A COMPARISON OF GLYCEROPHOSPHATE AND LACTATE METABOLISM IN LIVER AND MUSCLE OF THE RAT

by

David Gaudin, B.S.

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I. Preface

In recent years various pathways of carbohydrate metabolism in addition to the classic Embden-Meyerhoff pathway have been discovered. The reactions involved in Glycerophosphate (GP) metabolism constitute one such pathway. However, rather than being an entirely separate pathway, the metabolism of GP may be looked upon as a branching or side chain of the Embden-Meyerhoff glycolytic scheme. This reaction sequence involves the diversion of one of the triosephosphate molecules produced during glycolysis, in other words the diversion of dihydroxyacetone phosphate (DHAP) to GP. Several roles are generally attributed to this branching of the glycolytic pathway. First, under aerobic conditions, dihydroxyacetone phosphate and GP are believed to act catalytically in transporting hydrogen of cytoplasmic DPNH to the electron transport chain of mitochondria. Secondly, under anaerobic conditions, DHAP has been shown to compete with pyruvate as a hydrogen acceptor. Thirdly, GP is a connecting link between the pathways of carbohydrate and lipid metabolism.

The experimental work conducted as part of this thesis is concerned with the study of the relative amounts of lactate and GP accumulation in muscle under anaerobic conditions. Obviously, if one is to measure the increase in concentration of a substance anaerobically the starting point or in situ level of that substance under aerobic conditions must be known. Therefore, the in situ levels of both GP and lactate were determined and compared. Since GP accumulation in liver has already been demonstrated, quantitative comparisons of GP accumulation in muscle will be made with that in liver as well as

with lactate production in muscle.

Two experimental designs were utilized. In one, the in situ changes of both GP and lactate levels were compared in liver and muscle under both aerobic and anaerobic conditions. The second experimental design involved the Warburg experiment. Here, GP levels in the tissues and appearance of both lactate and GP in the Warburg medium were compared, again under both aerobic and anaerobic conditions. GP was measured in both tissues and Warburg medium, because, unlike lactate, its concentrations in intracellular and extracellular fluids have not been shown to be in equilibrium.

Since GP levels have been reported to parallel pyruvate levels in diaphragm after a seven second tetanus it was interesting to determine whether or not the concentration of GP appearing in the Warburg medium was the same as pyruvate concentrations, the level of which has already been demonstrated in this laboratory under the same experimental conditions. The relative amounts of GP production by skeletal muscle and diaphragm were also determined in the Warburg experiments.

The relationship between the reactions involving GP and glycolysis are shown on the following chart.

F-6-P	fructose-6-phosphate
F-1,6-diP	fructose-1,6-diphosphate
G-1-P	glucose-1-phosphate
G-6-P	glucose-6-phosphate
DHAP	dihydroxyacetone phosphate
GP	L(-) glycerophosphate
GAP	D-glyceraldehyde-3-phosphate
1,3-diP Gly	1,3-diphosphoglycerate
3P Gly	3-phosphoglycerate
2P Gly	2-phosphoglycerate

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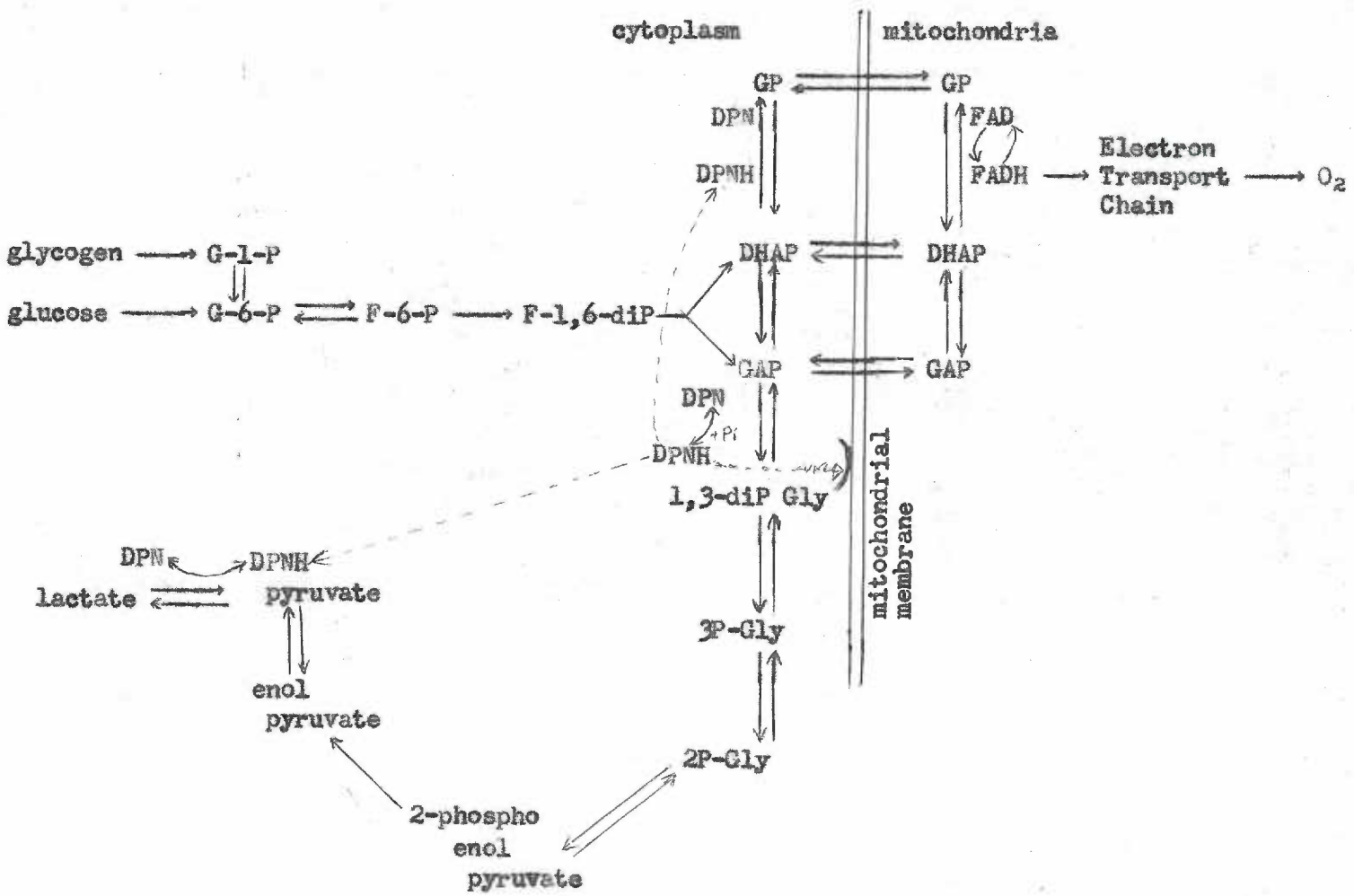
The Relation Between Glycolosis and the Glycerophosphate Cycle



II. Introduction

1. Historical

Glycerophosphate dehydrogenases are widely distributed throughout the plant and animal kingdoms. Since respiratory activity of mammalian tissues towards glycerophosphate (GP) was first discovered by Meyerhof in 1919 (111), two glycerophosphate dehydrogenases have been found in mammalian tissues. The enzyme studied by Green in 1936 (62, 64) is found in mitochondria, whereas that studied by von Euler in 1937 (167) is localized in cytoplasm (179). The cytoplasmic enzyme is DPN-linked (167), and catalyzes the reduction of cytoplasmic dihydroxyacetone phosphate (DHAP) to GP. GP found in the mitochondria can be oxidized to DHAP by a mitochondrial glycerophosphate dehydrogenase. This dehydrogenase is a flavoprotein. Very few flavoprotein dehydrogenases are known, succinic dehydrogenase being one of them (175). Both of the glycerophosphate dehydrogenases are believed to be universally present in mammalian muscle. In addition to being present in mammals, the glycerophosphate dehydrogenases are also found in birds, amphibians, and fish; the pigeon, toad, and goldfish being representative examples (50). Similarly, the two enzymes have been demonstrated in insect tissues and have the same cytological localization as their mammalian counterparts (140, 141). Yeast also has a GPDH (DPN) (117). This enzyme is similar to that found in mammalian muscle even to requiring an unusual cofactor which will be discussed later (162, 168). Somewhat different glycerophosphate dehydrogenases are to be found in

lower forms of plant and animal life. For instance, S. faecalis, an organism devoid of the cytochromes, has a soluble GP oxidizing enzyme which catalyzes the direct oxidation of GP by molecular oxygen. This is a flavine adenine dinucleotide (FAD) linked enzyme which is specific for α-GP, and will not catalyze reactions of β-GP or 1, 2-propanediol-1-phosphate (82). Trypanosoma rhodesience, a blood parasite, contains a GP oxidizing enzyme associated with the particulate portion of the cell. However, the Trypanosoma have no mitochondria or cytochrome chain. This enzyme catalyzes the direct oxidation of GP by molecular oxygen, and is present in sufficient activity to account for a major portion of the respiration of intact Trypanosoma (61). Lactate is not a major product of glycolysis in Trichinella spiralis larva, which contain an active glycerophosphate dehydrogenase (1).

Green described the properties of an enzyme from rabbit skeletal muscle which is specific for L(-)α glycerophosphate. He found the enzyme to be an anaerobic dehydrogenase from the particulate portion of the cell. However, he was not able to bring the enzyme into solution in an active form. Green also attempted to identify the product of the reaction and obtained a substance which he identified as an aldotriose phosphate. Although he believed the product to be glyceraldehyde-3-phosphate the possibility that it might be DHAP was not ruled out. Green, however, did not consider the stereochemistry of the reaction (62). This type of investigation was left to Fischer and Baer (54), who concluded that living tissues must attack GP at the

number two carbon to produce DHAP. Since the product is active in the glycolytic system, the mitochondrial flavin adenine dinucleotide linked glycerophosphate dehydrogenase (GPDH) (FAD) could not attack GP at the number one carbon. Such a reaction would produce L-glyceraldehyde-3-phosphate instead of the glycolytically active D-glyceraldehyde-3-phosphate. Fisher and Baer's conclusions were, however, of an indirect nature. Tung, Anderson, and Lardy (165) rather than using a crude preparation as had Green (63), managed to bring the enzyme into solution with deoxycholate and then free it from contaminating enzymes. In this way they were able to provide conclusive experimental proof that DHAP is really the product of GP oxidation. At the same time they were able to explain Green's finding of GAP by showing that triose phosphate isomerase can interconvert DHAP and glyceraldehyde-3-phosphate even in the presence of a carbonyl trapping agent such as hydrazine. Also, a crude mitochondrial preparation such as Green used must have contained triose phosphate isomerase, this latter enzyme having been demonstrated in mitochondria (26).

Fiske and Subbarow (55) in 1929 were the first to isolate GP from tissues under conditions which precluded the possibility of its formation from lecithin or other phospholipid. These workers were surprised to find the α- form of GP. Previously lecithin had been thought to consist primarily of the β- form. The β-isomer of GP has since been shown by Baer and Kates (6, 7) to be formed during the alkaline hydrolysis of phospholipids by a reversible migration of the

phosphate radical between the α- and β- positions. Meyerhof and Green (113) obtained preparations of α-GP as pure as 98% by repeated barium salt precipitations.

GPDH (DPN) of rabbit muscle was once considered to catalyze the interconversion of 1,2-propanediol-1-phosphate and acetol phosphate (115, 154). However, the earlier work was done with crude enzyme preparations. Huff (79), using more highly purified enzymes, reports that GPDH (DPN) oxidizes GP 1000 times faster than 1,2-propanediol-1-phosphate, and suggests that oxidation of the latter compound normally is catalyzed by a different enzyme. D-erythrulose-1-phosphate, on the other hand, is reported to be reduced by GPDH (DPN) at 10% of the rate of the natural substrate DHAP (43).

As in many other cases the metabolism of GP was not investigated for many years following its initial discovery. At first GP was considered to play only a minor role in lipid metabolism. From a quantitative point of view, GP can provide only a very small portion of the energy obtained from the oxidation of fats. Only in recent years has GP been implicated in carbohydrate metabolism. The early discoverers of the role of GP in carbohydrate metabolism felt that GP plays an important role only in insect metabolism, and that it is the product of a relatively unimportant side reaction in mammals. This point of view has slowly disappeared as more work has been done on mammalian muscle and other tissues. Today the opinion is expressed that lactate production can represent the greater proportion of anaerobic glycolysis only in tumors.

2. Glycerophosphate Metabolism of Insects

The flight muscles of insects have many unique characteristics which make a study of them of general interest. In addition, many workers have been interested in comparing the metabolic processes of insects with those of mammals. The flight muscles of insects are among the most efficient in the animal kingdom. Not only are they able to pass through the relaxation-contraction cycle at tremendous rates (over 1000 contractions per second) but they can work continuously for long periods of time (161). The flight muscles of insects, like their mammalian counterparts, depend on an adequate supply of carbohydrates and fats for their supply of energy. In recent years, however, evidence has been found that the metabolism of a number of insects, and possibly of a majority, differs markedly from that of the vertebrates. Locusts, for example, are able to utilize fats almost exclusively as an energy supply, especially during long flights. Lepidoptera seem to be even more specialized in this direction. They are thought to convert their carbohydrates into fats before utilization, even though their caloric intake is almost entirely in the form of carbohydrates. In addition to insects which utilize primarily fats or carbohydrates as an energy source, other insects take an intermediate position.

