic dehydrogenase. It is possible also that the heat treatment alters the structure of the stroma sufficiently to abolish the inhibitory properties.

The first explanation that comes to mind for the decreased activity in the presence of the stroma, is the possible

TABLE X

Spectrophotometric Assay of Lactic Dehydrogenase Activity. (Normal Erythrocytes)

Fraction	Volume used	Δ O.D./340 mμ	% of Activity in Fraction
SFH	0.1 ml.	0.121	100
Stroma	0.5 ml.	0.000	0
SFH + Stroma	0.1 ml. 0.5 ml.	0.113	94

Cuvette contents: Normal rabbit erythrocyte fractions[#]; 1.0 mgm. DPN; 1.0 ml. 0.1 M $Na_4P_2O_7$; 0.0016 M dl-lactate; 0.06 M nicotinamide; volume made up to 3 ml. with 0.154 M KCl. Carried out at room temperature, for four minutes.

TABLE XI

Effect of Heat on the Inhibition of Lactic Dehydrogenase by the Stroma

Fraction	μl. CO_2 per sample	% of Activity of SFH
SFH	334	100
Stroma	24	7 (?)
SFH + Stroma	252	75
SFH + Stroma (heated)	324	97

Flask contents: 0.3 ml. SFH and/or 0.5 ml. stroma fractions (normal erythrocytes); 0.0154 M $KHCO_3$; 0.2 ml. of a 0.64 M neutralised NaCN solution; 0.033M nicotinamide; 1 mgm. DPN; 0.08 M dl-lactate; 0.2 ml. of a neutralised 11% solution of $K_3Fe(CN)_6$ in the side arm; volume made up to 3 ml. with 0.154 M KCl.
Gas phase: 95% N_2 + 5% CO_2. Temperature 37.5°C.

[#]The stroma of the mature erythrocyte, often exhibits no lactic dehydrogenase activity. In this respect it differs from the stroma of the reticulocyte. The stroma from both types of cell, however, possess the property of inhibiting the activity of the SFH.

action of the DPNase of the stroma (184). This explanation, however, is not satisfactory for two reasons: first, the inhibition did not increase with time. Referring to Figure 15, (page 111), it is evident that the ratio of the activity of the combined SFH and stroma fractions to the sum of the individual activities of these two fractions, computed every ten minutes, gave a constant value of 0.54, with the exception of the ratio of the 50-minute values, which was 0.53.

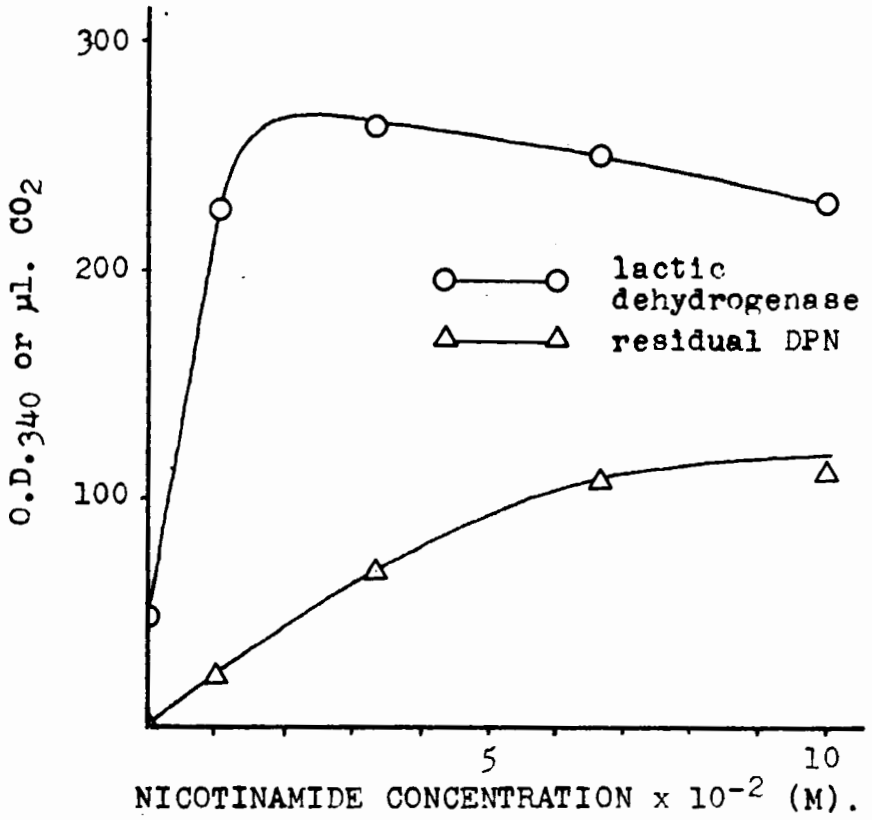
The second argument against the implication of DPNase is evident from the estimation of the amount of DPN left at the end of the reaction period during which the activity of lactic dehydrogenase was followed. The amount of DPN remaining was related to the concentration of nicotinamide used to inhibit the DPNase (189). The results of one experiment are shown in Figure 17.

The measurement of DPN in the medium of the flasks in which lactic dehydrogenase had been assayed, proved to be impossible due to the interference by ferricyanide with the method. For this reason a duplicate set of flasks was incubated along with the first set, and was used for the separate estimation of DPN. It was necessary to omit from the medium in these flasks, both ferricyanide and lactate. Lactate was excluded because, if present, it would bring about the reduction of the DPN, and with no ferricyanide present to reoxidise it, the reduced DPN cannot serve as substrate for DPNase (187).

Figure 17 indicates that the activity of lactic dehydrogenase is at a maximum with nicotinamide present in concen-

FIGURE 17

Influence of Nicotinamide on DPNase and on Lactic Dehydrogenase.



Flask contents: for lactic dehydrogenase: 0.2 ml. SFH and 0.3 ml. stroma (normal erythrocytes#); 0.2 ml. neutralised 11% solution of $K_3Fe(CN)_6$; otherwise as for Table XI. Both the stroma and the ferricyanide were placed in the side arm.

For DPN estimation: as above but omitting $K_3Fe(CN)_6$ and lactate.

#The enzyme preparation here was obtained from normal blood rather than from reticulocyte-containing blood. This is permissible, since the mature erythrocyte and the reticulocyte, as will be seen later, have the same DPNase activity.

trations of about 2×10^{-2} M. The concentration of nicotinamide necessary for maximum protection of the DPN in the reaction mixture, on the other hand, is 10^{-1} M or higher.

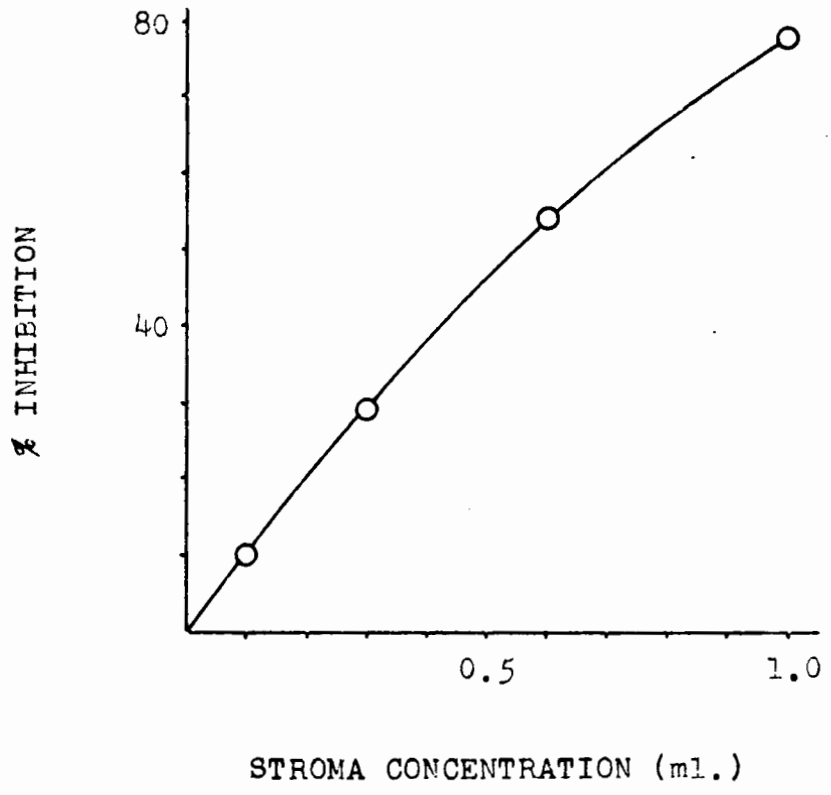
Assuming that the amounts of DPN measured afford an accurate indication of the amount in the corresponding vessels used for the estimation of lactic dehydrogenase activity, it then appears that very small quantities of DPN are adequate for the maintenance of maximum dehydrogenase activity. With 3.3×10^{-2} M nicotinamide, and incubation at 37.5°C. for one hour, only 20 percent of the added DPN was recovered.

The inhibition of lactic dehydrogenase at the higher concentrations of nicotinamide has been discussed previously by Alivisatos and Denstedt (186).

The effect of varying the inhibitor (i.e. stroma) concentration upon the activity of the SFH was tested, while the concentration of the SFH was kept constant. Plotting the concentration of the stroma versus the percentage inhibition a line is obtained which appears to be straight up to the 55 percent inhibition level, and then begins to fall off (Figure 18). If the values corresponding to the data in Figure 18 are plotted as V/V_i against (I), where V is the velocity in the absence of inhibitor, V_i the velocity in the presence of inhibitor, and (I) the concentration of inhibitor in ml., an exponential curve is obtained (Figure 19). This relation is an abnormal one, since the plot should give a straight line

FIGURE 18

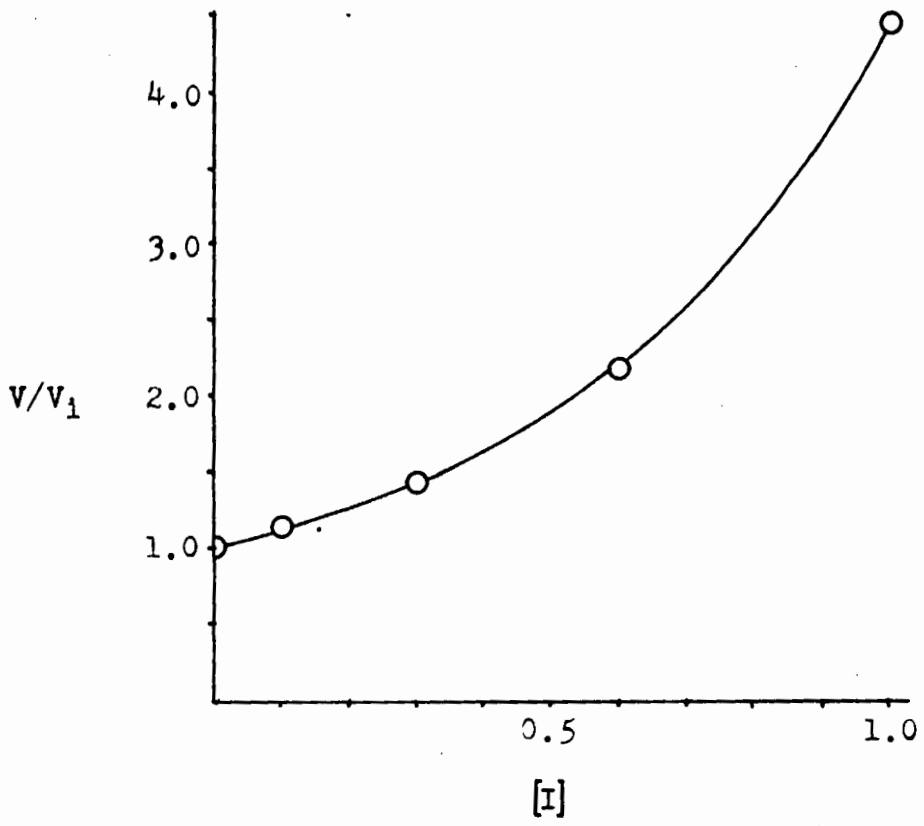
Inhibition of Lactic Dehydrogenase by Stroma.



Flask contents: 0.5 ml. SFH + Stroma (75% reticulocytes); rest as in Table XI.

FIGURE 19

Inhibition of Lactic Dehydrogenase by Stroma.



Flask contents: as in Fig. 18.

relationship whether the inhibition is competitive or noncompetitive. If the inhibition is noncompetitive, on plotting V/V_i against (I), a straight line cutting the abscissa at 1, and having a slope of $1/K_i$ should be obtained. If the inhibition is of the competitive type, on the other hand, a similar straight line cutting the abscissa at 1 would be obtained, but with a slope of K_m/K_i $(K_m + (S))$, where K_m is the Michaelis constant for the enzyme, K_i, the dissociation constant of the enzyme-inhibitor complex, and (S) the substrate concentration.

As a further investigation of this anomalous type of inhibition, a determination of the Michaelis constant for the lactic dehydrogenase of the reticulocyte SFH, both in the presence and in the absence of stroma, was attempted. The results of this experiment are represented in Figures 20 and 21. The K_m as calculated from Figure 20, for dl-lactate, is approximately 8×10^{-2}. Here again, as with the plot of Figure 19, the inhibition by stroma does not obey the laws of the Michaelis-Menten equations for any of the common types of inhibition. This same type of curve is obtained with the activation of aconitase by glutathione (168), or the activation of inorganic pyrophosphatase by Mg^{++} ions[#], when 1/V is plotted against 1/S. V, in each case, is the velocity of the enzymatic

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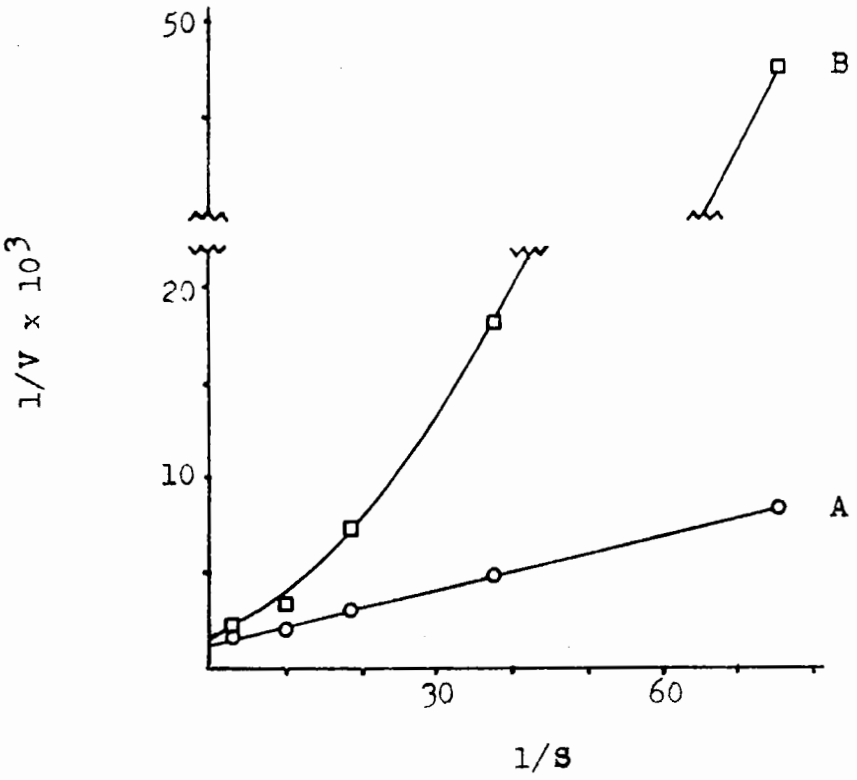
[#]The curve for the activation of inorganic pyrophosphatase is obtained by calculation from values given by Bloch-Frankenthal (186).

FIGURE 20

Lactic Dehydrogenase: Km and Effect of Stroma.

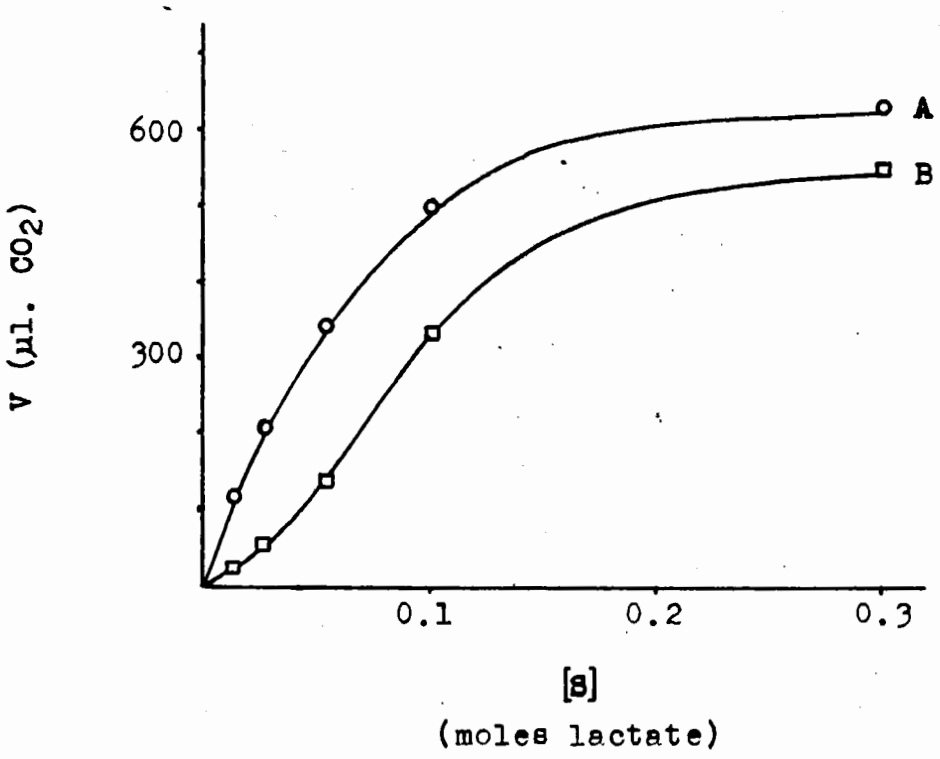


A SFH
B SFH + Stroma

Flask contents: 0.3 ml. SFH, 0.8 ml. Stroma (50% reticulocytes); otherwise as in Table XI.

FIGURE 21

Effect of Stroma on Lactic Dehydrogenase.



A SFH
B SFH + Stroma

Flask contents: 0.3 ml. SFH; 0.8 ml. Stroma (50% reticulocytes); otherwise as in Table XI.

reaction, and S is the concentration of glutathione or Mg^{++}, according to the case.