In 1956 Zebe and McShan (182) undertook a series of experiments designed to compare the two metabolic types, using honeybees and locusts as examples of the carbohydrate and fat utilizing insects

respectively. A surprising feature was found. The flight muscles of both types of insects contained a very low lactic dehydrogenase (LDH) activity. The similarity of results suggested that perhaps this low LDH activity is characteristic of insect flight muscle in general. In order to verify this hypothesis, similar studies were conducted on a wide variety of insects. The LDH activity proved to be low in all insect flight muscles studied.

Many of the initial studies of GP metabolism were done with insect tissues. Early workers, interested in comparing the metabolism of insects with that of mammals, discovered that the amount of lactate produced anaerobically by insect tissues was not sufficient to account for either the glycogen and glucose utilized (2, 44, 59, 94), or the oxygen debt (44). When carbon dioxide evolution from a bicarbonate medium was used as a measure of the acid produced during the anaerobic incubation of cockroach muscle, the amount of lactate produced was insufficient to account for the carbon dioxide produced (13, 164). Glycogen and glucose of insect flight muscle are known to be utilized quite rapidly even under anaerobic conditions. The relative unimportance of the reduction of pyruvate to lactate in cockroach muscle is demonstrated by the fact that the anaerobic production of pyruvate can exceed that of lactate (80). Since the reduction of pyruvate to lactate is not sufficient to account for the hydrogen produced by the oxidation of glyceraldehyde-3-phosphate (GAP) some other reaction had to be considered. One such suggestion was that fatty acids might be

formed (59). Other workers suggested the possible accumulation of an intermediate phosphate ester (164). A phosphate ester highly resistant to alkaline hydrolysis was found to accumulate during the anaerobic incubation of cockroach muscle, and the suggestion was made that it might be GP (99). Although initially some confusion was caused by the incorrect chromatographic identification of the accumulating intermediate as a phosphoglyceric acid, it has since been positively identified as GP (37, 176).

In addition to the low lactic dehydrogenase (LDH) activity, comparative studies of the carbohydrate and fat utilizing insects have revealed another characteristic feature of insect flight muscle, namely its extraordinarily high glycerophosphate dehydrogenase (GPDH) (DPN) activity (141, 181, 182). Although there is considerable species difference, the high GPDH (DPN) activity is characteristic of all insect flight muscle studied. In comparing insect and mammalian tissues, Zebe and McShan (182) concluded that a reciprocal relationship exists between LDH and GPDH (DPN) activities.

ENZYME ACTIVITIES

	GPDH (DPN)	LDH
Insects (range)	164 - 800	3 - 40
Rat leg	29	440

It can be seen from the above data that, in a quantitative sense, insects do not possess the typical glycolytic scheme as we have come to know it in mammalian tissues, even though glycolytic enzymes other

than LDH are present in high activity. The possibility that insect flight muscles might not need an anaerobic mechanism was considered. Under such a system immediate and complete oxidation of metabolites would be accomplished without accumulation of some partially oxidized intermediate. However, this system does not take into consideration the high resistance of some insects to oxygen deficit (71, 125, 129). On the other hand, the high activity of both the cytoplasmic and mitochondrial glycerophosphate dehydrogenases suggested that the reduction of DHAP and oxidation of GP might be of considerable physiological importance. Although the glycolytic pathway of insects is qualitatively similar to that of mammals (38, 137), the quantitative difference is well brought out by the anaerobic conversion of hexose diphosphate to equimolar amounts of GP and pyruvate by muscle of the American cockroach (37). This has led to the suggestion that anaerobic glycolysis in insects should be represented by the following general equation (136):

Glucose + Inorganic phosphate (Pi) ——→ GP + Pyruvate

instead of the general equation for anaerobic glycolysis as we have come to know it in mammalian tissues:

Glucose ——→ 2 Lactate

Yet, as will be shown, the conversion of glucose or glycogen to GP and pyruvate is much less efficient in terms of ATP production than is their conversion to lactate only.

Recently evidence has been obtained suggesting that the accumula-

tion of GP cannot account for the major portion of anaerobic glycolysis in the housefly, Musca domestica. When houseflies are kept anoxic for three hours in a nitrogen atmosphere, GP accumulates for only the first thirty minutes. Glycogen, on the other hand, continues to be used for over an hour. The observed increase in GP content occurs primarily in the thoracic muscles (71). The increase in GP content of the flight muscles is accompanied by an increase in Pi and a decrease in both ATP and arginine phosphate. When the fly is moved from the nitrogen atmosphere back into normal room air, the GP content of flight muscles returns to about normal in five minutes. However, recovery of activity by the fly is not correlated with either GP or ATP levels. Rather, it is correlated with arginine phosphate levels and occurs after about thirty minutes in room air (129).

Insects possess both a mitochondrial and a cytoplasmic GPDH, just as do mammalian tissues. In fact, it was through a consideration of the equilibria of the two reactions catalyzed by these enzymes involving GP, as well as an appreciation of the cellular localization and possible interaction of the two glycerophosphate dehydrogenases, which led to the formulation by insect biochemists of our present concept of the GP-cycle (53, 148).

Of particular interest is the tremendous rate at which insect flight muscles can oxidize GP (33, 35, 53, 65, 139, 148). Just as in mammalian muscle, the oxidation product of GP by insect tissues is dihydroxyacetone phosphate (DHAP) (140). We find two reactions occur-

ring. First, in the cytoplasm, there is the reduction of DHAP (reaction 1). Secondly, in the mitochondria, there is the subsequent oxidation of GP coupled to oxidative phosphorylation (reaction 2). Particularly noteworthy is the fact that the sum of the two reactions represents the oxidation of cytoplasmic DPNH plus oxidative phosphorylation.

1. $DHAP + DPNH \longrightarrow GP + DPN$

2. $GP + 2\ ADP + 2\ Pi + O_2 \longrightarrow DHAP + 2\ ATP + H_2O$

SUM: $DPNH + 2\ ADP + O_2 + 2\ Pi \longrightarrow DPN + 2\ ATP + H_2O$

The oxidation of one GP is believed to result in the formation of two ATP's, P/O ratios ranging from 1.1 to 1.4 having been obtained (142, 143). These ratios are in agreement with work done on mammalian muscle. The GPDH (FAD) of insects is coupled to a cytochrome chain very similar to that of mammals (33, 34).

The equilibrium of the mitochondrial GPDH reaction lies so far towards the complete oxidation of GP that addition of DHAP at forty times the physiological concentration has no measurable effect on respiration by mitochondrial preparations (148). Saktor and coworkers observed the tremendous rate of GP oxidation by insect flight muscle mitochondria. However, their preparations would not oxidize pyruvate or members of the TCA-cycle at comparable rates. Ratios of the rate of GP oxidation to the rate of succinate oxidation as high as 10 to 1 were observed (33, 139). However, such extreme ratios were not consistently found, ratios of 2 to 1 (143) and 6.7 to 5.7 (142) also

being found. Saktor et al. suggested, as a result of these observations, that GP is the only substrate towards which mitochondria show sufficient activity to account for the tremendous increase in respiration observed when insects go from rest to flight. As a consequence of this they suggested that the TCA-cycle might not be of primary importance as a source of substrate for the respiratory chain of flight muscle (33, 139). Gregg, Heisler and Remmart are critical of Saktor's work and of his conclusions (65). These workers found oxidation rates of GP by flight muscle of the housefly (Musca domestica) which were even higher than those obtained by Saktor. But, and more importantly, the preparation used by Gregg et al. was able to oxidize pyruvate even more rapidly than GP. They concluded that "Since pyruvate can be oxidized completely by mitochondria and gives both a high P/O ratio and a high oxidation rate, it would appear to be a more logical source of energy for muscular contraction than GP, the oxidation product of which (DHAP) has been shown to accumulate in the mitochondria".

GP may play a more important role than simply being another substrate for mitochondrial oxidation. Just as do their mammalian counterparts, the flight muscle mitochondria of insects exhibit a relative impermeability towards DPNH (146). Comparison of the possible pathways of DPNH oxidation, which include 1) direct mitochondrial oxidation, 2) oxidation by pyruvate in the presence of LDH, leading to the accumulation of lactate, 3) oxidation via the GP-cycle, 4) the acetoacetate cycle (48, 49), and 5) reduction of oxalacetate by

a cytoplasmic malic dehydrogenase, indicates that, in flight muscle, GP provides the major vehicle for the passage of hydrogen from cytoplasmic DPNH across the mitochondrial membrane (146). In the thoracic muscles of the housefly, GP is normally present in considerable quantity, amounting to about 20% of the total acid soluble phosphate (177).

3. A Comparison of Glycerophosphate and Lactate Metabolism in Mammalian Tissues

DHAP has been, in a sense, considered to be a dead end compound of metabolism. Meyerhof, after observing the very high activity of GPDH (DPN) in mammalian tissues, expressed the opinion that "The large amount of triose phosphate isomerase present in muscle and the extremely large turnover number exhibited by this enzyme may be thought of as a mechanism developed by muscle cells to deal with the metabolically unfavorable triose phosphate equilibrium". At equilibrium about 95% of the triose phosphate is present as DHAP (112, 114). However, this equilibrium, when viewed in light of our present knowledge of the GP-cycle, lends further importance to the role of the GP-cycle, by providing it with a large amount of reducible substrate (DHAP). Under these circumstances the ability of the GP-cycle to oxidize cytoplasmic DPNH would not be restricted by a limiting amount of the substrate DHAP.

An examination of the possible scheme of reactions into which L-α-GP can enter reveals some fundamental differences between GP metabolism and lactate metabolism. Lactate, since it can reenter the main metabolic pathways only by a reversal of the reaction through which it

was formed, represents a dead end compound in mammalian metabolism. GP on the other hand not only can be oxidized by the mitochondrial GPDH (FAD), but is required for the biosynthesis of triglycerides and phospholipids (45, 90, 120, 152), and is an intermediate in glycerol metabolism (75). Alterations in the rate of GP formation would therefore be expected to result in changes in lipid metabolism.

Numerous studies have indicated that the membranes of freshly isolated intact mitochondria are relatively impermeable to DPNH (21, 36, 98, 126, 146), while some smaller molecules can pass through much more easily. A consideration of the equilibria of the DPN-linked GPDH of cytoplasm and the FAD-linked GPDH in mitochondria indicates that the GP-cycle can provide an effective pathway for the transfer of DPNH resulting from the oxidation of glyceraldehyde-3-phosphate in cytoplasm to the electron transport chain of mitochondria (25). The DPN-linked reaction greatly favors GP formation, the equilibrium being about $1:10^4$ in favor of GP formation (180), while the equilibrium of the intramitochondrial reaction lies far towards DHAP production.

The exact pathway taken by DHAP produced in mitochondria is still somewhat uncertain. This DHAP may diffuse out of the mitochondria in equilibrium with cytoplasmic DHAP or may be converted to glyceraldehyde-3-phosphate by a mitochondrial triose phosphate isomerase (26, 62, 157), the resulting glyceraldehyde-3-phosphate feeding directly into the glycolytic pathway. Green (63) has pointed out that "only enzymes pertinent to the exercise of mitochondrial function are present to any

significant extent in the mitochondria". In either event, the GP-cycle provides a mechanism by which the oxidative processes of mitochondria can affect the rate of glycolysis (25). Such a control would be seriously curtailed in malignant tissues with their very low GPDH (DPN) activity (25).

A comparison of the thermodynamic and equilibrium constants of reactions catalyzed by LDH and GPDH (DPN), rather than revealing any very significant differences, indicates that there is a very striking similarity between the properties of the two enzymes. From the table (Page 19), it can be seen that the constants do not greatly favor either enzyme in a competition for the substrates DHAP or pyruvate and DPNH.

Evidence has been presented by Hohorst et al. (77) showing that the steady state equilibria of the L/P and GP/DHAP systems are very close to mass action equilibria in the normal rat liver. The relation between the L/P and GP/DHAP ratios in situ can be expressed by the equation:

$$(L/P):(GP/DHAP) = 1.6K_{GP}/K_{lact},$$

K representing the mass action equilibrium constants of the corresponding DPN coupled reactions. It was felt that if the above condition actually represents a functional relationship, this would still be true when alterations in the L/P and GP/DHAP ratios occur. Even under the extreme conditions of ischemia (77), alloxan diabetes, and bilateral adrenalectomy (78), there are no measurable deviations of

EQUILIBRIUM AND KINETIC CONSTANTS OF REACTIONS CATALYZED BY LDH AND GPDH (DPN)

CONSTANT	LDH	Reference	GPDH (DPN)	Reference
K at 22°C pH 7.0	3.3×10^{-12}	118	$5.5\text{-}5.8 \times 10^{-12}$	11, 32, 180
REDOX POT.	-0.190v	32	-0.192v	32
E'_o, pH 7 25°C				
MICHAELIS CONST. at pH 7, 22°C				
for R_1R_2CO	1.5×10^{-5}	67	4.6×10^{-4}	180
" $R_1R_2CH_2OH$	5.3×10^{-4}	67	1.1×10^{-4}	180
" DPN	2.5×10^{-4}	67	3.8×10^{-4}	180
" DPNH	3.5×10^{-6}	67	$5\text{-}6 \times 10^{-6}$	25

these DPN linked reactions from mass action equilibria. Work done by Saktor et al. (147) is in agreement with that of Hohorst et al. However, not all workers are in agreement regarding the direction of the changes observed by Hohorst (159). In addition, it has been demonstrated that the accumulation of GP and lactate in liver parallels the activity of GPDH (DPN) and LDH (42).