From the shape of the curve in Figure 21, it seems possible that with the low concentrations of lactate, this substance may be removed from the medium by the stroma, either by being bound, or possibly by undergoing some reaction which does not give rise to the production of acid, and hence, evolution of CO_2 (Reaction 34, page 78). Neither possibility has as yet been confirmed.

A possible explanation for the absence of inhibition by stroma in the case where lactic dehydrogenase was assayed spectrophotometrically (Table X), is that the concentration of Stroma was too small. Higher concentrations could not be used because of the limitations imposed by the method. The fact that the pH used in the spectrophotometric experiments differed from that used in the manometric method, may also have influenced the results.

The problem of the inhibition of the dehydrogenase activity by the stroma, is encountered also in the case of malic dehydrogenase. A solution to this question has not yet been found.

(iv) Malic Dehydrogenase

As with lactic dehydrogenase, the assay of this enzyme gave unusual results in that the sum of the activities of the stroma and SFH was much greater than the activity of the unfractionated hemolysate. As indicated in Table XII, the sum of the activities of the SFH and stroma, was 203 percent of the activity of the whole hemolysate, while the activity of the recombined fractions was the same as that of the unfractionated preparation.

TABLE XII

Distribution and Activity of the Malic Dehydrogenase of Reticulocytes

Fraction	Q_{CO_2}	% of Activity in Fraction
Whole hemolysate	6.5	100
SFH	8.8	124
Stroma	70.0	79
SFH + Stroma	7.1	107

Flask contents: As for Table IX, except that 0.1 M l-malate was substituted for the lactate.

The spectrophotometric assay in alkaline pH, was used for a single study of the distribution of malic dehydrogenase; the values obtained with the stroma were lower than

those by the manometric method. In a corresponding experiment with stroma prepared from mature erythrocytes, no activity was detectable as had been also the case with lactic dehydrogenase. Activity was measurable in the case of stroma prepared from reticulocytes and the activity was increased in material which was kept in the cold for several days, (Table XIII).

TABLE XIII

Distribution of Malic Dehydrogenase in the Reticulocyte and the Mature Erythrocyte (Spectrophotometric Assay)

Reticulocyte			Mature Erythrocyte		
Fraction	$\frac{\Delta \text{ O.D.}}{340 \text{ m}\mu}$	% of SFH Stroma	Fraction	$\frac{\Delta \text{ O.D.}}{340 \text{ m}\mu}$	% of SFH Stroma
SFH + Stroma	0.253	100	SFH + Stroma	0.089	100
SFH	0.230	91	SFH	0.085	96
Stroma	0.057	22	Stroma	0.000	0
Stroma (fresh)	0.085		Stroma (fresh)	0.006	
Stroma# (stored)	0.147		Stroma# (stored)	0.020	

#Stored for 5 days

#Stored for 10 days

Cuvette contents: 0.02 ml. enzyme fraction (reticulocyte enzyme: 50% reticulocyte); 1.0 ml. 0.1 M $Na_4P_2O_7$; 0.06 M l-malate; 0.033 M nicotinamide; 1 mgm. DPN; volume made up to 3.0 ml. with water.
Temperature: room temperature.

The inhibitory effect of the stroma on the malic dehydrogenase was not studied further, since it was reasonable to suppose that the effect was the same as that observed with lactic dehydrogenase.

Since the conditions were different in the assays done by the spectrophotometric and the manometric methods, no significance can be ascribed to the difference between the values obtained by the two methods.

(v) Glucose-6-Phosphate Dehydrogenase

Warburg and Christian (60, 61) and Dickens (62), long ago showed that this enzyme is extremely active in the erythrocyte of the mammal. The enzyme catalyses the oxidation of glucose-6-phosphate according to the following equation:

36) Glucose-6-phosphate + TPN^+ $\longrightarrow$ 6-phospho- δ -gluconolactone + TPNH + H^+

The lactone, in the presence of lactonase, undergoes hydration to 6-phosphogluconate.

The glucose-6-phosphate dehydrogenase catalyses, the first step in the hexose monophosphate shunt, but the role of this system in the metabolism of the erythrocyte is not known. It has not yet been conclusively shown that a system for the reoxidation of reduced TPN produced in the above mentioned reaction, is operative in the intact erythrocyte. However, in view of the strong activity of this dehydrogenase in

the red cell, one is inclined to infer that this enzyme has some function in the metabolism of the cell.

The activity and distribution of this enzyme in the reticulocyte was studied by the spectrophotometric method, following the reduction of TPN, at 340 mμ. The results are presented in Table XIV.

TABLE XIV

Distribution and Activity of Glucose-6-Phosphate Dehydrogenase in the Reticulocyte

Fraction	$\frac{\Delta \text{ O.D.}340}{\text{mgm. dry wt./hr.}}$	% of Activity in Fraction
Whole hemolysate	2.23	100
SFH	2.72	110
Stroma	0.00	0

Cuvette contents: 0.02 ml. enzyme fraction (72% reticulocytes); 0.04 M phosphate buffer at pH 7.4; 0.0026 M glucose-6-phosphate; 0.02 ml. crude nucleotide preparation as a source of TPN; 0.04 M nicotinamide; volume made up to 2.5 ml. with 0.154 M KCl.
Temperature: 25°C.

The assay of this enzyme was usually over a period of ten minutes, during which time the rate of the reaction remained constant. All the activity in this case can be seen to be contained in the soluble fraction (SFH) of the reticulocyte.

c) Hydrases

(i) Fumarase

This enzyme was shown by Quastel (191) to be present in a relatively active form in the mammalian erythrocyte. Alivisatos (190), in our laboratory, showed that in the rabbit erythrocyte, the activity was present in the cytoplasmic fraction.

In the reticulocyte most of the activity is obtained in the soluble fraction, but some activity remains in the stroma even after repeated and thorough washing of the residue. The assay for fumarase is a spectrophotometric procedure and depends on the measurement at 240 mμ, of the absorption due to the ethylenic bond formed by the dehydration of malate to fumarate.

The equilibrium of the reaction favours the formation of malate from fumarate (162), but because of the relative ease with which fumarate can be estimated, it is convenient to use malate as the initial substrate (Table XV).

The sum of the activities of the fractions is 89 percent of the activity of the unfractionated hemolysate. The specific activity of the stroma is sufficiently high compared to that of the whole hemolysate to warrant the conclusion that the observed activity is an accurate measure of the true activity of this fraction. The same may be said of the activity of the SFH.

TABLE XV

Distribution and Activity of Fumarase in the Reticulocyte

Fraction	Δ O.D.240 mgm. dry wt./hr.	% of Activity in Fraction
Whole hemolysate	0.272	100
SFH	0.207	68
Stroma	0.550	21

Cuvette contents: 0.04 ml. enzyme fraction (55% reticulocytes); 0.06 M phosphate buffer at pH 7.4; 0.06 M l-malate; volume made up to 2.5 ml. with 0.154 M KCl.
Temperature: 25°C.

(ii) Aconitase

This enzyme, like fumarase, is a hydrase, but its presence in the mature erythrocyte has not been conclusively demonstrated.

In the reticulocyte aconitase is distributed between the soluble and insoluble fractions in much the same manner as in the chicken erythrocyte (174). In the reticulocyte, the particulate fraction shows the stronger activity. The distribution is given in Table XVI.

The aconitase activity in the reticulocyte is only about 1/20th of that of fumarase as measured by the change in the optical density at 240 mμ per mgm. dry weight per hour, even though the aconitase reaction was carried out at 37°C. while that with fumarase was carried out at 25°C. (Cf. page 81, under Methods).

TABLE XVI

Distribution and Activity of Aconitase in the Reticulocyte

Fraction	Δ O.D.240 mgm. dry wt./hr.	% of Activity in Fraction
Whole hemolysate	0.02	100
SFH	0.01	43
Stroma	0.18	73

Reaction mixture: 0.7 ml. enzyme fraction (81% reticulocytes); 0.05 M phosphate buffer at pH 7.2; 0.12 M citrate; volume made up to 2.1 ml. with 0.154 M KCl.
Temperature: 37°C.

d) Other Enzymes

(i) Hexokinase

This enzyme is of key importance in metabolism since it catalyses the phosphorylation of glucose. It is especially important to the erythrocyte, which apparently derives all its energy from the breakdown of glucose through the glycolytic system. The common reactions involving hexokinase are the following:

37) $\left.\begin{matrix}\text{glucose}\\ \text{fructose}\\ \text{mannose}\end{matrix}\right\}$ + ATP $\xrightarrow[\text{hexokinase}]{Mg^{++}}$ $\left.\begin{matrix}\text{glucose-}\\ \text{fructose-}\\ \text{mannose-}\end{matrix}\right\}$ 6-phosphate + ADP

The phosphate esters thus formed are of "low-energy" type and the reaction equilibrium tends to be so far to the right that the reaction may be regarded as being non reversible.

Hexokinase from yeast has been crystallised (194, 195), but as yet, the crystallised enzyme has not been prepared from animal sources.

The properties of hexokinase in plant and animal tissues have been extensively studied. ATP and Mg^{++} ions are essential for hexokinase activity. In brain the enzyme is confined in its distribution, to the mitochondria (196), as is true for plant hexokinase (197). The enzyme in the mature erythrocyte of the mammal is soluble and confined to the SFH (196).

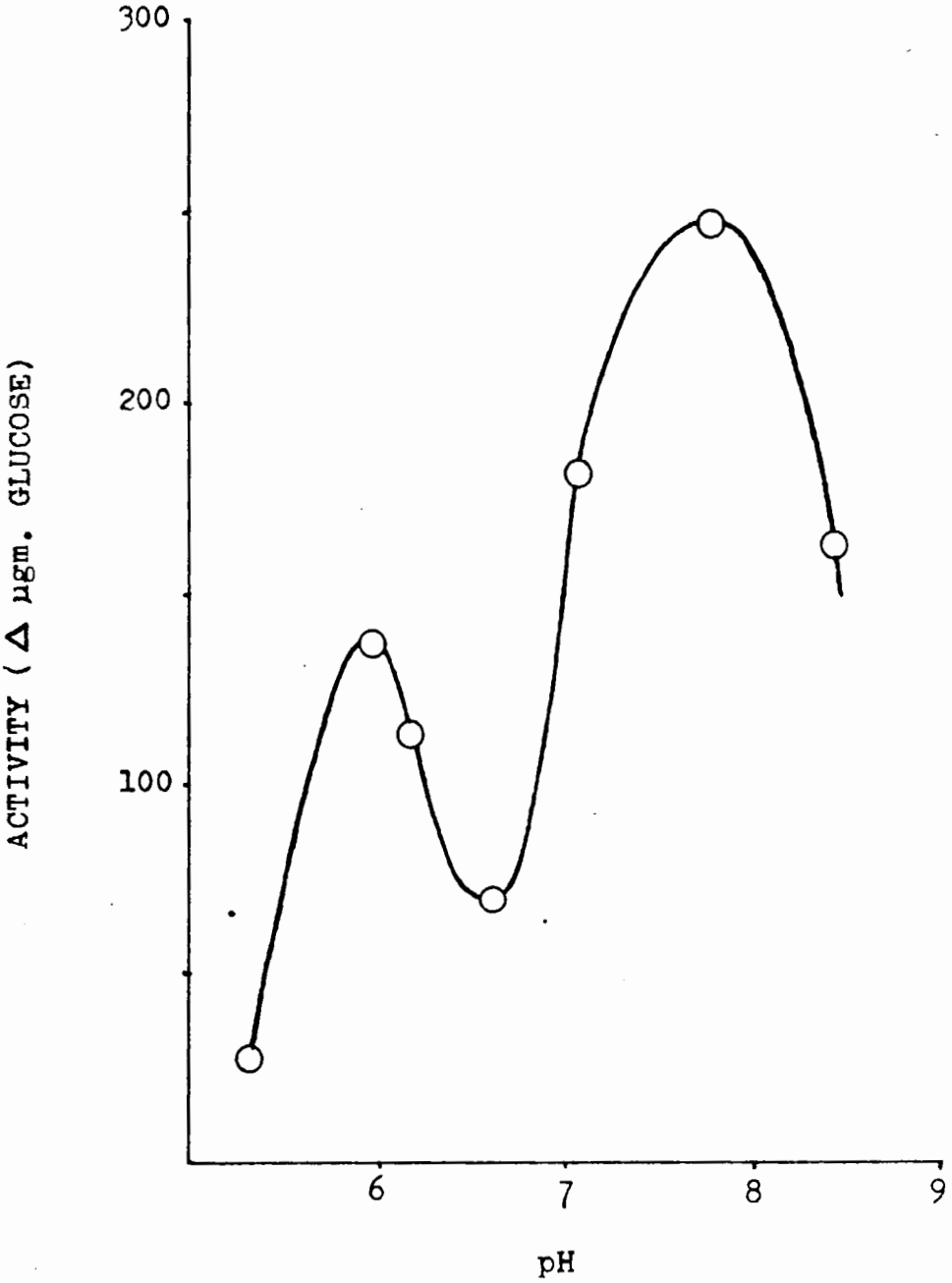
During studies on the hexokinase of the mature erythrocyte of the rabbit[#] it was found that glucose, fructose, mannose and galactose can be phosphorylated, as evidenced by the disappearance of reducing sugar, in the presence of added ATP and Mg^{++}. It is possible that glucose, fructose and mannose are phosphorylated by a relatively nonspecific hexokinase, but galactose is not a substrate for hexokinase. A specific galactokinase (193) may also be present in the red cell.

The hexokinase of the erythrocyte has two pH optima with regard to the phosphorylation of glucose. The two maxima occur at about 6 and 7.8. The hexokinase activity at pH 7.8 is about twice that at 6. (Cf. Figure 22).

[#]The results on the mature erythrocyte from rabbit blood reported here, were obtained with the collaboration of Mr. S. Kashket, and will be reported more fully in a thesis to be submitted by him for the degree of Doctor of Philosophy.

FIGURE 22

Hexokinase pH Curve.



For the measurement of hexokinase activity of the reticulocyte the experiments were performed at pH 8 where the activity is greatest. Table XVII indicates the distribution and activity of hexokinase in the rabbit reticulocyte, and also, for comparison, the values for the mature rabbit erythrocyte.

TABLE XVII

Distribution and Activity of the Hexokinase of the Reticulocyte and of the Mature Erythrocyte

Fraction	μMoles glucose used / mgm. dry wt./hr.		% of Activity in Fraction	
	Retic.	Normal	Retic.	Normal
Whole hemolysate	0.17	0.023	100	100
SFH	0.12	0.018	67	72
Stroma	0.22	0.000	12	0

Reaction mixture: 0.2 ml. enzyme preparation (reticulocyte preparations contained 50% reticulocytes); 0.013 M phosphate buffer at pH 8; 0.027 M glycylglycine buffer at pH 8; 0.005 M $MgCl_2$; 0.01 M KF; 0.005 M ATP; 0.002 M glucose; volume made up to 1.5 ml. with 0.154 M KCl.
Temperature: 37°C.

The difference between the hexokinase of the two cell preparations is not only quantitative but also qualitative. The hexokinase in the reticulocyte is much more active than that of the mature cell, and furthermore, the stroma of

the reticulocyte has definite hexokinase activity, whereas in the mature erythrocyte the activity is confined to the soluble fraction. The sum of the activities of the individual fractions in the two cases reported in Table XVII were 79 percent and 72 percent of the activities of the whole hemolysates of the reticulocyte and mature erythrocyte, respectively.

The anomaly in the distribution of the hexokinase is difficult to explain. The mature rabbit erythrocyte depends on the utilization of glucose for its energy. Since glucose presumably must be phosphorylated in order to pass through the cell membrane (240), and the membrane appears to contain no hexokinase, the question arises: how does the glucose gain entrance into the cell? There is yet no answer to this question, but it is conceivable that the membrane of the erythrocyte is constructed in such a way that the soluble hexokinase inside the cell may permeate the membrane, and thus be able to phosphorylate the hexose entering the cell from the external medium#.

The mature erythrocytes are capable of taking in and utilising glucose at approximately 1/3 of the rate shown by the SFH. It is an odd observation that intact reticulocytes, which without doubt, have hexokinase in the cell membrane, do not always utilise glucose during incubations at 37°C.; at

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#For discussion of this topic the reader is referred to the Ph. D. Thesis by S. Kashket.

least they do not cause the disappearance of reducing material, or the appearance of lactate and pyruvate, all of which are criteria of glucose utilization. In the majority of cases, no disappearance of glucose from the external medium could be demonstrated. Intact chicken erythrocytes likewise take up no measurable amount of glucose when stored at 5°C.,# even though they too contain hexokinase in the particulate fraction. In the case of the chicken red cell, however, the hexokinase of the particulate fraction may not be confined to the stroma, since the fraction includes the nuclei.

(ii) Pyrophosphatase

Inorganic pyrophosphatase is probably present in all living cells and is active in the erythrocytes of mammals (198).

The enzyme acts specifically on free inorganic pyrophosphate and has no action on organic phosphate esters. The enzyme catalyses the hydrolysis of the pyrophosphate bond, in the presence of Mg^{++} ions, to produce the liberation of two molecules of orthophosphate. The pyrophosphate bond being a high energy bond, the equilibrium of the reaction favours hydrolysis.

In the erythrocyte, the pyrophosphatase is activated by Mg^{++} ions (198). According to Naganna et al. (198),

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#Shown in experiments by Dr. H. McLennan in these laboratories in 1947.

Ca^{++} ions inhibit the enzyme competitively as referred to Mg^{++} as substrate. Malkin (200) in our laboratory, has shown that the inhibition is of the noncompetitive type when pyrophosphate is considered the substrate. The strongly inhibitory effect of fluoride on this enzyme (198), has been shown by Malkin (200) to be of the rather uncommon type known as "quadratic inhibition" (211).

The activity of the pyrophosphatase in the reticulocyte is considerably greater than that of the mature erythrocyte. However, the distribution of the enzyme in the two types of cell is the same, that is, the activity is confined to the SFH. Table XVIII indicates the distribution of

TABLE XVIII

Distribution and Activity of Inorganic Pyrophosphatase in the Reticulocyte

Fraction	$\frac{\mu Moles\ orthophosphate}{mgm.\ dry\ wt./hr.}$	% of Activity in Fraction
Whole hemolysate	5.20	100
SFH	4.24	79
Stroma	0.40	1

Reaction mixture: 0.05 ml. enzyme fraction (81% reticulocytes) dialysed for 2 hours against running tap water (5°C.); 0.001 M $Na_4P_2O_7$; 0.02 M $MgCl_2$; made up to volume with 0.1 M TRIS buffer at pH 7.5.
Temperature: 37°C.

the activity of the reticulocyte pyrophosphatase as measured by the liberation of inorganic phosphate during incubation of the enzyme preparation with inorganic pyrophosphate#. The percentage of the activity of the whole hemolysate recovered in the fractionated preparation was 80 percent.