Quite different evidence, in keeping with the thought that more structural relationships should be ascribed to reactions occurring in cytoplasm, has been presented. Hoberman and D'Adamo (75) indicate that a larger portion of glyceraldehyde-3-phosphate dehydrogenase is coupled to GPDH (DPN) than to LDH, suggesting molecular aggregates in cytoplasm formed by pairs of dehydrogenases. Bloom (22) has found a pool of DPNH relatively common to the glyceraldehyde-3-phosphate dehydrogenase and GPDH (DPN) reactions. When lactate-2-H^3 or glycerol-2-H^3 is incubated in the presence of rat kidney or liver slices, the $DPNH^3$ formed from glycerol-2-H^3 is more extensively used for glycogen synthesis than is $DPNH^3$ originating from lactate-2-H^3. The glyceraldehyde-3-phosphate dehydrogenase reaction is primarily responsible for the incorporation of deuterium from glycerol-2-D into the glucosyl residues of glycogen (75). In the case of liver, the proposed coupling favors the incorporation of tritium from glycerol-2-H^3 over tritium from lactate-2-H^3 by a factor ranging from 10 to 38. In kidney this factor is 3.0 to 3.3. In kidney the incorporation of tritium from lactate-2-H^3 into fatty acids is 2 to 3 times as extensive as the

tritium from glycerol-2-H^3. No such favored incorporation of tritium into fatty acids occurred in liver. It should be noted that, in all cases, the greatest proportion of tritium was incorporated into water. The work of Bloom may have considerable bearing on the importance of the GP-cycle. Such a cycle serving to transport hydrogen from cytoplasmic DPNH into the mitochondria for oxidation, would be expected to result in the transfer of tritium from glycerol-2-H^3 almost entirely into water. Therefore the tritium of glycerol-2-H^3 would be expected to be a better precursor of water than the second hydrogen of lactate. Consequently it would be expected to be a poorer precursor of glycogen hydrogen. The reason for this is that intramitochondrially formed DPNH is not utilized for reductive biosynthesis in cytoplasm. However, the results did not follow these expectations. In view of the fact that most of the tritium of glycerol-2-H^3 and lactate-2-H^3 was converted to water, the possibility remained that the tritium found in glycogen came from tritiated water. This possibility was ruled out when liver and kidney, incubated in the presence of tritium oxide, incorporated far less tritium into glycogen than when glycerol-2-H^3 or lactate-2-H^3 were present. Other workers have found that the distribution of deuterium in glycogen arising from D_2O in such a case is far different than when it arises from lactate (75). These results led Bloom to conclude that the GP-cycle is of much less importance in rat liver and kidney than in other tissues (53).

Klingenberg and Bucher (87) are critical of the conclusions of Bloom. The suggestion is made that the difference in the rate of

appearance in glycogen of the tritium from the number two position of glycerol and lactate can be explained by the different stereospecificity exhibited by the enzymes toward DPN. Lactic dehydrogenase is specific for the α-isomer of DPN (102) while glyceraldehyde-3-phosphate dehydrogenase is specific for the β-isomer, as is GPDH (DPN) (102, 103). DPNH, having obtained its tritium from lactate, might therefore have to be subjected to some "shuttle between an α- and β-dehydrogenase" before transferring that hydrogen to glyceraldehyde-3-phosphate.

The accumulation of GP might possibly have an advantage over lactate accumulation since during the subsequent oxidation there is no requirement for DPN which might be in short supply. A more practical consideration favoring lactate accumulation lies in the relative amounts of energy available from the formation of lactate and GP. During the anaerobic reactions involved in the conversion of glyceraldehyde-3-phosphate to pyruvate, two ATP are produced. Since two glyceraldehyde-3-phosphates can be formed from each glucose molecule, four ATP's can be formed during the anaerobic conversion of glucose to lactate. But, two ATP are required, one each to phosphorylate glucose and fructose-6-phosphate, leaving a net energy production of two ATP from glucose or three ATP from glycogen. On the other hand, if one of the triose phosphates produced during glycolysis is diverted and allowed to accumulate as GP, only one glyceraldehyde-3-phosphate molecule can be produced for each glucose. Therefore there will be two less ATP per molecule of glucose, assuming that each glucose is converted quantitatively. There would then be a net formation of only one

ATP per glucosyl unit of glycogen and none from glucose. Therefore, from an energy standpoint, it would seem to be of advantage to a cell to convert glucose and glycogen to lactate rather than to GP.

In the previous comparisons of GP and lactate metabolism under anaerobic conditions, only the competitive nature of the two systems has been mentioned. However, it is suggested that the two metabolic pathways may have a complementary rather than a competitive relation (87). It should be remembered that when glucose is converted anaerobically to pyruvate and lactate via the glycolytic pathway, each mole of pyruvate formed is accompanied by a mole of DPNH requiring oxidation. However, during the anaerobic conversion of glucose or glycogen to GP and pyruvate, the DPNH formed by the oxidation of glyceraldehyde-3-phosphate is reoxidized by the DHAP. This system can bring about an increased pyruvate concentration within a tissue under anaerobic conditions while providing for the continued oxidation of DPNH. The conversion of glucose to GP and pyruvate can raise the pyruvate concentration without producing a corresponding increase in DPNH. Such an increased pyruvate concentration maintains the redox potential of the glycolytic system and permits glycolysis to continue for a longer period of time. This in turn permits a greater amount of ATP to be formed during anaerobic glycolysis. Now, since the lactate to pyruvate ratio is large (135), the accumulation of GP and pyruvate would allow for the production of more lactate and therefore of more ATP than was lost through the diversion of three carbon units to GP.

Besides direct mitochondrial oxidation, the GP-cycle is but one

of several possible routes which have been proposed for the oxidation of cytoplasmic DPNH. All of the proposed cycles capable of transporting hydrogen of DPNH into the mitochondria have two common properties. First, there is a metabolite which can be reduced by cytoplasmic DPNH to a product which can easily pass through the mitochondrial membrane. Secondly, this product must be a substrate for mitochondrial oxidation. Since mammalian cells normally contain only a catalytic amount of DPN (106) they must possess a means for continually oxidizing the DPNH formed, thereby permitting glycolysis to continue. Catalytic quantities of acetoacetic acid will stimulate the oxidation of DPNH by freshly isolated rat liver mitochondria without previous hypotonic treatment or addition of exogenous cytochrome c. Therefore, Devlin and Bedell (48) have proposed an acetoacetate cycle similar to the GP cycle. This cycle would make use of the beta hydroxybutyric dehydrogenase found in cytoplasm (95) and in mitochondria (48, 99). Another suggested possibility is the oxidation of DPNH and reduction of oxalacetate by the cytoplasmic malic dehydrogenase followed by the mitochondrial oxidation of malate (46, 66, 138, 146). The relative contribution of each of these routes has been determined for insect flight muscle. The GP cycle provides the major vehicle for the transportation of hydrogen from cytoplasmic DPNH across the mitochondrial membrane in this tissue (146). No similar work in mammalian muscle has been found. In a malignant cell, the malate and oxalacetate cycles are not available to any significant extent (24).

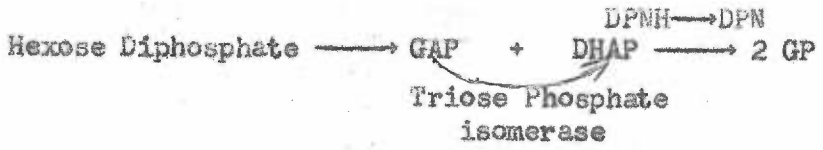
4. Glycerophosphate Metabolism of Individual Tissues

a) Liver

From a quantitative standpoint, the metabolism of GP is probably of greatest importance in the liver. Here we find a greater concentration of GP per unit weight than in any other tissue (100), as well as both very active cytoplasmic (25, 42, 179) and mitochondrial (40, 62) GPDH. A possible exception is in lymph nodes, where LDH/GPDH (DPN) ratios of 0.5 have been found (25). Ciaccio, Keller and Boxer (42) demonstrated that in normal liver under anaerobic conditions, very nearly equal amounts of GP and lactate accumulate, both in homogenates and in situ. Boxer and Shonk (25) have found LDH/GPDH (DPN) ratios ranging from 1.3 - 2.6 in normal rat livers. Even though the GPDH(DPN) activity is generally felt to be exclusively extraparticulate, Ciaccio and Keller (41) report that catalytic quantities of GP or DHAP stimulate the oxidation of exogenous DPNH by well washed hamster liver mitochondria without the need for externally added GPDH(DPN). This suggests that both enzymes of the GP-cycle are located within hamster liver mitochondria. However, these same workers reported in another paper (40), without mentioning the species of animal used, that GP does not stimulate the oxidation of exogenous DPNH without the addition of GPDH(DPN) or exogenous cytochrome-c.

Racker (127) has attributed the inability to measure DPNH formed by the oxidation of glyceraldehyde-3-phosphate to the rapid oxidation of DPNH by DHAP in the presence of GPDH(DPN) in liver. When attempts were made to measure the activities of hexokinase and phosphohexokinase by the addition of the suitable substrate and measurement of the subsequent formation of DPNH, failure was attributed to the presence of GPDH(DPN). Slater (160) avoided this difficulty by employing iodo-

acetic acid at concentrations which inhibit glyceraldehyde-3-phosphate dehydrogenase but not GPDH(DPN) and measuring the decrease in DPNH concentration as a result of the following reactions.



Young and Pace (179) also utilized iodoacetic acid to inhibit glyceraldehyde-3-phosphate dehydrogenase when analyzing for GPDH(DPN) of muscle.

Enzyme activities of GPDH(DPN) in livers of embryonic and young rats expressed as % of the adult level for seventeen day fetal, newborn, and nine day old rats are 10, 45, and 114 respectively. Enzymatic changes in the fetal and neonatal liver are directed in favor of an increased capacity for lipid synthesis and lactate utilization rather than lactate formation (30).

During fasting, changes in GP content of rat liver parallel the levels of liver glycogen (101, 128). In order to determine whether changes in the phosphorus distribution are due to the withdrawal of a particular portion of the diet, rats were maintained for twenty hours on normal mixed diets, glucose alone, casein alone, or olive oil after previously having been on a normal mixed laboratory diet (128). Liver GP was considerably lowered in rats maintained on casein or olive oil, as it was in fasted rats. Glucose alone was able to produce the same effect on the distribution of phosphorus compounds in the liver as a normal laboratory diet. Therefore changes in the phosphorus distribution during fasting are ascribed to the lack of carbohydrates in the

diet. The GP content of the liver reached a minimum within twenty four hours. The distribution of phosphorus compounds was the same at forty eight hours as at twenty four hours. It was felt that a minimum of about 16 mg% GP is required for normal liver function, while the increment of about 12 mg% found in fed rats may have a specific function related to carbohydrate metabolism.

b) Muscle

For many years it has been known that muscle is not a homogeneous tissue containing only one kind of fiber. Rather, two types of fibers are found, referred to generally as red and white fibers. The red fibers contain a relatively higher concentration of oxidative enzymes and mitochondria, and a relatively lower activity of glycolytic enzymes than do white fibers. Histological evidence indicates a difference in the size of the fibers, the red being somewhat smaller than the white (50, 51). There is, however, not an absolute distinction between red and white fibers in terms of size or color, intermediate sizes and color existing.

Histochemical localization of GPDH(DPN) and of GPDH(FAD) indicates that there is at least a partial separation of the two enzymes between the two types of fibers. GPDH(FAD) activity is primarily associated with the larger or white fibers and is weak or absent in the smaller or red fibers. Conversely, the GPDH(DPN) activity is greater in the red than in the white fibers (121). Such a separation of the required components of the GP-cycle does not support suggestions that the GP-cycle may be of considerable importance in muscle. It

does, however, add evidence in support of the thesis that the two types of fibers rely on substantially different pathways in obtaining their supplies of energy for muscular contraction.

A review of the literature presents a rather confusing picture of the activities of both the cytoplasmic and the mitochondrial GPDH in mammalian muscle. Frequently the ratios of LDH/GPDH(DPN) are expressed. These ratios have ranged from a high of 15:1 (182) to a low of 1:1 (179). Surprisingly enough, these ratios were both found in skeletal muscle from the same kind of animal, the rat. Similarly, wide variations are reported for the mitochondrial enzyme. The mitochondria from human skeletal muscle are reported to oxidize GP very slowly if at all (6, 52). Rat skeletal muscle mitochondria, on the other hand, have been found which will oxidize GP more rapidly than succinate (88). As might be expected, those who find a high activity of one of these enzymes ascribe considerable importance to the GP cycle (144), while those finding low activities consider the reduction of DHAP and oxidation of GP to be rather unimportant side reactions (37, 53, 182). Unlike the more extensively studied GP metabolism of insect flight muscles, no accumulation of GP has been demonstrated in mammalian skeletal muscle except during the seven second tetanus of a rat diaphragm (93). However, the diaphragm is not truly representative of skeletal muscle in general, both qualitative (122) and quantitative (16) differences having been demonstrated.

A relaxing factor activity was described by Marsh (108, 110) which is associated with the microsomal fraction of muscle. More recently this relaxing factor has been found to be soluble and dialyzable,

indicating that it is of relatively low molecular weight (27), and not a protein as had formerly been thought. Later, Marsh isolated the material chromatographically. Rf values indicated that it might be GP. However, when GP alone was tested it was found to have no factor activity. But, GP plus inorganic phosphate, ATP and Mg^{++} developed full relaxation activity (109). Parker, on the other hand, reported that GP is not identical to the soluble relaxing factor. He then set out to determine whether GP might be a precursor of the relaxing factor. He reasoned that if GP were a precursor of the relaxing factor, the microsomes should be capable of synthesizing GP. However, they can not synthesize GP (119). Bendall and coworkers are critical of the technique of Marsh, saying that GP does not exhibit relaxing factor activity under conditions where the natural relaxing factor of granules is known to be active, and does not inhibit myofibrillar ATPase (19). As yet, none of this work appears to have been confirmed, and, the identity of the relaxing factor system has not been positively established.

c) Red blood cells

The red blood cells are known to convert glucose almost quantitatively to lactate (13). When Bartlett (14) subjected trichloroacetic acid extracts of partially purified red blood cells to column chromatography, no appreciable phosphorus was reported in the position expected for GP. The trace found was not positively identified as GP. It was concluded that the absence of GP is characteristic of the red blood cells.

d) White blood cells

Partially purified suspensions of leucocytes, essentially free of erythrocytes, can be obtained from whole citrated horse blood (171, 174). However, these preparations are contaminated by platelets and contain polymorphonuclear leucocytes, lymphocytes, and monocytes. Attempts at further purification result in inactivation of the leucocytes.

Early experiments demonstrated the accumulation of an unidentified organic phosphate intermediate during the anaerobic breakdown of glycogen to lactic acid in leucocyte preparations. Addition of sodium fluoride to the reaction mixture inhibited the formation of lactic acid, but not glycogen breakdown or the formation of the then unidentified intermediate (174). The formation of this intermediate could not be demonstrated with intact cells, but only with cells which had been treated to destroy the integrity of the cell wall, such as by treatment with hypotonic solutions (173, 174). It is also interesting to note that a decrease in inorganic phosphate was demonstrated under such conditions (174). At no time was GP identified as the accumulating product in these early experiments. Later, however, it was identified chromatographically as a normal metabolite in leucocytes (173).

In a study of the oxidizing enzymes of leucocyte-platelet mixtures (172), the oxygen consumption was considerably higher with GP as a substrate than with either succinate or glucose. GPDH(FAD) is the most active respiratory enzyme in leucocyte-platelet suspensions, succinic dehydrogenase being the next most active. A comparison of

this leucocyte-platelet mixture with a rat liver homogenate showed just the opposite to be true for liver, where succinic dehydrogenase exceeded GPDH(FAD) activity by several hundred percent.

ul O_2 uptake/hr/mg N

	endogenous	succinate	GP
LIVER	15.0	140	18.0
LEUCOCYTE-PLATELET MIXTURE	37.0	42.3	55.0

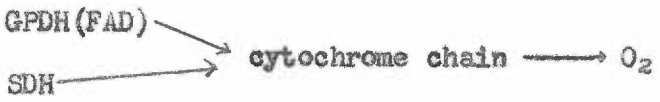
taken from Wagner (172)

Wagner concluded that GP plays a very important role in the carbohydrate metabolism of white blood cells, but not in that of liver.

e) Brain

It would seem that GP metabolism constitutes a very important aspect of brain metabolism. Brain contains an active GPDH(DPN), and is probably the richest source of GPDH(FAD) yet found (62, 89, 149, 179). A rather unusual relationship between succinic dehydrogenase and GPDH(FAD) is believed to exist. Ringler and Singer (131, 133, 132), while studying the inhibition of both succinic dehydrogenase and GPDH(FAD), found that both enzymes are inhibited to the same extent by antimycin-A. Also, the oxidation of both succinate and GP is stimulated to the same extent by cytochrome-c in cytochrome deficient mitochondrial preparations. They then carried their investigations further by studying the relative rates of oxidation of GP and succinate, both singly and together. When both substrates were oxidized at the same time, instead of their having an additive effect on respiration, the two substrates proved to be competitive. Ringler

and Singer took this to mean that succinic dehydrogenase and GPDH(FAD) may be structurally and functionally interrelated rather than independent enzymatic entities. It was postulated that both enzymes might be served by the same cytochrome chain in brain mitochondria.



The oxidation of GP proved to be competitively inhibited by the addition of DHAP, which is in oppositition to the results obtained with insect flight muscle mitochondria (53).

f) Heart

The literature concerhing GP metabolism in heart muscle presents a somewhat less confusing picture than that of skeletal muscle. Some workers report that heart muscle mitochondria have little or no oxygen consumption when incubated in the presence of GP and form only trace amounts of DHAP (47). Green, on the other hand, found a small but appreciable amount of GPDH(FAD) activity in heart muscle (62). Only trace amounts of GPDH(DPN) activity are reported for heart (179). In no case is the reported activity of either enzyme of the GP-cycle equal to the activities found in the other major organs such as brain, muscle, liver, or lung. Because of the low enzyme activities, the GP-cycle is felt to be of little importance in heart muscle.

g) Kidney

Studying the whole kidney, Young and Pace (179) report on the presence of GPDH(DPN). Histological techniques have demonstrated the presence of GPDH(DPN) and localized it in the various types of cells (72). A high activity is found in the ascending and descending tubules of the outer medulla and cortical collecting ducts; moderately strong

activity in juxtaglomerular cells, distal convoluted tubules, vascular endothelium, and medullary collecting duct epithelium; weak activity in glomerular epithelium, and interstitial cells; and no activity in proximal convolutions or vascular smooth muscle. Interesting changes occur in these activities during experimental hypertension and hormonal imbalance which are mentioned in the section on hormones.

h) Skin

When fructose-1,6-diphosphate is incubated in the presence of fluoride and trace amounts of DPN and inorganic phosphate, there is a quantitative conversion of the fructose-1,6-diphosphate to an equimolar mixture of phosphoglyceric acid and GP under anaerobic conditions (20). This conversion required 60 minutes, and indicates that the GPDH(DPN) activity of rat skin is very low.

Turnover studies of cytochrome-c in different tissues with GP, succinate, pyruvate, and malate showed that cytochrome-c turnover is relatively constant with succinate and even more constant with pyruvate and malate, but less so with GP. It was suggested that the TCA-cycle plays a basic role in the metabolism of different tissues while GP oxidation is more clearly associated with some special function (89).

5. The Fate of Glycerophosphate Injected into the Blood Stream

Of particular interest regarding the metabolism of GP is the fate of the material once it has entered the blood stream. When GP^{32} is injected into the blood stream of a rat or a rabbit, a rapid decrease in GP^{32} concentration in serum occurs (81). The GP^{32} is rapidly hydrolysed, the $P^{32}i$ concentration of blood increasing rapidly. The half time for this hydrolysis is about 20 minutes. A comparison of the specific activity of P^{32} in blood serum and urine showed the

specific activity to be higher in urine. This was attributed to high activity of a kidney phosphatase releasing Pi from the ester into urine. GP^{32} was found to be less readily diffusible into liver and other soft tissues than inorganic phosphate. A slightly different picture is presented by Popjak and Muir (124) who suggested that unhydrolysed GP does not enter liver cells from blood plasma either in vivo or in vitro. Also, unhydrolysed GP^{32}, arising from P^{32}-labeled phospholipids, when injected into the maternal blood stream will not pass through the placenta membrane (124). As is characteristic of the PO_4 esters of glycolysis, parenterally administered GP is incorporated more rapidly into growing rat incisor than is $P^{32}i$ (81). In addition to incisor GPDH(DPN), activity is also found in bone, a preosseous cartilage (3).

6. GPDH(DPN) Activity of Malignant Tissues

A comparison of GPDH(DPN) activities in a number of malignant tissues with their normal counterparts shows a decrease in the activity of this enzyme in most malignant tissues studied (25, 42, 144, 178).

Bone marrow cells were aspirated from the femur, tibia, and humerus from both normal and leukemic mice, and analyzed for LDH and GPDH(DPN) activities (144). The course of the leukemia was followed hematologically from the time of implantation till the time of death, as were the dehydrogenase activities. There was a close parallel between the progression of leukemia and the ratio of LDH/GPDH(DPN). A similar situation exists in lymphocytes and lymph nodes (25).

GPDH(DPN) and LDH Activities of Isolated Lymphocytes and Lymph Nodes

	LDH µmoles DPNH/ min/ml/mg prot.	GPDH(DPN)	Ratio LDH/GPDH(DPN)
Isolated Lymphocytes:			
Normal	0.66	0.083	8
Leukemic	1.43	0.014	100
Lymph Nodes:			
Normal	1.51	0.167	9
Leukemic	2.38	0.023	95

Taken from Boxer and Shonk (25)

As can be seen from the above table, the relation between the dehydrogenases in lymph nodes and lymphocytes changed markedly with the progression of leukemia. Similar changes occurred in the marrow and spleen of leukemic animals.

Since DPNH produced in the cytoplasm is not believed to be oxidized directly by the mitochondria (36, 98, 146), but rather through the mediation of some cycle such as the GP cycle or acetoacetic acid cycle (48, 49), Saktor and Dick (144) concluded that the complex hydrogen systems in leukemic tissues are balanced differently from those in normal hematopoietic tissues. In addition, the mitochondrial GPDH is also lowered in leukemic tissues, suggesting that the GP cycle may not account for a significant portion of the hydrogen transport.

In a similar series of experiments, Ciaccio, Keller and Boxer (42) also found GPDH(DPN) activity decreased and LDH activity increased in

malignant tissues. Homogenates of normal liver and of Novikoff hepatoma were maintained anaerobically during incubation in the presence of added hexose diphosphate by continuously bubbling 95% N- 5% CO_2 through the incubation mixture. When normal liver was used, in which the LDH and GPDH(DPN) activities were about the same, the increase in GP content paralleled the lactate accumulation. In the Novikoff hepatoma, with its decreased GPDH(DPN) activity, the relative accumulations of GP and lactate were very nearly proportional to the activities of GPDH(DPN) and LDH respectively.

The results of similar experiments in situ were essentially the same as with homogenates. Again there was decreased production of GP and increased production of lactate in the malignant tissues.

In a study by Boxer and Shonk of 26 transplanted rodent tumors, four human tumors growing in cortisonized hamsters, and a carcinogen induced tumor, GPDH(DPN) activity was found to be low or absent (25). Tissues studied were liver, muscle, brain, kidney, spleen, lymph nodes, and adipose tissue of rats, mice and hamsters. In normal animals the LDH/GPDH(DPN) ratios varied between the extremes of 0.5 - 7.0. These ratios were increased from ten to several hundred-fold in the malignant tissues, ratios as high as 870 being reached (145). The increased ratios are primarily due to decreases in GPDH(DPN) activity. LDH activity either remained the same or rose slightly. In either case,

the relative change in LDH activity is small compared to the change in GPDH(DPN). Boxer and Shonk (25) stated that this alteration in GPDH (DPN) is in keeping with generally observed enzymes found in tumors. This is the extreme opposite to the enzyme pattern found in insect flight muscle (182). Characteristic of the carbohydrate (CHO) metabolism of malignant tissues is a decrease in the enzyme activities which provide alternate pathways for CHO utilization or permit a reversal of glycolysis, the result being an undeviating conversion of glycogen and glucose to pyruvate and lactate. A decrease in GPDH(DPN) activity fits this generally observed pattern of enzyme alterations (24, 25).

The opposite picture is found in blood plasma and in ascites fluid, when GPDH(DPN) activity is greatly elevated (151).