The role, if any, of inorganic pyrophosphatase in the normal metabolism of the mature erythrocyte is not known.

(iii) DPNases

It has long been known that the DPN in cells is inactivated when the cells are broken down. The inactivation can be brought about by any of three enzymes known as DPNases, which are known to exist in cells.

The first of these enzymes is a nucleosidase promoting the breakdown of the nicotinamide-ribose bond and liberating nicotinamide in stoichiometric proportion to the number of moles of either DPN (201) or TPN (203) split. This DPNase is strongly inhibited by nicotinamide (184, 190, 204, 205) and does not split the reduced form of the DPN or TPN (187). In the rabbit this enzyme is localised entirely in the stroma (184, 190), and apparently is confined to the outer surface or region of the membrane.

Kornberg and Lindberg discovered the existence of a second type of DPNase (210), which is not inhibited by

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#The assays involved in this enzyme study were kindly performed by Dr. Rex Malkin.

nicotinamide. This enzyme is a pyrophosphorylase and catalyses the reaction:

38) $DPN + PP_i \rightleftharpoons NMN + ATP$

The enzyme has been purified from yeast and hog liver (206), and the kinetics of the purified enzyme have been thoroughly studied. Unlike the reaction catalysed by the nucleosidase, that catalysed by the pyrophosphorylase is reversible (207).

There is also a third type of DPNase which is a pyrophosphatase. It is actually a hydrolase which splits the pyrophosphate bond of DPN and TPN, to form NMN and either AMP, or ADP with the second phosphate group on the 2' position of the ribose (209).

The last two kinds of DPNase have not been found in the erythrocyte of mammals.

In the reticulocyte the nucleosidase has been investigated and some experimental results are shown in Table XIX. The experiments also were designed to test the possibility of the presence of more than one DPNase. The measurement of DPNase activity is achieved by estimating the amount of DPN remaining at the end of a definite period of incubation of the DPN with the enzyme. As with the mature erythrocyte, all the DPNase activity of the reticulocyte is present in the stroma, and it appears that the only DPNase present in the reticulocyte is the nucleosidase. This is indicated by

TABLE XIX

Distribution and Activity of DPNase of Reticulocytes

Fraction	μMoles DPN split / mgm. dry wt./hr.		% of Activity		% of Activity accounted for	
	ADH	CN^-	ADH	CN^-	ADH	CN^-
Whole hemolysate	0.43	0.46	100	100		
SFH	0.00	0.00	0	0		
Stroma	4.28	4.45	90	88	90	88
			% inhibition			
Whole hemolysate nicotinamide	0.06	0.06	87	88		

Reaction mixture: 0.06 ml. enzyme fraction (81% reticulocytes); 0.8 mgm. DPN; 0.04 M phosphate buffer at pH 5: nicotinamide where present, 0.06 M; volume made up to 1 ml. with 0.154 M KCl.
Temperature: 37°C.
DPN measured by the alcohol dehydrogenase (ADH) and cyanide (CN^-) methods.

the good agreement between the measurements by the alcohol dehydrogenase and cyanide methods. The former method measures the intact DPN present, while the latter method measures only the nicotinamide-ribose bond. If either of the DPN pyrophosphatases were present, the DPNase activity as measured by the alcohol dehydrogenase method would be greater than

when measured by the cyanide method, since the breakdown of DPN at the pyrophosphate bond would not alter the reactivity of the fragment with cyanide. That nicotinamide inhibits the breakdown of DPN by the same amount according to both methods of assay, is further proof of there being only one DPNase in the reticulocyte.

VII

MATURATION OF THE RETICULOCYTE

The activity of the various enzymes referred to in the previous section was determined in several reticulocyte preparations, and the mean for the results obtained with each enzyme was calculated. A similar procedure was adopted for the corresponding enzymes in the mature erythrocyte. In this way the activity of each enzyme in the two types of cell could be compared.

Since the proportion of reticulocytes in the different preparations varied considerably (from 50 to more than 90 percent reticulocytes), it was considered desirable to adjust the assay values to what they would have been if the specimens had consisted entirely of reticulocytes. The appropriate correction was applied for the activity of the normal erythrocytes present in the sample.

It was assumed for the purpose of this correction, that the dry weight of the reticulocyte is identical with that of the normocyte. The reticulocyte has a lower specific gravity (12) than the normocyte. On the other hand it also has a larger volume (30). The most important justification for the above assumption, however, is the fact that reticulocytes are supposed to possess their full complement of hemoglobin (7), and since this substance makes up most

of the dry weight of the red cell, it is probable that any error involved in the above mentioned correction, would be small.

The results obtained are summarised in Table XX. They give an idea of the overall changes in the enzyme activity which may be expected to occur during the transition from the reticulocyte to the mature erythrocyte.

The correlation between the disappearance of the stainable material of the reticulocyte, with the changes in the RNA content of the blood and the changes in the activity of several enzymes was attempted in a series of four experiments. The enzymes which disappear or become weaker in activity during the maturation of the reticulocyte were of special interest.

For the purpose of these experiments, heparinised blood containing reticulocytes, was incubated at 37°C. to which glucose had been added, to a final concentration of 0.05 M, to sustain cell metabolism. Penicillin was added to inhibit bacterial growth. Samples were taken at zero time and thereafter at two-hour intervals for a total of twelve hours. The reticulocyte count was determined as soon as the samples were taken and the red cells were centrifuged, washed and stored in the cold overnight. The assays of enzymatic activity in all the samples were performed the next day. Just before proceeding with the assays, the red cell samples were hemolysed and fractionated as required,

TABLE XX

Comparison of Activities of Enzymes in the Reticulocyte and the Normocyte

Enzyme	Preparation	Reticulocytes			Normocytes		Reticulocyte / Normocyte
		N[1]	Actual Q[2]	Corrected Q[3]	N	Q	
DPNase	Stroma	5	5.2 ± 1.5[4]	5.2 ± 1.5	3	4.9	1.1
Lactic dehydrogenase	SFH	5	14.7 ± 2.7	17.7 ± 4.1	4	10.3 ± 2.5	1.7
Glucose-6-phosphate dehydrogenase	SFH	2	1.02	1.25	2	0.68	1.8
Isocitric dehydrogenase	W.H.	4	2.3 ± 0.2	3.0 ± 0.1	4	1.6 ± 0.3	1.9
Malic dehydrogenase	SFH	6	6.8 ± 2.2	9.1 ± 2.4	4	3.4 ± 0.3	2.7
Fumarase	W. H.	7	0.44 ± 0.17	0.66 ± 0.14	4	0.05 ± 0.01	13.2
Hexokinase	W. H.	4	0.26 ± 0.03	0.41 ± 0.11	4	0.03 ± 0.01	13.7
Pyrophosphatase	SFH	3	4.5	5.8	2	0.3	19.3
Succinic dehydrogenase	Stroma	4	19.1 ± 4.3	33.0 ± 3.4	3	0	∞
Aconitase	W. H.	2	0.021	0.024	2	0	∞
Cytochrome oxidase	Stroma	4	3.1 ± 1.0	5.5 ± 2.1	3	0	∞

(1) N Number of samples.
(2) Units of Q values: as described in the discussion of the individual enzymes.
(3) Q corrected to 100% reticulocytes.
(4) S.D., when given. Calculated for enzymes having more than 3 samples. Others, arithmetic mean.

as described under Methods. The results of these experiments are recorded in Table XXI and in Figures 23-26.

Table XXI shows the overall changes which have taken place in the twelve hours of incubation while Figures 23-26 show the changes which are taking place during the incubation.

From the values it can easily be appreciated that there is no relationship between the progressive changes occurring in the activity of the enzymes and those taking place in the reticulocyte count. Lactic and malic dehydrogenases, and DPNase, which in the mature cell are nearly as active as in the reticulocyte (Table XX) showed no change in activity, or only a small decrease, in all the experiments in which they were tested. The aconitase activity fell to 11 percent of its original activity during an experiment in which the decrease in the reticulocyte count was only 13 percent. In another experiment, the reticulocyte count decreased by 28 percent of its original activity and the decrease in the aconitase was only 52 percent. Succinic dehydrogenase and cytochrome oxidase, which like the aconitase are absent from the normocyte, underwent a smaller change than did fumarase and pyrophosphatase both of which persist in the mature cell.

The proportion of reticulocytes in the original blood specimen, or the extent to which the reticulocytes matured morphologically appears to have had little bearing on the rate or the extent of the changes that occurred in

TABLE XXI

Changes in the Enzyme Activity of Reticulocytes during Incubation at 37°C.

Incubation	1	2	3	4
Original % of reticulocytes	94	43	74	60
Final % of reticulocytes	81	23	53	10
Final RNA (% of original)	82	75	71	-
Extent of hemolysis at the end of the experiment	2	2	2	1
Enzymes Studied				
Hexokinase	60	66	75	-
Aconitase	11	-	48	-
Succinic dehydrogenase	61	43	42	-
Fumarase	63	70	83	79
Cytochrome oxidase	-	47	-	47
Pyrophosphatase	16	-	22	-
Lactic dehydrogenase	-	90	87	100
Malic dehydrogenase	-	-	100	80
DPNase	-	-	-	81

Assays of the enzymes: as described in the section on Methods, and the discussion of the individual enzymes.
Other assays as described under Methods.
Incubation time: 12 hours.

FIGURE 23

Incubation 1.