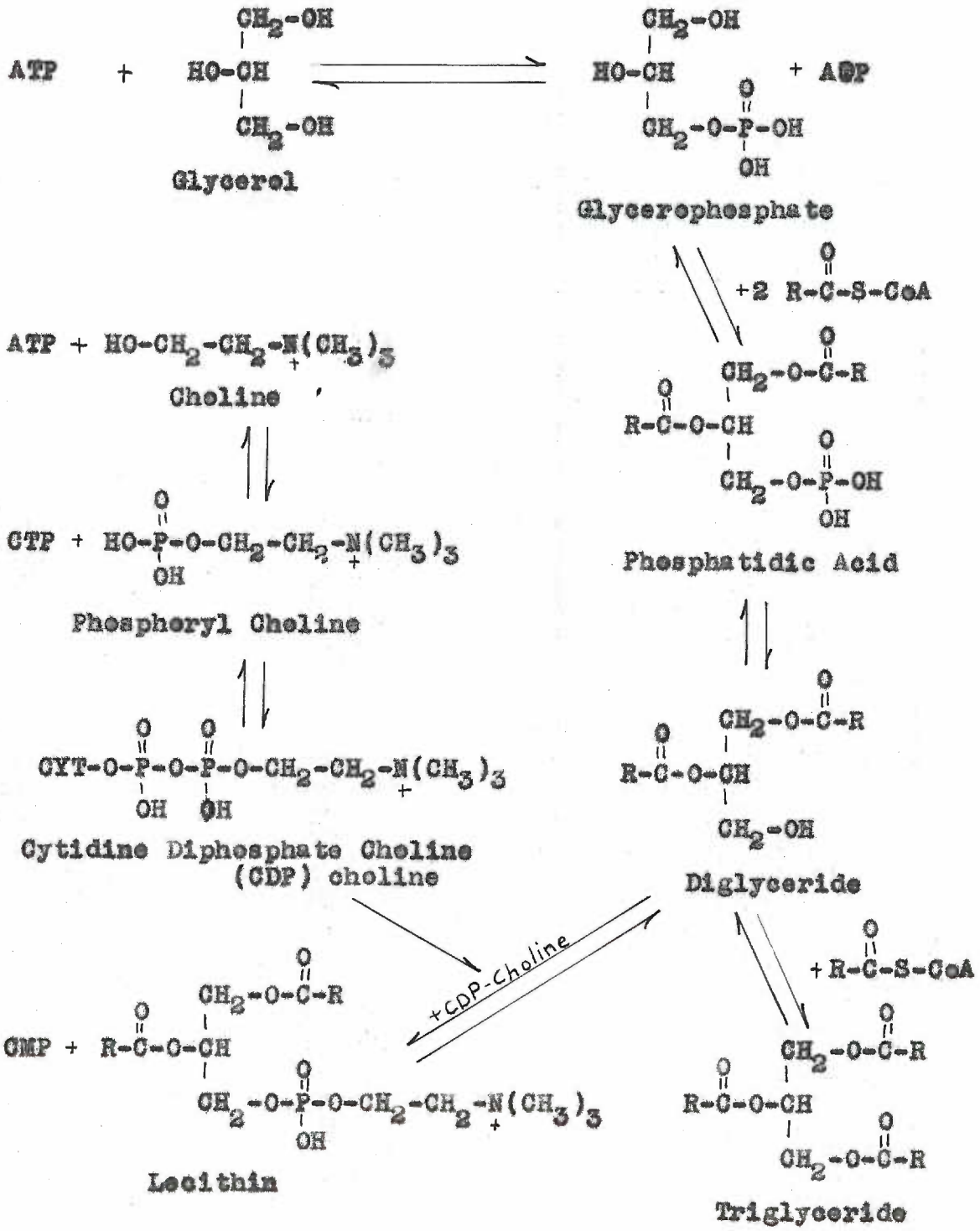
7. The Role of Glycerophosphate in Lipid Metabolism

A wide range of lipid phosphates related to GP is believed to occur universally in the higher plants, animals, and most primitive organisms (104). Of these there are two possible types, the alpha and beta isomers. These phosphate esters are very resistant to either acid or alkaline hydrolysis. In this respect they differ greatly from the glycerides of fatty acids. For instance, when GP is heated in 6 N HCl at 100°C it is hydrolyzed at the rate of 1.5 - 3.0% per hour (8). The hydrolysis of phospholipids in 1 N alcoholic NaOH at 37°C for one

hour results in the release as Pi of only 0.33% of the phosphate of GP (31). By way of comparison, the triose phosphates are completely hydrolyzed in 1 N NaOH in 20 minutes at 17°C (9). It is this resistance of GP to hydrolysis which is primarily responsible for early reports of the occurrence of both α- and β- forms of phospholipids in nature. That this concept might be in error is shown by the discovery that the phosphate group can migrate reversibly between the α- and the β- position during hydrolysis, giving an equilibrium mixture with proportions depending on the pH (7, 8). For a long time there has been definite proof for the natural occurrence of α-GP. Optically active α-GP can be obtained from the hydrolysis of phospholipids (8). But, since β-GP is optically inactive, there can be no such evidence for either its presence or absence. There is, however, no reliable evidence that lipids based on the β-isomer occur naturally. In addition it has been shown that rat liver has no metabolic activity towards the β-isomer (84).

Since it was first suggested by Zilversmit and coworkers (183), considerable evidence has accumulated showing GP to be an obligatory precursor for the formation of fats and phospholipids. The incubation of cell-free extracts of rat liver in the presence of GP^{32} has been accompanied by the rapid incorporation of inorganic P^{32} into phospholipid (92, 91). However, the possibility remained that the GP^{32} might be hydrolyzed and the resulting inorganic P^{32} incorporated into phospholipid via some other compound. This possibility was ruled out by demonstrating a higher specific activity of P^{32} in the phospholipid

The Role of Glycerophosphate in Lipid Metabolism



fraction than in the inorganic fraction (89). Also, if GP is an intermediate in the biosynthesis of phospholipid, the incorporation of inorganic P^{32} into the phospholipid should be greatly reduced by the addition of unlabeled diluting GP. This effect has also been demonstrated (85, 84). GP is the required substrate for the formation of phosphatides, glycerol not acting directly (86). Glycerol must first be phosphorylated by ATP in the presence of glycerokinase before being incorporated into fats or phospholipids (28, 58). The resulting GP can be esterified rapidly with long chain fatty acids by liver preparations. Sixteen and eighteen carbon fatty acids are the most rapidly esterified (90, 150). Free phosphatides, formed from GP, are also found in liver (45, 120, 152). The metabolic scheme on the preceding page has been proposed for the formation of fats and phospholipids (83).

A slightly different problem of lipid formation concerns the conversion of hydrolysed or partially hydrolysed fat in the intestines into triglycerides found in lymph. When C^{14}-glucose is injected into a duodenal pouch of rats or hamsters, C^{14}-glycerol and C^{14}-GP are among the major products formed (163). The C^{14}-glycerol is presumably formed by the action of intestinal phosphatases on GP. Other isotope experiments have shown that the glycerol arising from the hydrolysis of ingested fat is not utilized for the resynthesis of lymph triglycerides (130). Further confirmation of this lies in experiments with labeled fructose-1,6-diphosphate. Added glycerol has been found not to reduce the amount of labeled fructose-1,6-diphosphate incorporated into glyceride-glycerol by cell free intestinal mucosa homogenates of swine.

By way of contrast, unlabeled DHAP or GP markedly decrease the amount of C^{14}-fructose-1,6-diphosphate incorporated into glyceride-glycerol. Glycerol cannot be converted to GP by intestinal mucosa, which contain no glycerokinase. It is for this reason that glycerol is not a precursor of glyceride-glycerol in intestinal mucosa. Buell et al. (29) concluded, from the above evidence and the knowledge of the role played by GP in triglyceride formation of other organs, that GP is an immediate precursor of glyceride-glycerol in intestinal mucosa.

Because of the link provided by GP between carbohydrate and fat metabolism, it is interesting to note that the activities of both enzymes of the GP cycle are increased in tissues of obese hyperglycemic mice as compared to the tissues of normal mice (56).

8. The Effect of Hormones on Glycerophosphate Metabolism

Studies on the effect of thyroid hormone on dehydrogenase activity of mitochondria have been conducted by Lee, Takemori and Lardy (97). Significant in their findings is the fact that GP oxidation is increased about three- to five-fold in liver mitochondria of thyroid fed rats. The rate of succinate oxidation was initially about nine times that of GP, however, the increase in the rate of GP oxidation caused by thyroid feeding was several times that of succinate, the final ratio being about 2 to 1. Then the increased oxidative capacity was produced by feeding dessicated thyroid, succinate oxidation reached a maximum two days after the feeding was started, but the rate of GP oxidation continued to increase from a three-fold increase at two days to a five-fold maximum at ten days. If, after ten days of thyroid feeding, the rats were returned to a normal diet, the increased

oxidative rate of the mitochondria toward GP, succinate, choline, glutamate, and β-hydroxy butyrate returned to about normal. The increase in the rate of GP oxidation was greater than the increase in oxidative rate with any other substrate. Administering the thyroid hormone subcutaneously had similar effects, except that the response occurred more rapidly.

In order to determine whether or not the observed results reflected an increase in the permeability of the mitochondrial membrane, the dehydrogenase activities from sonically disrupted mitochondria were determined. Activities of GPDH(FAD) and succinic dehydrogenase were found to parallel the increased oxidative rates of GP and succinate by intact mitochondria. Feeding ethionine to the experimental rats in addition to dessicated thyroid blocked the effect of the thyroid. It was concluded that the feeding of dessicated thyroid to the rats results in the formation of new protein, namely the enzymes, rather than merely activation of inactive forms of the enzymes.

Further evidence in support of this conclusion lies in the fact that liver and kidney of thyroidectomized rats oxidize GP at 60% and 16% respectively of the normal rate. If triiodothyronine is injected into the thyroidectomized rats, the oxidation rate of GP is brought back to or above normal.

Feeding normal rats thyroid substance can increase the concentration of GPDH(FAD) as much as ten- to twenty-fold in liver and kidneys, less in heart, uterus, skeletal muscle, diaphragm, and adipose tissue, and not at all in brain, lungs, spleen, testes, and ovary (96). It may be significant that the group of tissues which does not increase

its oxygen consumption following the administration of thyroid hormone is the same group in which GPDH(FAD) activity is not increased. By way of comparison, GPDH(DPN) activity is not increased by thyroid feeding.

GPDH(FAD) activity of rat liver is increased by bilateral adrenalectomy (70). This increase can be reversed by the administration of epinephrine, corticosterone, aldosterone, or hydrocortisone. However, if the adrenals are demedullated and the cortex allowed to regenerate, the initial increase gradually disappears. The adrenal cortical secretions are considered to enhance the effect of inhibitors which limit substrate oxidation by nonphosphorylating rat liver mitochondria. Also, an increase in GPDH(FAD) activity has been demonstrated histologically in kidney following bilateral adrenalectomy. Of interest here is the fact that GPDH(FAD) activity parallels the renin content of the juxtaglomerular cells in both experimental hypertension and adrenal insufficiency (72).

9. The Cofactor of GPDH(DPN)

GPDH(DPN) contains a cofactor of a rather unusual nature. The enzyme does not have the absorption spectrum of a pure protein, but rather, has a prosthetic group absorbing at 260 mμ (18, 170, 168). Van Eys (170), after comparing this unusual cofactor with known coenzymes concluded that it is not DPN, nor is it identical with any other cofactor which he studied. This cofactor cannot be removed by recrystallization following $(NH_4)_2SO_4$ precipitation, or by dialysis. However, it can be removed from the enzyme by treatment with charcoal (120). Before treating the enzyme, the charcoal must be washed, first

with hot hydrochloric acid, and then with tris-versene buffer pH 8.1. If either of these washings is omitted the enzyme is inactivated, presumably by heavy metal contamination from the charcoal. However, following this inactivation the enzyme can be reactivated completely by the subsequent addition of versene. Following the separation of enzyme and cofactor, the enzyme retains all of its activity.

Three suggestions have been made regarding the nature of the unknown cofactor by van Eys (170). First considered was the possibility of it being a contaminant. This possibility was discounted when the cofactor was found to be present 1) after preparation by different modifications of the methods of Beisenhertz (18) and Baronowski, 2) in commercially prepared samples of the enzyme (C. F. Boeringer and Sons, Mannheim, Germany), and 3) was present in proportion to the enzyme activity. Furthermore, chemical and chromatographic tests indicate that the same compound is present in both rabbit and monkey muscle. It is also found in yeast associated with GPDH(DPN) activity. In other words, the nonprotein component is present in GPDH(DPN) from different sources and prepared by different methods.

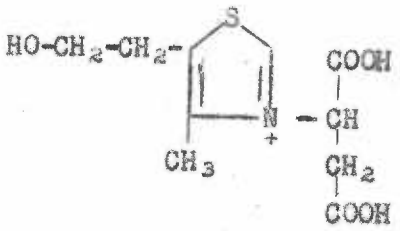
Secondly, it was suggested that the nonprotein component might have a function not connected with the catalytic properties of the protein as we currently understand them. This would indicate an as yet unknown function, or possibly an altered function in a complete metabolic system.

The third suggestion made was that the nonprotein component might have some catalytic function which could be replaced by versene.

Ankel and coworkers (4) have identified the nonprotein component by high voltage electrophoresis and spectral measurements as ADP-ribose or a closely related derivative. The absorption curve of the cofactor is very similar to commercially prepared ADP-ribose. Both commercially prepared ADP-ribose and the nonprotein component obtained from GPDH(DPN) migrate at the same rate under high voltage electrophoresis at the different pH's of 3.5, 7.0, and 11.0. Mild alkaline hydrolysis results in the formation of inorganic phosphate, ribose, ADP, and AMP. Chemical analysis shows that the nonprotein component contains two ribose groups and two phosphates for every adenine.