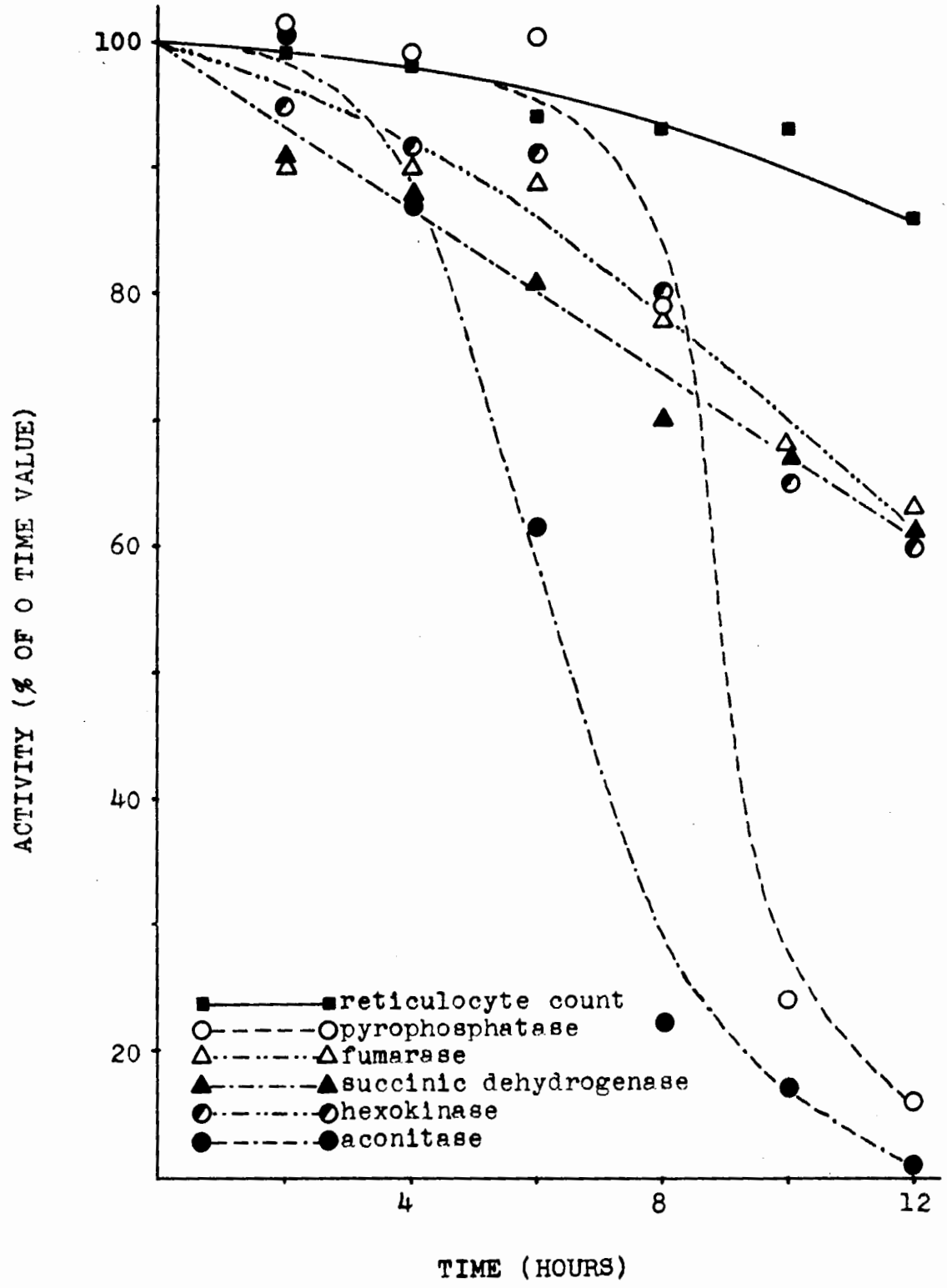


FIGURE 24

Incubation 2.

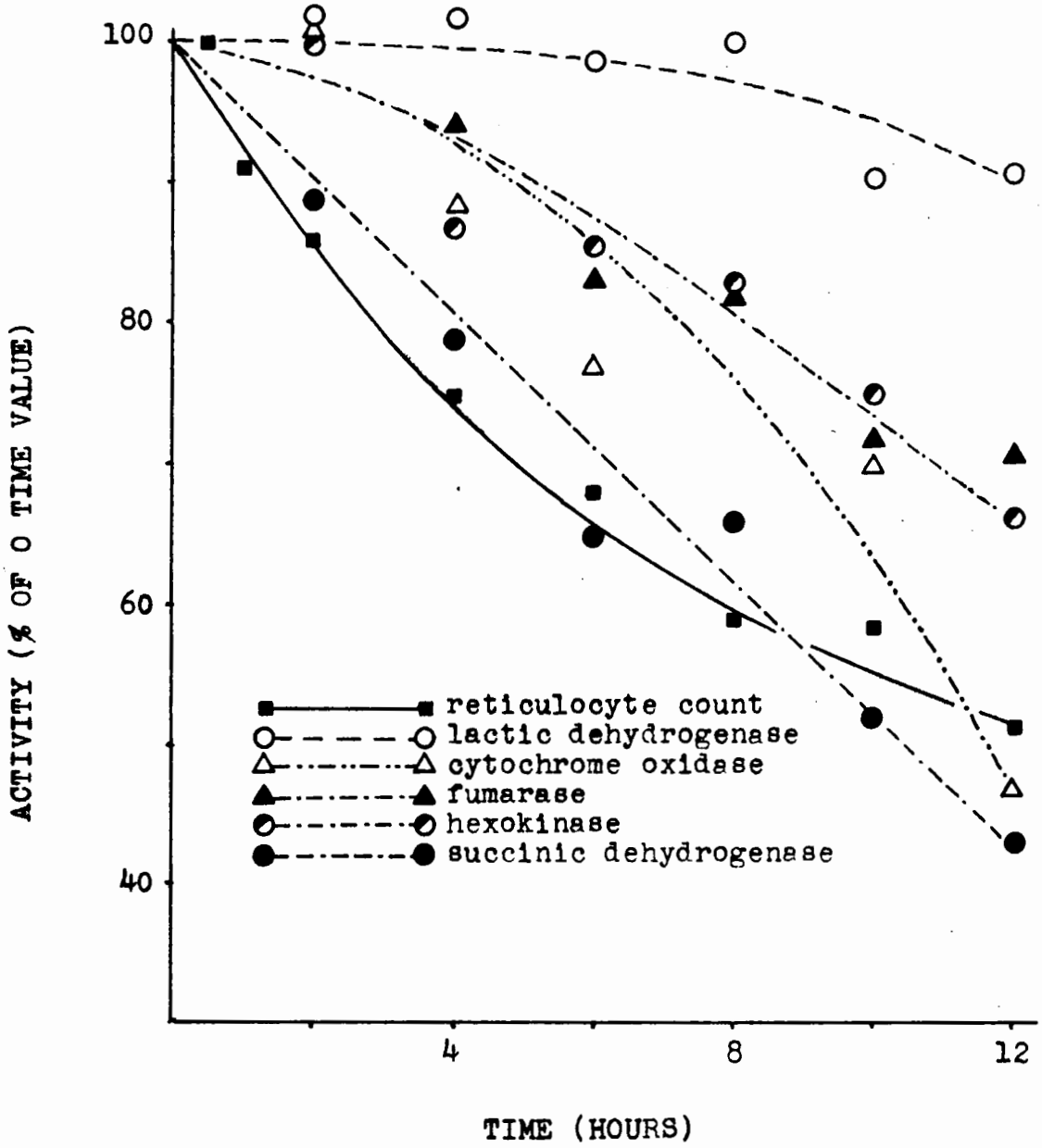


FIGURE 25

Incubation 3.