A comparison of the properties of GPDH(DPN) before and after separation from the nonprotein component shows that the crystal form and sedimentation constant remain the same. Both forms bind one mole of DPN per 70,000 grams of protein, and respond in the same way to the addition of ethylenediamine tetraacetate (EDTA). After removal of the nonprotein component there are more free SH groups on the protein. However, there is no detectable change in enzyme activity.

Van Eys (168), on the other hand, concluded that the spectral properties of the grouping point to a quaternary thiazole compound. Upon alkaline hydrolysis he obtained aspartic acid and a polydeoxypentose. The compound isolated can replace the thiazole moiety of thiamine in the growth of Leuconostoc mesenteroides. From this and other data, van Eys suggested the following as the most probable structure and gave it the trivial name "thiamic acid".



3(4-methyl-5(beta hydroxy ethyl) thiazolyl) succinic acid

This compound containing a thiamine group, was synthesized and found to be identical with the naturally occurring one in all chemical tests, and could reactivate charcoal treated yeast or muscle GPDH(DPN). Additional evidence tending to support this claim lies in the fact that thiamine deficiency produces a striking decrease in GPDH(DPN) activity of liver, muscle, and brain (169). The observed decrease is not due to a diminished food intake, since there is not a comparable decrease in enzyme activity in pair-fed non-thiamine deficient controls. When the deficiency was produced by feeding the antimetabolite oxythiamine or pyrithiamine, the decrease in GPDH(DPN) activity was proportional to the dose administered. This decrease in activity is believed to be a true effect of thiamine deficiency and not of starvation. Protein biosynthesis is possible even in extreme thiamine deficiency (5). Also, LDH activity did not decrease as much as GPDH(DPN), and aldolase activity was unaffected.

However, no data have yet been presented to shed any light on the true role of the nonprotein component of GPDH(DPN). No mention has been made as to whether or not the cofactor can replace either partially or wholly the thiamine requirements of higher animals. And, it has not been possible to activate a muscle deficient in GPDH(DPN) activity with supernatant from a control muscle. Many similar derivatives of thiazole have been found which will substitute for thiamine in the growth of numerous

bacteria. It would certainly be of interest to determine whether such thiazole derivatives can alleviate any of the symptoms of thiamine deficiency in mammals or any of the higher animals. As yet no work seems to have been done on this problem. Also, the cofactor reported by van Eys is the only such thiazole derivative so far found in mammalian tissues.

Since both groups of investigators point to the absorption maximum at 260 mμ as evidence for the structure of the nonprotein component, it should be pointed out that adenine (76), thiamine (73, 79), and various derivatives containing the quaternary thiazole group (134) all absorb maximally in this region.

III. METHODS

1. Animals

Female Sprague-Dawley rats maintained on Purina Chow were used in all experiments. Before their use in an experiment, the rats were kept in the laboratory for at least 4 to 5 days. Both food and water were fed ad libitum during this period. Three hours before tissue samples were taken for analysis of in situ or anoxic levels of GP and lactate, the rats were fed 5 ml of milk by stomach tube. All other food was taken from the rats at this time. When tissues were to be taken for a Warburg experiment, the rats were given 5 ml of milk by stomach tube about 18 hours beforehand. As before the other experiments, all food was taken from the rats at the time of the stomach tubing.

2. Methods of Obtaining Tissue Samples for Analysis of in situ GP and Lactate Levels

a) Muscle

Skeletal muscle samples were obtained by a method previously

developed in this laboratory (17). Ten minutes before the samples were to be taken, the rat was anesthetized with 7.5 mg of nembutal per 100 gm of body weight. At the end of this ten minute period the hind legs were skinned, care being used not to damage underlying tissue. The legs were then immersed in a previously prepared bath of ethanol maintained at a temperature of about -70°C with dry ice. After the legs were frozen (4 minutes), they were removed from the bath, quickly cut off with bone shears, and stored in a dry ice chest.

b) Liver

In order to obtain liver samples for the analysis of in situ GP and lactate levels, the liver was exposed ten minutes after the injection of 5 mg of nembutal per 100 gm of body weight. A lobe of the liver was raised with forceps and a previously prepared slush of dry ice in ethanol packed on top of and on all sides of the liver. After a few minutes the frozen liver was quickly cut out with bone shears, wrapped in aluminum foil, and stored in a dry ice chest.

3. Grinding of Tissues in Box

Frozen muscle and liver were quickly transferred to a box (17) maintained at a temperature of about -45°C. Low temperatures were maintained by lining the sides of the box with tanks filled with dry ice. These tanks were vented into the room to prevent the accumulation of CO_2 within the box. Once inside the box, all visible fat pads were trimmed from the leg. Then, the desired muscle was cut from the leg with bone shears and placed in a mortar lined with dry ice. When liver was used, it was wiped free of adhering alcohol, cut into several small pieces and dropped into the mortar. A steel pestle was attached to a drill press located above the

box. The tissue sample was first crushed with the motor off. Fascia, left relatively intact by this preliminary crushing, was removed with forceps from the remaining tissue which was then ground to a fine powder with the drill press. There were three grinding periods. The first was for one minute, and the others for two minutes each. Between each grinding period the frozen tissue powder was scraped from the bottom of the mortar and mixed. The resulting frozen tissue powder was then passed through a fine screen into previously weighed Potter-Elvehjem homogenizing tubes containing 2 ml of 0.5 N perchloric acid. Screening the powder removed pieces of fascia and small pieces of tissue which may have escaped the grinding. Tissue samples were also added to weighing bottles for the determination of dry weights and percent nitrogen. The homogenizing tubes were taken from the cold box and the lower portion of the tubes containing the frozen perchloric acid immersed in warm water. When this lower portion of the acid had thawed, the tubes were placed on the Vortex mixer. The warm perchloric acid melted the frozen acid above it and then quickly mixed with frozen tissue, also thawing it. This procedure permitted mixing the tissue with the perchloric acid without first thawing the tissue for an appreciable period of time. The tubes were then reweighed to obtain the weight of added tissue. At this point the tissue could be stored frozen in the refrigerator. It was desirable to obtain skeletal muscle samples weighing at least 300 mg so as not to be below the limit of sensitivity of the analytical method.

4. Method of Obtaining Anoxic Tissues. Muscle and Liver:

For the preparation of both liver and muscle samples, the rat was killed by decapitation. At five and ten minute intervals samples of gastrocnemius or liver were taken and placed in previously weighed Potter-Elvehjem homogenizing tubes containing 0.5 N perchloric acid. Tissue samples were also taken for the determination of dry weight and percent nitrogen.

5. Warburg experiments

For Warburg experiments the rat was anesthetized by intraperitoneal injection of 3.5 mg of nembutal per 100 gm of body weight. Ten minutes after injection the rat was decapitated. The diaphragm was obtained by cutting around it near its outer edge. Central portions of the diaphragm were cut away and discarded. While muscle slices were being obtained, the diaphragm was soaked at room temperature in a Krebs-Ringer bicarbonate buffer, pH 7.4, containing 150 mg of glucose per 100 ml.

Strips of adductor muscle were obtained by first skinning the leg and then exposing the muscle through an incision. The adductor muscle was then dissected free of surrounding tissue. A small portion of the muscle was grasped with forceps near its insertion at the knee. The portion of muscle held in the forceps was cut from its tendinous insertion and the muscle cut longitudinally with a sharp pointed scalpel. Smaller strips of muscle no more than two millimeters in thickness were then obtained by gently teasing the larger strips apart with forceps. All visible fat pads were trimmed from the muscle and it was

placed in the soaking medium. The soaking medium was continually gassed with 95% O_2 - 5% CO_2. Red and white fibers were separated and placed in different soaking flasks. When all of the desired muscle had been obtained it was removed from the soaking medium and placed in small piles on a piece of filter paper. Red and white muscle strips were placed in separate piles containing from 200 to 300 mg of tissue. Blotting the muscle on filter paper removed excess water carried over from the soaking medium which would otherwise have placed subsequent weighings in error. When the excess water was blotted off the tissue the muscle strips were transferred to previously weighed Warburg flasks containing three milliliters of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 150 mg of glucose per 100 ml. Warburg flasks to be incubated aerobically were then gassed with 95% O_2 - 5% CO_2 for about one minute. Flasks to be incubated anaerobically were gassed with 95% N_2 - 5% CO_2. The Warburg flasks were reweighed to obtain the tissue weight and then connected to the Warburg manometers and placed in the Warburg bath which had been brought up to the operating temperature of 37°C. After 15 minutes the equilibrium flasks were removed from the bath. Vents on the remaining flasks were closed and the flasks left in the bath for two hours more.

Tissues taken from the equilibrium flasks were used for dry weight and percent nitrogen determinations. Tissues incubated for two hours were removed from the flasks, blotted on filter paper to remove adhering medium and transferred to previously weighed Potter-Elvehjem homogenizing tubes, containing 2 ml of 0.5 N perchloric acid. Media from both equilibrium and two hour flasks were transferred to test

tubes and stored in a dry ice chest for later analysis of GP and lactate production.

6. Homogenization of Tissues

Tubes which had been frozen in the refrigerator were thawed and then homogenized. The homogenization was continued for about one minute after the last remaining particles of large size had disappeared. The tubes were kept immersed in ice water throughout the entire procedure. Adhering homogenate was then rinsed off the pestle and into the tube with one milliliter of 0.5 N perchloric acid, bringing the total volume of homogenate up to three milliliters. After mixing the homogenate well on the Vortex mixer, it was allowed to stand at room temperature for one hour to fully extract the GP. The tubes were centrifuged for ten minutes at 3,000 rpm, and the clear supernatant decanted into another test tube. This supernatant may be stored frozen in the refrigerator. The same homogenization procedure was used for all tissues.

7. Preparation of Protein Free Filtrates

Just over 130 mg of K_2SO_4 were added to the perchloric acid extract to precipitate the ClO_4^- ion as the insoluble $KClO_4$. The extract was allowed to stand for ten minutes, then centrifuged for ten minutes at 3,000 rpm, and the supernatant decanted into another test tube. About 100 mg of $CaCO_3$ were added to this supernatant to neutralize the acid extract. $CaCO_3$ must be added a little at a time so as to prevent excess foaming. The tubes were allowed to stand ten minutes following the last addition of $CaCO_3$, then centrifuged ten minutes at 3,000 rpm, and the supernatant was decanted into another test tube. Very small

quantities of salts were added by this procedure. The K^+ of K_2SO_4 was precipitated as the insoluble $KClO_4$, and the $SO_4^=$ later precipitated as the insoluble Ca^{++} salt. Also, any excess $CaCO_3$ added was insoluble.

For the preparation of a protein free filtrate of Warburg medium 0.1 ml of concentrated perchloric acid was added to 2.5 ml of Warburg medium which was then allowed to stand ten minutes, centrifuged ten minutes, and the supernatant decanted into another test tube. The rest of the procedure was the same as for perchloric acid extracts of tissue homogenates. That the procedure was adequate for the removal of protein is indicated by the fact that the filtrates gave a negative sulfosalicylic acid test. A particular advantage of this procedure, and the reason it was chosen, is that there is no dilution of the samples.

8. Analysis of GP

In the present study of GP metabolism, the procedure for the analysis of GP was based on the enzymatic methods reported by Bublitz and Kennedy (23), Beltralik and Noll (28), and Ciaccio (39). Each of these groups of workers used hydrazine as a trapping agent for the DHAP formed during the reaction. It was reported by Boltralik and Noll that GPDH(DPN) is rapidly inactivated by hydrazine, and that this inactivation can be prevented by the addition of bovine serum albumin to the reaction mixture. Bublitz and Kennedy did not report any such inactivation and did not include serum albumin in their reaction mixture. Yet, all of these workers report linear results over a wide range of GP concentrations and yields of DPNH in excess of 90%. In order to determine whether or not such preventive measures would be

necessary, a series of standards were run both with and without added serum albumin. A pH of 10.2 was chosen, this being the optimum pH of the enzyme for the oxidation of GP (180). Glycine was chosen as a buffer since it is known not to inhibit the enzyme (153), as would a phosphate buffer (98). The reaction mixture contained 0.05 M glycine buffer, pH 10.2; EDTA, 0.001 M; DPN, 5 μmoles; GPDH(DPN), 20 μg; L-α-GP in one of three concentrations: 0.125 μmoles, 0.062 μmoles, or 0.031 μmoles, in a three milliliter reaction mixture. The optical density of the samples was read at 340 mμ against reagent blanks without GP. Highly variable results were obtained both with and without added serum albumin. The production of DPNH varied widely between a high of 95% and a low of zero. Percentage production of DPNH was calculated from the known amount of GP added to the reaction mixture and the extinction coefficient for DPNH at 340 mμ, which is 6.22×10^6 cm^2/mole (76).

In an attempt to obtain consistently higher productions of DPNH, standards were run at varying DPNH and hydrazine concentrations. This had little effect since DPNH production remained highly inconsistent.

Rather than solve any problems, this latter procedure brought out another problem which had been largely overlooked. As the concentration of either DPN or hydrazine was increased, the optical densities of the blanks increased at a far greater rate than could be accounted for simply by adding the optical densities of the reactants. Some unknown reaction was occurring which formed a product absorbing at 340 mμ. This reaction is presumably between DPN and hydrazine as indicated by the results shown in the table below.

The reaction mixture contained 200 μmoles of hydrazine, adjusted to pH 10.2 with NaOH, and 5 μmoles of DPN. Optical densities were read against distilled water at 340 mμ on a Beckman DU spectrophotometer.

Time (min.)	O.D.
1	.164
3	.182
6	.189
10	.196
35	.221
60	.238

Although the nature of the absorbing product is not known, it is not DPNH, as indicated by the absorption curve of the above reaction mixture.

Hydrazinolysis of the protein probably did not play a role in the inactivation of the enzyme because of the long time and high temperatures required for the reaction to take place (69).

Because of the inconsistent results obtained while using hydrazine as a trapping agent for DHAP, semicarbazide was substituted for the hydrazine. Also, it was felt that the high pH of 10.2 might be harmful to the enzyme. Therefore, the pH of the reaction mixture was lowered to 9.2. This pH is intermediate between those of 9.1 and 9.4 used by Boltralik and Noll (23), and by Bublitz and Kennedy (28) respectively. Standards run at the lower pH of 9.2 still were not linear in the upper ranges, but were linear in the lower ranges at which the analyses were carried out. The high yields of DPNH claimed by others have not

been obtained. However, the results using semicarbazide as a trapping agent at pH 9.2 are consistent and reproducible. It was on the basis of consistency and reproducibility that the method was considered to be satisfactory for the analysis of GP. The more consistent and reproducible results can not be ascribed entirely to the decreased pH. Standards run using hydrazine as a trapping agent remained inconsistent even at the lower pH of 9.2.

The final reaction mixture which was decided upon contained the following:

1. glycine buffer, final conc. 0.1 M, pH 9.2
2. EDTA, final conc. 0.001 M
3. GPDH(DPN), 20 µg
4. DPN, 3.5 µmoles
5. semicarbazide, 200 µmoles
6. GP, 0.046 and 0.023 µmoles
7. water to make 1.1 ml

A reagent blank without GP was also prepared. After thirty minute incubation in a water bath at 37°C, the optical densities of the standards were read against the reagent blank on a Beckman DU at 340 mµ.

Boltralik and Noll found it necessary to add 95% ethanol at the end of the incubation period to stabilize the reaction mixture and the final optical density which was obtained (23), but Bublitz and Kennedy did not (28). Therefore, a time curve was plotted without the addition of alcohol. The final optical density proved to be stable for at least ninety minutes. Because of the stability of the final product, nothing needs to be added to further stabilize the reaction mixture.

These findings are consistent with the fact that both of the products, DPNH and the hydrazone of DHAP, and the reactants DPN and GP are stable at the pH of 9.2 (68, 105, 107). The presence of either hydrazine or semicarbazide does not affect the stability of the final optical density.

Analysis of the Glycerophosphate Content of Tissues and Warburg Medium:

For the analysis of the GP content of tissues other than anoxic livers, 0.5 ml of protein free filtrate were added to a reaction mixture containing 1) 0.3 ml of a solution of EDTA plus glycine buffer, pH 9.2 final concentration 0.1 M; 2) 0.05 ml of a DPN solution, 3.5 μmoles; 3) 0.05 ml of GPDH(DPN) solution, 20 μg. Then, 0.2 ml of semicarbazide solution (200 μmoles), adjusted to pH 9.0-10.0, was added and the reaction mixture well mixed. Tissue blanks without GPDH(DPN) were prepared with each sample in addition to a water blank.

Occasionally a slight turbidity developed when the protein free filtrate was added to the alkaline reaction mixture. Therefore all tubes were centrifuged following a thirty minute incubation at 37°C. The turbidity was probably due to the precipitation of phosphates from the tissues in the alkaline reaction mixture. The optical densities of all samples were read in a Beckman DU spectrophotometer at 340 mμ against their individual tissue blanks.

When anoxic liver samples were analysed for their GP contents, 0.1 ml aliquots of the protein free filtrates were used instead of the 0.5 ml aliquots used for the other tissues because of the much higher GP content of liver. Water was added to bring the total volume of the reaction mixtures up to 1.1 ml.

9. Analysis of Lactate

The procedure for the determination of lactate used here was a modification of the method developed by Barker and Summerson (10)

A. Preparation of standards and blanks:

One milliliter of a stock solution of lithium lactate containing 200 μg of lactate per milliliter was added to ten milliliters of water, to form the working standard containing 18.18 μg of lactate per milliliter. 1.0 and 0.5 ml aliquots of the working standard were added to 1.0 and 1.5 ml of water respectively. Then, 0.5 ml of saturated $CuSO_4$ was added. Blanks containing 2 ml of water plus 0.5 ml of saturated $CuSO_4$ were also prepared.

B. Dilution of Warburg medium:

At the same time as the blanks and standards were prepared 0.2 ml of medium from equilibrium flasks, 0.1 ml from 2 hour aerobic flasks, and 0.1 ml from 2 hour anaerobic flasks were each added to 2 ml of water and 0.5 ml of saturated $CuSO_4$ in individual test tubes.

C. Homogenates of anaerobic tissues:

0.1 ml of a protein free filtrate from a homogenate of an aerobic tissue was added to 2 ml of water plus 0.5 ml of saturated $CuSO_4$.

D. Dilutions for in situ lactates:

For the determination of the in situ content of lactate, different dilutions must be used because of the small amounts of lactate present.

1. Standards.

One ml of the stock solution was added to 25 ml of water, forming the working standard containing 7.69 μg of lactate per ml. Two standards were prepared, one containing one ml of the working standard and

the other containing 0.5 ml of the working standard plus 0.5 ml of water. To each of these dilutions was added 0.25 ml of saturated $CuSO_4$.

2. Protein free filtrates.

0.4 ml of the protein free filtrates of the perchloric acid extract of the tissues was added to 0.6 ml of water and 0.25 ml of saturated $CuSO_4$. All tubes could then be stored frozen in the refrigerator.

E. Lactate analysis:

At the time of analysis two standards and three or four samples were thawed. In the case of anoxic tissues or Warburg medium filtrates, two scoops of lime containing 0.5-0.6 gm were added to each tube. One scoop containing 0.25-0.3 gm of lime was added to the smaller volumes used in determining in situ lactate levels. Each tube was mixed well on the Vortex mixer, allowed to stand for 30 minutes, and then centrifuged 15 minutes at 3,000 rpm. The clear supernatant was decanted into another test tube. 0.2 ml of the copper-lime filtrate from the Warburg medium or 0.2 ml of the protein free filtrate from the ischemic tissues was added to 0.6 ml of water in 19x150 mm cuvettes. Then 3 ml of concentrated sulfuric acid were added while continually shaking the tube in an ice bath. All tubes were capped with plastic covers and then placed in a boiling water bath for five minutes. At the end of the five minutes, the tubes were returned to the ice bath and allowed to cool. After about five to seven minutes in the ice bath, one drop of 6% cupric sulfate and one drop of a solution of p-hydroxydiphenyl were added to each tube. The mixture was well stirred on a vortex mixer. The tubes were then placed in a constant

temperature water bath at 27°C for forty minutes. After the incubation period the optical density of the tubes was read on a Coleman Jr. spectrophotometer at 565 mμ against the water blank.

10. Preparation of Special Reagents

1) DL-α-GP (Eastern Chemical Corp.)

stock solution contained 10 μmoles GP/ml

10 μmoles GP/ml = 2.34 mg/ml or

58.5 mg L-α-GP/25 ml or

117 mg of DL-α-GP/25 ml

A working standard was prepared by diluting the stock solution 1:21. The stock solution was stored frozen in the refrigerator in a number of separate test tubes, each tube being thawed only just before it was to be used in preparation of the working standard. A fresh working standard was prepared each time the analysis was run.

2) DPN, obtained from Sigma Chemical Co.

50 mg of DPN were dissolved in a milliliter of distilled water and stored frozen in the refrigerator.

3) EDTA plus Glycine buffer (both obtained from Eastman-Kodak)

1.35 gm of glycine plus 62 mg of EDTA were dissolved in 50 ml of water and adjusted to a pH of 9.2 with concentrated NaOH, using a pH meter.

4) Semicarbazide (Eastman-Kodak, reagent grade)

0.67 gm of semicarbazide were dissolved in water. The solution was then neutralized with concentrated NaOH to a pH between 9.0 and 10.0. A more accurate neutralization was not necessary since this pH range is not within the buffering range of the semicarbazide. The

semicarbazide was always prepared fresh just before each analysis.

5) GPDH(DPN) - (Boehringer and Son, Mannheim, Germany)

The enzyme was obtained as a crystalline suspension in $(NH_4)_2SO_4$ solution. One milliliter suspension containing 10 mg of the enzyme was diluted to a volume of 5 milliliters (2mg/ml), forming the stock solution. One ml of this stock solution was then diluted to 5 ml, forming the working solution. Small aliquots of the stock solution were kept frozen in the refrigerator, and not thawed until used. The stock solution was also kept frozen in the refrigerator.

IV. RESULTS AND DISCUSSION

In the comparison of sample means, using the "t" test, the 95% confidence interval was used as the minimum interval for the determination of the significance of a difference between sample means. In other words, the differences between sample means are not considered significant unless the p values are less than 0.05.

In order to permit comparison of the lactate and GP levels in the various tissues studied, the results are expressed in terms of μmoles of GP or lactate present in one gram of tissue on a wet weight basis. Dry weight and nitrogen content of the tissues are also given. However, because of the relatively large magnitude of the differences observed between the aerobic and anaerobic tissues, expressing the GP and lactate production in terms of the nitrogen content of the tissues would not alter any of the conclusions. Since there is a large difference in the molecular weights of GP and lactate, the frequently used units of milligrams per gram of tissue will not be used. Thus,

each μmole of GP or of lactate which accumulates represents a μmole of hydrogen accepted by DHAP or pyruvate respectively.

In situ experiments:

The average in situ level of GP in the gastrocnemius of the rat is 0.16 ± 0.04' μmoles per gram of tissue (table 1). This value compares favorably with that obtained by Klingenberg et al. (87). GP and lactate levels were also determined in skeletal muscle five and ten minutes after the rats had been killed by decapitation (table 2). After five minutes the level of GP had risen from an aerobic level of 0.16 ± 0.04 μmoles to 1.1 ± 0.02 μmoles per gram of muscle wet weight (table 2), a seven-fold increase. At the end of ten minutes the GP concentration had not increased significantly over the level at five minutes, reaching 1.2 ± 0.06 μmoles per gram of muscle. Because there was no significant difference between the GP levels after five or ten minutes of extreme ischemia the two samples have been grouped together giving a combined sample with an average GP content of 1.2 ± 0.03 μmoles per gram of muscle.

By way of comparison, the lactate levels of muscle rose from an in situ level of 1.8 ± 0.02 (table 1) μmoles per gram of muscle to 21.5 ± 2.1 μmoles per gram after five minutes of ischemia (table 2). The average lactate concentration after ten minutes of extreme ischemia was 21.3 μmoles per gram. Therefore the two lactate samples were also grouped together. In other words, both GP and lactate production reached a maximum within five minutes under the conditions

'standard error

table 1.

Glycerophosphate and lactate levels in liver and muscle frozen *in situ*[1]

	muscle	n[2]	liver	n[2]
GP	0.16 ± 0.04[3]	12	0.70 ± 0.12	12
lactate	1.8 ± 0.02	7	2.4 ± 0.4	8
L/GP	11		3.4	

1. GP and lactate levels are expressed in terms of umoles per gram of tissue.
2. Number of experiments
3. Standard error

table 2.

Glycerophosphate and lactate content of muscle and liver after five and ten minutes of ischemia.[1]

	5 min.	n[2]	10 min.	n[2]	p[3]	five and ten min. groups combined
			muscle			
GP	1.1 ± 0.02[4]	6	1.2 ± 0.06	6	>.10	1.2 ± 0.03
lactate	21.5 ± 2.1	6	21.3 ± 2.2	6	>.10	21.4 ± 1.4
L/GP	19		18			19
			liver			
GP	2.6 ± 0.2	6	2.6 ± 0.1	6	>.10	2.6 ± 0.1
lactate	11.9 ± 1.0	6	13.0 ± 0.4	6	>.10	12.4 ± 0.6
L/GP	4.6		5.0			4.8

1. GP and lactate levels are expressed in terms of umoles per gram of tissue.
2. Number of experiments
3. Standard error
4. Probability based on paired analysis

of extreme ischemia (table 2).

The ratios of the average lactate to the average GP content of muscle frozen in situ (aerobic conditions) was 11, while after five and ten minutes of ischemia the ratios were 19 and 18 respectively. No significant difference can be demonstrated between the ratios of GP and lactate of the tissues frozen in situ and of those subjected to severe ischemia because of the wide variation in ratios. However, it is apparent that there is a large increase in GP levels of skeletal muscle of the rat during an oxygen deficit.