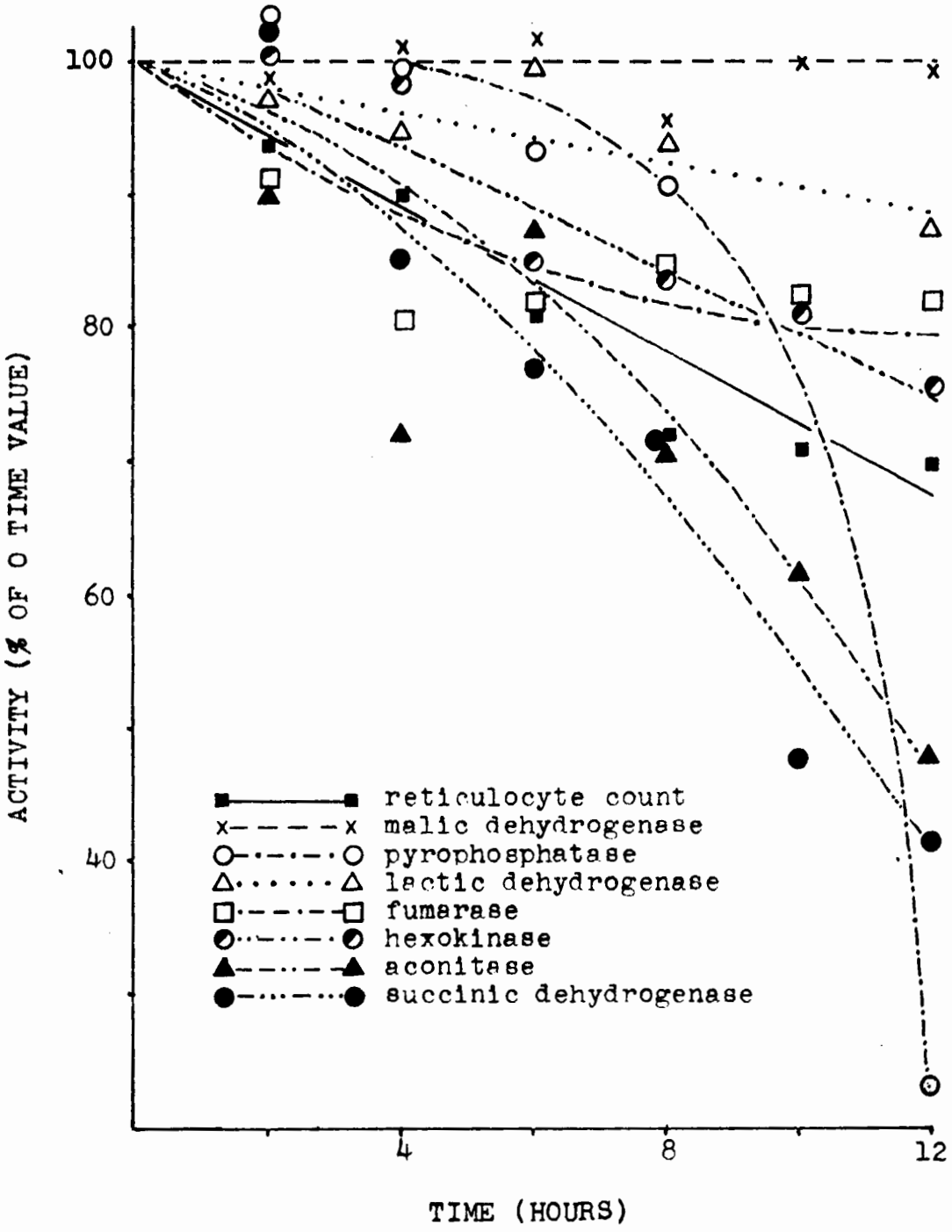
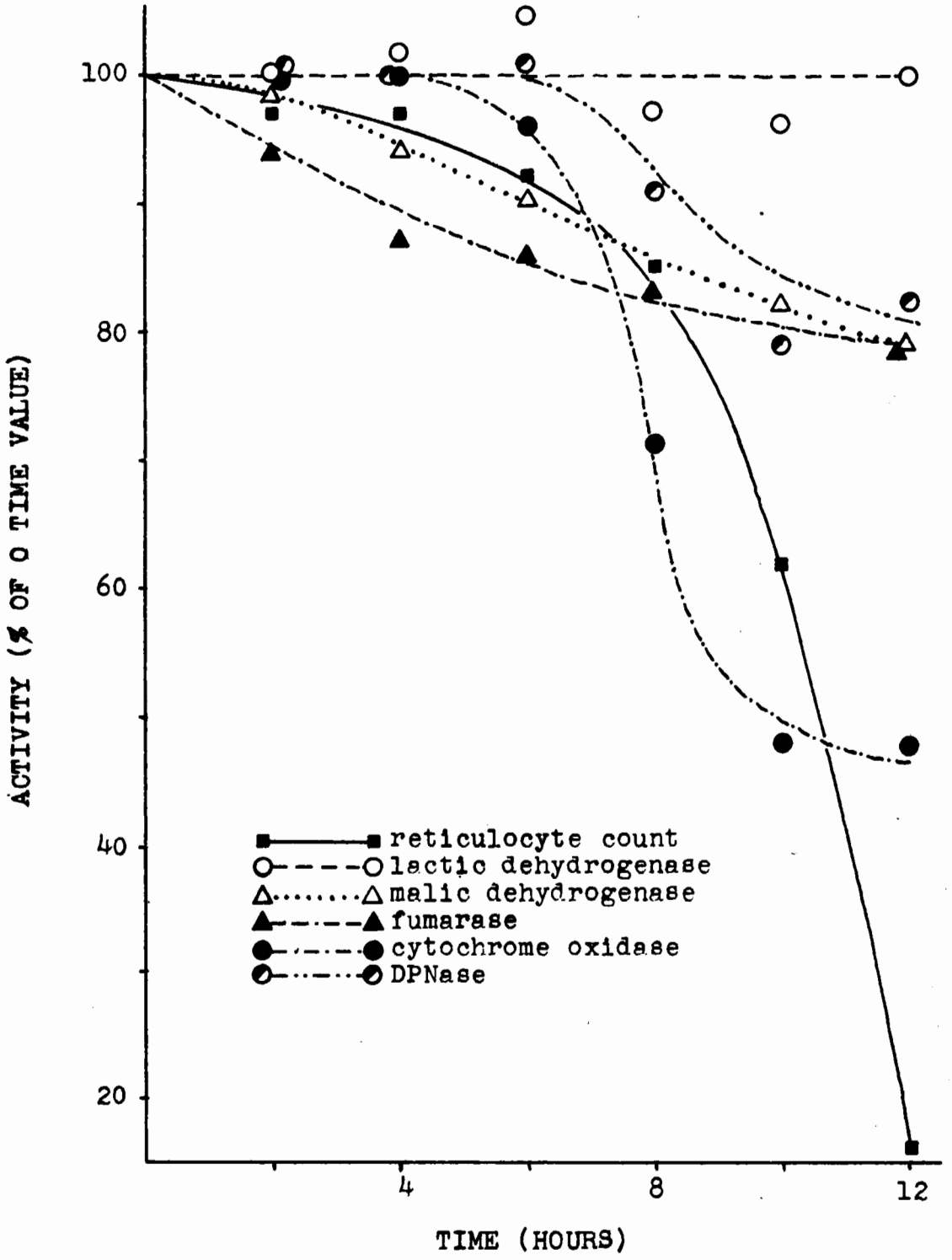


FIGURE 26

Incubation 4.



the activity of the enzymes. Furthermore, one cannot ascribe a "pacemaker" role to any of the enzymes, that is to say, there was no evidence that the behaviour of any of the enzymes consistently dominated or regulated the time or character of the behaviour of the others.

There appears to be no consistency in the manner in which the changes in the enzyme activity took place during the incubation from one experiment to another, or between the different experiments.

The experiments revealed no relationship between the rate of disappearance of the stainable material from the cells, i.e. morphological maturation, and the decrease in the enzymatic activity, i.e. enzymological maturation.

In the circulating blood the concentration of RNA is proportional to the number of reticulocytes (17, 22). During the incubation of blood containing a high proportion of reticulocytes on the other hand, the rate or extent of the decrease in the concentration of RNA does not always parallel the rate of disappearance of the reticulocytes.

The lack of correlation observed may possibly have been due to the conditions employed for the experiments. As has already been pointed out, cells which, according to staining and counting techniques appear to be morphologically mature may not necessarily be found to be mature if examined by more sensitive methods, such as for example, examination by phase contrast microscopy.

VIII

DISCUSSION AND CONCLUSIONS

It is now possible to say with certainty that the mature mammalian erythrocyte does not respire. That the cell has no tricarboxylic acid cycle in its enzymological make-up is evidenced, not only by the almost complete lack of oxygen consumption, but also by the fact that several of the enzymes involved in the tricarboxylic acid cycle are lacking. Among the missing ones are succinic dehydrogenase and aconitase, and cytochrome oxidase which is necessary for respiratory activity.

The reticulocyte on the other hand, exhibits a significant oxygen consumption which compared with that of other tissues is of relatively small magnitude. The evidence obtained from studies with inhibitors, and the demonstration of enzymes from the tricarboxylic acid cycle make it safe to conclude that the oxygen consumption of the reticulocyte represents true respiratory activity.

Sherwood Jones et al. (181) have shown that citrate accumulates in rat reticulocytes poisoned by fluoroacetate. The latter substance is converted enzymatically to fluorocitrate which is a specific and potent inhibitor of aconitase (235). The same authors have shown that the respiratory activity of the reticulocyte is abolished in the presence of 10^{-4} M cyanide. We have confirmed the inhibition of the respiratory activity

by malonate, and we have shown that this substance prevents the regeneration of ATP in the cell. DNP, which is known to uncouple oxidative phosphorylation, likewise prevented the formation of ATP in the cells.

Cytochrome oxidase and all the enzymes directly involved in the citric acid cycle, with the exception of pyruvic and α-ketoglutaric oxidases, and oxalsuccinic carboxylase, have been demonstrated in the reticulocyte. The presence of the pyruvic oxidase system is evident from the results already referred to, of Sherwood Jones et al. (181). The α-ketoglutaric oxidase system appears to be similar to the pyruvic oxidase, with the exception of the "dehydrogenase". Furthermore, the fact that hemoglobin is synthesised in the reticulocyte (45) from δ-aminolevulinic acid, and this in turn is formed from glycine and active succinate (possibly succinyl-CoA) (236), makes it probable that α-ketoglutaric oxidase is also present in the reticulocyte.

The spontaneous decarboxylation of oxalsuccinate has been shown by Kornberg et al. (234) to occur quite readily especially in the presence of any of various cations.

The citric acid cycle is generally considered to be associated with the mitochondrial structure, and the activity of the enzymes involved in this system, with the exception of aconitase and isocitric dehydrogenase, is associated with these particles. Rubinstein (174) in our laboratory,

has demonstrated the presence of the tricarboxylic acid cycle in the avian erythrocyte, which contains no demonstrable mitochondria. The oxidative cycle in this cell appears to be associated with the nuclear material (174). The tricarboxylic acid system in the reticulocyte, which lacks the nucleus and has no demonstrable mitochondria, most probably is resident in the stroma, since this fraction contains, not only all the cytochrome oxidase and succinic dehydrogenase, but also variable amounts of the other enzymes of the cycle. The stroma fraction contains only 21 and 33 percent of the activity of fumarase and isocitric dehydrogenase, respectively. It is possible that in the intact cell, the greater proportion of the enzymes is found in the stroma, and that some of the enzyme activity may become dissociated from the stroma, by solubilization, during the process of preparation of the enzyme fractions.