In liver, as in muscle, no further increase in either lactate or GP occurred after five minutes of extreme ischemia (table 2). However, rather large differences have been observed in lactate and GP content between skeletal muscle and liver of the rat. The in situ GP content of muscle is much less than that of liver. The levels found were 0.16 ± 0.04 and 0.70 ± 0.12 μmoles per gram respectively (table 1). In the anoxic liver, the average GP content reached 2.6 ± 0.01 μmoles per gram as opposed to 1.2 ± 0.03 μmoles per gram in muscle (table 2). However, the accumulation of GP in the liver represented a much higher proportion of the hydrogen accepted as compared to hydrogen acceptance due to lactate formation than these figures indicate. This difference in proportion is due to the fact that the level of lactate in the anoxic liver was not as high as that in skeletal muscle. The accumulation of lactate in the gastrocnemius reached an average of 21.4 ± 1.4 μmoles per gram as compared to only 12.4 ± 0.06 μmoles per gram in liver under anaerobic conditions. In muscle the percent of the hydrogen accepted

anaerobically which entered the GP molecule was only six percent of that which entered lactate, while it was twenty-one percent in liver. Therefore, it can be seen that DHAP plays a quantitatively more important role as a hydrogen acceptor in liver than in skeletal muscle.

Lactate to GP ratios (L/GP) of aerobic liver in which both GP and lactate were measured did not differ significantly from the L/GP ratios of anoxic livers (tables 1, 3), demonstrating the parallel increase of GP and lactate in the liver. This parallel increase is in agreement with the work of Ciaccio, Keller and Boxer (42).

Warburg experiments:

In the Warburg experiments an attempt was made to compare GP and lactate production of red and white muscle and diaphragm under both aerobic and hypoxic conditions. The tissues in the Warburg flasks which were gassed with 95% N_2 - 5% CO_2 were severely deficient, but not completely lacking in, oxygen. Therefore these tissues will be referred to as being hypoxic rather than anoxic. The Warburg flasks to be maintained under hypoxic conditions during the two hour incubations were gassed for about one minute with the nitrogen-carbon dioxide mixture. This procedure almost certainly did not displace all of the oxygen in the flasks. Some air may also have been admitted when the flasks were placed on the Warburg monometers.

In the aerobic flasks, the GP concentration of the red and white muscle was 0.12 ± 0.02 μmoles per gram and 0.06 ± 0.02 μmoles per gram respectively (table 3). Because of the low optical densities obtained as a result of the low GP levels and the small size of the tissue samples available for the Warburg experiments, the data are not considered

table 3.

The glycerophosphate content[1] of adductor muscle fiber groups and diaphragm following two hours incubation in the Warburg[2] under either aerobic[3] or hypoxic[4] conditions.

	aerobic	n[5]	hypoxic	n	p
red muscle	0.12 ± 0.02[6]	6	0.53 ± 0.05	6	<.002[7]
white muscle	0.06 ± 0.02	6	0.22 ± 0.04	6	<.002[7]
diaphragm	0.29 ± 0.02	7	0.73 ± 0.03	6	<.001[8]

1. Content in terms of umoles per gram of muscle wet weight
2. Warburg medium consists of a Krebs-Ringer bicarbonate buffer pH 7.4 plus 150 mg of glucose per 100 ml.
3. Flasks gassed with 95% O_2- 5% CO_2
4. Flasks gassed with 95% N_2- 5% CO_2
5. Number of experiments
6. Standard error
7. Probability based on paired analysis
8. Probability based on unpaired analysis

suitable for a statistical comparison of the GP contents of red and white muscle. However, under hypoxic conditions the total GP accumulation was over twice as large in red muscle as in white (table 4). These observations are in agreement with those of Pearse (121), who demonstrated, by histochemical techniques, a higher activity of GPDH(DPN) in red muscle than in white. Not only do red and white muscle differ from each other in terms of GP content, but they also differ from diaphragm. The diaphragm, with an aerobic GP content of 0.29 μmoles per gram contains over twice the GP of red muscle and over six times that of white (table 3). Under hypoxic conditions the GP content of diaphragm rose from an aerobic level of 0.29 to 0.73 μmoles per gram, as compared to 0.53 and 0.22 μmoles per gram respectively for red and white muscle under hypoxic conditions.

Warburg medium:

The relative amounts of GP and lactate appearing in the Warburg medium present a very different picture from the contents of these substances in situ. While the lactate diffuses readily from all muscle preparations, no significant amount of GP was measurable in the Warburg medium of adductor fiber groups under aerobic conditions (table 5). Thus, we see here another fundamental difference between the GP and lactate metabolism of skeletal muscle. In addition, there is a significant difference between skeletal muscle and diaphragm in this respect. Diaphragm under aerobic conditions released an average of 0.14 μmoles of GP per gram of tissue per hour into the medium.

The restricted ability of GP to diffuse into the medium remained even under hypoxic conditions (table 7), though the concentration of GP

table 4.

Comparison of the glycerophosphate contents[1] of red and white muscle following incubation in the Warburg[2] under hypoxic conditions[3].

white muscle	red muscle	n[4]	p[5]
0.22 ± 0.04[6]	0.53 ± 0.05	6	<.01

1. Contents are expressed in terms of umoles per gram of muscle wet weight.
2. Warburg medium consisted of a Krebs-Ringer bicarbonate buffer pH 7.4 plus 150 mg of glucose per 100 ml.
3. Gassed with 95% N_2- 5% CO_2
4. Number of experiments
5. Probability based on paired analysis
6. Standard error

table 5.

A comparison of the difference from zero of the glycerophosphate production[1] appearing in the Warburg medium[2] following two hours incubation of adductor fiber groups and diaphragm under aerobic[3] conditions.

tissue	GP production[1]	n[4]	p[5]
red muscle	0.01 ± 0.01[6]	6	> .10
white muscle	zero	6	> .10
diaphragm	0.14 ± 0.02	6	< .01

1. Production is expressed in terms of umoles of GP per gram of muscle wet weight per hour.
2. Warburg medium consists of a Krebs-Ringer bicarbonate buffer pH 7.4 plus 150 mg of glucose per 100 ml.
3. Gassed with 95% O_2- 5% CO_2
4. Number of experiments
5. Probability based on paired analysis
6. Standard error

within all three types of muscle when undergoing hypoxia is greater than in the medium. The amount of GP within a muscle undergoing hypoxia was greater than that which appeared in the Warburg medium, even though the volume within which the GP was confined was much smaller than that of the medium (tables 4, 6). At no time did the GP concentration inside the tissue approach equilibrium with that outside in the medium. The small amount of GP appearing in the Warburg medium is in sharp contrast to the accumulation of lactate. The ratio of lactate to GP in the Warburg medium under hypoxic conditions was over 1,000 for red muscle, over 3,000 for white muscle, and was 62 for diaphragm. The lower L/GP ratio obtained with diaphragm is primarily due to a greater release of GP into the medium, although the lactate production by diaphragm under hypoxic conditions was less than for either red or white muscle.

Since the Warburg medium is essentially continuous with the extracellular fluids, the volume within which GP is contained in the tissues is very small compared to the volume of the medium. It would seem that in order for the GP to be kept within the cell during the conditions of an oxygen deficit, the cell wall must either remain impermeable to the GP or the GP must be bound to a substance such as a protein which can not diffuse out of the cell. Under the conditions of severe hypoxia to which the muscle samples were subjected it does not seem likely that the cell could sustain active processes to keep GP within the cell wall. However, this possibility can not be ruled out entirely. The role generally ascribed to GP in transporting hydrogen across the mitochondrial membrane depends on the presence of the small

table 6.

Comparison of glycerophosphate and lactate production[1] appearing in the Warburg medium[2] after two hours incubation under aerobic[3] or hypoxic[4] conditions.

	red muscle	white muscle	n[5]	p[6]
	glycerophosphate			
aerobic	0.01 ± 0.01[7]	zero	6	not compared
hypoxic	0.06 ± 0.01	0.02 ± 0.01	6	<.05
	lactate			
aerobic	7.5 ± 0.5	10.6 ± 1.4	6	<.002
hypoxic	64.3 ± 4.6	62.9 ± 3.2	6	>.10

1. Production in terms of umoles of GP per hour per gram of muscle wet weight. Lactate production equals the lactate present after 135 minutes incubation minus that present after the 15 minute equilibration period.
2. A Krebs-Ringer bicarbonate buffer pH 7.4 plus 150 mg of glucose per 100 ml.
3. Gassed with 95% O_2- 5% CO_2
4. Gassed with 95% N_2- 5% CO_2
5. Number of experiments
6. Probability on the basis of paired analysis
7. Standard error

table 7.

A comparison of the glycerophosphate and lactate production[1] appearing in the Warburg medium[2], aerobic[3] vs hypoxic[4] conditions.

	aerobic[3]	n[5]	hypoxic[4]	n[5]	p
			glycerophosphate		
red muscle	0.01 ± 0.01[6]	6	0.06 ± 0.01	6	<.01[7]
white muscle	zero	6	0.03 ± 0.01	6	<.05[7]
diaphragm	0.14 ± 0.02	6	0.72 ± 0.05	8	<.001[8]
			lactate		
red muscle	7.5 ± 0.5	6	64.3 ± 4.6	6	<.001[7]
white muscle	10.6 ± 1.4	6	62.9 ± 3.2	6	<.001[7]
diaphragm	8.9 ± 0.9	7	44.5 ± 3.2	7	<.001[8]

1. Production in terms of umoles of GP per gram of muscle wet weight. Lactate production equals the lactate present after 135 minutes incubation minus that present after the 15 minute equilibration period.
2. A Krebs-Ringer bicarbonate buffer pH 7.4 plus 150 mg of glucose per 100 ml.
3. Gassed with 95% O_2- 5% CO_2
4. Gassed with 95% N_2- 5% CO_2
5. Number of experiments
6. Standard error
7. Probability based on paired analysis
8. Probability based on unpaired analysis

readily diffusible molecule, GP. Any suggestion that GP can not diffuse out of the cell because it is bound to a protein would seem to refute the suggested role of GP in the GP cycle. In addition, homogenates of some tissues oxidize GP readily, suggesting that GP is not bound to a protein. It can not be said whether this also applies to muscle. However, there is no ready explanation for the virtual lack of GP appearance in the Warburg medium.

The results of the Warburg and of the in situ experiments both point to the increased quantity of GP in tissues during an oxygen deficit. However, the results of the Warburg and in situ experiments differ substantially in a quantitative sense. In the Warburg experiments the amount of GP produced under hypoxia was much less in proportion to the lactate production than in the in situ experiments (tables 2 and 7). The Warburg medium is essentially continuous with the extracellular fluids of the tissues. Therefore, when the tissues are placed in the Warburg medium under hypoxic conditions the volume into which the lactate can diffuse is greatly enlarged. However, the volume available to the GP remains essentially the same, since the GP remains primarily within the tissues. It would therefore seem that the design of the Warburg is strongly biased towards lactate production instead of GP production. For many years, the lactate appearing in the Warburg medium has been used as a measure of total anaerobic glycolysis. Although it has been demonstrated that this criterion is not true in liver and muscle in situ, the design of the Warburg experiment is such that the lactate production in the medium can be taken to represent the total anaerobic glycolysis without introducing a significant error (by

not considering the GP production). It would therefore seem that in situ experiments provide a more accurate quantitative estimate of the relative GP and lactate productions normally in tissues than do in vitro experiments. At the same time it must be noted that in situ experiments leave many variables uncontrolled, particularly the temperature and pH.

table 8.

Dry weight and total nitrogen content of the tissues

	dry weight[1]	n[2]	nitrogen[3]	n
		IN SITU TISSUES		
muscle frozen in situ	22.83 ± 0.62[4]	7	12.62 ± 0.03	5
ischemic muscle	24.47 ± 0.52	6	13.07 ± 0.02	6
liver frozen in situ	27.53 ± 0.42	9	11.03 ± 0.25	10
anoxic liver	30.63 ± 0.29	6	10.32 ± 0.20	6
		WARBURG TISSUES		
red muscle	19.56 ± 1.04	5	13.22 ± 0.26	6
white muscle	18.54 ± 0.38	5	13.10 ± 0.20	6
diaphragm	20.73 ± 0.82	11	12.83 ± 0.25	12

1. Percentage of the wet weight after about 24 hours at 90 C
2. Number of experiments
3. Grams of nitrogen per 100 grams of tissue dry weight
4. Standard error

V. SUMMARY

1. A review of the literature concerning GP metabolism is presented.
2. A method for the analysis of GP is described.
3. In situ experiments demonstrate that, as measured by the accumulation of GP, DHAP competes successfully with pyruvate for the DPNH produced during anaerobic conditions.
4. The extent of GP accumulation in skeletal muscle in situ is much less than in the liver of rats.
5. In Warburg experiments GP accumulates in diaphragm, and in red and white muscle during hypoxic conditions.
6. Neither red nor white adductor fiber groups release a measurable amount of GP into Warburg medium under aerobic conditions.
7. Diaphragm differs from red and white muscle fiber groups in that it releases appreciable amounts of GP into the Warburg medium.
8. Diaphragm forms more GP than red muscle which, in turn, forms more than white muscle under hypoxic conditions.

VI.

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