It may be that the mitochondria which cannot be demonstrated in the reticulocyte, became associated with the stroma during the maturation of the erythroblast and thus the structural relationship necessary for the function of the citric acid cycle, may be maintained.

It is probable, also, that the reticulocyte is a degenerating cell, in which the oxidative cycle is in the process of breaking down, possibly by solubilization of the enzymes. This would explain the observed presence of relatively large amounts of certain enzymes in the cytoplasm.

The observation that succinic dehydrogenase is detectable only in the stroma may be due to the conditions of the assay method. As already mentioned, the solubilization of succinic dehydrogenase destroys its capacity to transfer electrons and hydrogen ions to any of the common dyes (131). The same may be true for cytochrome oxidase. These two enzymes, therefore, could be present in the cytoplasm, but if so, would be there undemonstrated.

Comparing the mature erythrocyte, with the reticulocyte, on an enzymological basis, the latter is, in most cases, far more active. Succinic dehydrogenase, aconitase and cytochrome oxidase, can only be demonstrated in the reticulocyte, and fumarase is ten times more active in the reticulocyte than in the normocyte.

Of the enzymes studied, which are not associated with the tricarboxylic acid cycle, hexokinase and pyrophosphatase are both much more active in the reticulocyte; DPNase and the pyridine nucleotide-linked dehydrogenases, do not differ too greatly in their activities in the two cells.

It is apparent from this study that the enzyme activity disappears more readily from the stroma than from the cytoplasm. DPNase remains unchanged, while aconitase disappears from both fractions. Succinic dehydrogenase and cytochrome oxidase, malic and lactic dehydrogenases, and hexokinase, are generally absent from the stroma of the mature

erythrocyte. The last three mentioned enzymes, although absent from the stroma persist in the cytoplasm.

An extensive disruption of the metabolic systems of the reticulocyte takes place during the maturation of this cell. The capacity to carry on respiratory activity is lost. Concurrently, the activity of hexokinase is greatly diminished. Maturation, therefore, presents a picture of progressive metabolic deterioration. The process of failure continues, though at a slower rate, in the erythrocyte until by the 125th day, the cell is presumably no longer capable of maintaining its energy requirements and thus becomes nonviable.

The nucleus, which in somatic cells is supposed to control the reproduction and individuality of the cells, presumably has some regulatory influence on the synthesis of enzymes in the cell. Hence, the loss of the nucleus will necessarily have a pronounced effect on the metabolic activity of the cell. This would be evidenced by, among other things, a progressive decrease in the activity of the enzymes within the cell. Enzymes, as other proteins of the cell, will have a turnover rate, and in the absence of a system regulating the resynthesis of enzymes, a general failure of the metabolic systems of the cell will occur. Thus the metabolic degeneration of the reticulocyte, and later the normocyte is to be expected. Furthermore, the process is irreversible. The

reticulocyte having been without nucleus for less time than the normocyte would be expected to be, as in fact it is, more active enzymatically, than the normocyte.

In studies of the maturation of the reticulocyte, in which cells were incubated at 37°C. and the activity of several enzymes was tested periodically, the results obtained were very erratic. Even though the expected diminution was observed in the activity of certain enzymes, no consistent pattern of change was evident. From our results, even an approximation of the maturation time of reticulocytes, cannot be stated with any certainty. Furthermore, no correlation was evident between the changes in the reticulocyte count and the enzyme activities, or the RNA content of the samples. This is to be expected if one considers that the reticulocytes found in a sample inevitably differ in age, and that different samples will have different proportions of reticulocytes of various ages. The cell, to our manner of counting, remains a reticulocyte as long, and only as long, as some material is visible which stains with brilliant cresyl blue. Thus a young reticulocyte, with a high concentration of RNA can undergo a great decrease in the RNA content before it can be considered to have matured into a normocyte. In samples with a high proportion of young reticulocytes, therefore, one might expect on incubation, to obtain a change in the enzymatic activity of greater magnitude than the decrease in the reticulocyte count. The opposite would be true for

samples containing a preponderance of old reticulocytes.

The only accurate method of investigating the morphological maturation of reticulocytes would involve the segregation of these cells into different age groups, for the purpose of counting them. The different groups presumably, would be characterised by the amount of stainable material in the cell. The changes occurring with time, in each group, would have to be recorded, and finally the overall picture would have to be analysed. Such a procedure would inevitably be tedious, and would require a good deal of time and practice. Because of these difficulties it was not considered practical to adopt such a procedure for counting the reticulocytes.

Although it is generally accepted that the reticulocyte is an intermediate in the normal process of erythropoiesis, this has not been conclusively established. It may be that the reticulocyte appears only, or mainly, in states of hemopoietic emergency, in which the supply of erythrocytes is suddenly and drastically curtailed. In such emergencies the reticulocytes may be delivered into the circulation by the bone marrow, to ensure an adequate oxygen supply to the tissues, until the time when sufficient numbers of normal erythrocytes can be manufactured.

That reticulocytes lose their stainable material and assume characteristics similar to those of normocytes has been demonstrated innumerable times, but it has not been

shown, to the writer's knowledge, that these cells are truly normocytes.

The resistance of the hemoglobin of reticulocytes to denaturation by phenylhydrazine, casts some doubt on the notion that this cell is the precursor of the normocyte. It is possible, however, that the differences in the resistance to denaturation are attributable to certain differences in the permeability properties of the membranes of the two types of cell.

The question of the position in the erythropoietic process of the reticulocyte, is of considerable relevance to the present study, but is left to be answered by the histologist.

SUMMARY

1. The respiration of the reticulocyte is markedly increased by succinate but is inhibited by malonate and by fluoride. The synthesis of ATP in the cell was found to be impaired both by DNP and by malonate; even in the presence of malonate, the presence of glucose was adequate in maintaining the normal ATP level in the cell, thus showing that the glycolytic process alone is able to maintain the energy metabolism of the cell.
2. The reticulocyte preparations were fractionated into a particulate and a cytoplasmic fraction and the activity of several enzymes in these two fractions was determined, and compared with the activity of the corresponding enzymes in the normocyte.
3. Cytochrome oxidase, aconitase, and succinic dehydrogenase were found only in the reticulocyte; the activity of hexokinase, fumarase, and inorganic pyrophosphatase was much greater in the reticulocyte than in the normocyte, while that of the malic, lactic, isocitric, and glucose-6-phosphate dehydrogenases, and DPNase was about equal in the two types of cell.
4. The distribution of the enzymes between the stroma and the cytoplasm in the reticulocyte is variable. Of the enzymes studied, glucose-6-phosphate dehydrogenase and

pyrophosphatase are present only in the soluble fraction, while cytochrome oxidase, succinic dehydrogenase and DPNase are measurable exclusively in the particulate matter. The other enzymes are distributed in both ffactions. It seems probable, therefore, that the tricarboxylic acid cycle, if it exists in this cell, is situated in the particulate fraction.

5. The morphological maturation of reticulocytes, in vitro, at 37°C., and the concurrent changes in the activity of several enzymes were studied, but no consistent pattern of behaviour in the maturation process was observed.
6. The stroma fraction of the reticulocyte and the normocyte, was found to inhibit the lactic and the malic dehydrogenase activity of these cells.
7. The use of ferricyanide as a terminal electron acceptor in the spectrophotometric assay of DPN- and TPN-linked dehydrogenase systems, and also in the oxidation of succinate and tyramine was successful with liver homogenates. It was probably successful with reticulocyte preparations, but not so with hemolysates of mature mammalian erythrocytes.

APPENDIX 1

The possibility that the hemoglobin of the reticulocyte differs from that of the mature erythrocyte was checked in preliminary experiments by means of paper electrophoresis.

Hemoglobin was prepared according to the method suggested by Block et al. (230). Stroma-free hemolysate was treated with 0.4 volumes of toluene, shaken for ten minutes, centrifuged, and the top two layers removed and discarded. The rest was filtered through cotton, and the concentration of hemoglobin in the sample was adjusted to the desired level (123). The electrophoresis apparatus used was of the Durrum type.

Samples of hemoglobin from rabbit blood containing approximately 50 percent reticulocytes, showed no evidence of separation of the hemoglobin into components, in experimental runs of up to fourteen hours duration.[#] On electrophoretic analysis of the hemoglobin from a blood sample from a dog with 50 percent reticulocytes[##], the appearance of two bands was visible after fourteen hours.[#] This experiment has not been repeated, and a control experiment employing hemoglobin from mature dog erythrocytes has not been carried out.

[#]Constant current of 2.75 ma. per strip of Watman 3MM paper was employed. The buffer was veronal, at pH 8.6, and ionic strength 0.05.

[##]Donated by Dr. P. B. Stewart, of the Department of Physiology, McGill University.

The failure, in the first experiments, of two distinct bands to appear on the electrophoresis of the hemoglobin from rabbit reticulocytes, is not conclusive proof of absolute homogeneity of the pigment. It is possible for two types of hemoglobin to have the same electrophoretic mobility. This is true of the hemoglobin types A and F in human blood (231). These, however, can be distinguished by their differential liability to denaturation by alkali (75, 232).

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