Running title :

CARBOHYDRATE METABOLISM IN RAT SKELETAL MUSCLE

ABSTRACT

Wai Man Poon
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Department of Biochemistry

SOME STUDIES OF CARBOHYDRATE METABOLISM IN RAT SKELETAL MUSCLE

The factors controlling the rate of glycolysis in rat skeletal muscle homogenates and extracts were studied. Hexokinase was identified as the first rate-limiting step. In slightly acidic muscle extracts, the phosphofructokinase step was limiting. This was related to the amount of soluble and insoluble forms of the enzyme. The relative amounts of each form depended on the pH. The rate-limiting effect was shown to be eliminated when the muscle extracts were prepared in a slightly alkaline medium. The importance of the ATPase in the homogenates and extracts in controlling the rate of glycolysis was also shown. Increasing the amount of ATPase up to a certain level stimulates glycolysis by supplying ADP for the phosphoglycerate kinase and pyruvate kinase steps.

Various enzymatic and colorimetric methods for the assay of intermediates of the pentose phosphate pathway were studied. Transaldolase was partially purified from Candida utilis.

Rat skeletal muscle extracts have relatively high phosphoribose isomerase and phosphoketopentose epimerase activities, and were able to convert ribose-5-phosphate into ribulose-5-phosphate and xylulose-5-phosphate. However, the subsequent conversion of the pentose phosphates into other intermediates of the non-oxidative route of the pentose phosphate pathway was extremely slow, indicating that the transketolase step was limited. The muscle extracts were also not very effective in metabolizing fructose-6-phosphate and triose phosphate through the non-oxidative route of the pentose phosphate pathway.

P.T.O.

The ability of rabbit muscle phosphofructokinase to phosphorylate sedoheptulose-7-phosphate, forming sedoheptulose-1,7-diphosphate, was confirmed. A new enzymatic procedure for the assay of sedoheptulose-7-phosphate was reported.

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Wai Man Poon

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Department of Biochemistry, McGill University

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TABLE OF CONTENTS

Page

Page

Page

ABBREVIATIONS

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
IMP	Inosine monophosphate
NAD	Nicotinamide adenine dinucleotide, oxidized
NADH	Nicotinamide adenine dinucleotide, reduced
NADP	Nicotinamide adenine dinucleotide phosphate, oxidized
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
G-6-P	Glucose-6-phosphate
F-6-P	Fructose-6-phosphate
FDP	Fructose-1,6-diphosphate
G-3-P	Glyceraldehyde-3-phosphate
DHAP	Dihydroxyacetone phosphate
Gl-1-P	Glycerol-1-phosphate
1,3-di-PGA	1,3-diphosphoglyceric acid
3-PGA	3-Phosphoglyceric acid
2-PGA	2-Phosphoglyceric acid
PEP	Phosphoenolpyruvate
Pyr	Pyruvate
R-5-P	Ribose-5-phosphate
Ru-5-P	Ribulose-5-phosphate
Xu-5-P	Xylulose-5-phosphate
S-7-P	Sedoheptulose-7-phosphate
Er-4-P	Erythrose-4-phosphate
6-PGA	6-Phospho-gluconic acid
SDP	Sedoheptulose-1,7-diphosphate
Cr-P	Creatine phosphate
Pi	Inorganic phosphate
EDTA	Ethylene diamine tetraacetate (tetra-sodium salt)

I.U.	International unit: μmoles substrate converted/minute
HK	Hexokinase, ATP:D-hexose 6-phosphotransferase (2.7.1.1)
PGI	Phosphoglucose isomerase, D-glucose-6-phosphate ketol-isomerase (5.3.1.9)
PFK	Phosphofructokinase, ATP:D-fructose-6-phosphate 1-phosphotransferase (2.7.1.11)
TIM	Triose phosphate isomerase, D-glyceraldehyde-3-phosphate Ketol-isomerase (5.3.1.1)
Gl-1-P DH	Glycerol-1-phosphate dehydrogenase, L-glycerol-3-phosphate: NAD oxidoreductase (1.1.1.8)
G-3-P DH	Glyceraldehyde-3-phosphate dehydrogenase, D-glyceraldehyde-3-phosphate:NAD oxidoreductase (1.2.1.12)
PGK	Phosphoglycerate kinase, ATP:D-3-phosphoglycerate 1-phosphotransferase (2.7.2.3)
PGA mutase	Phosphoglyceromutase, D-phosphoglycerate 2,3-phosphomutase (5.4.2.1)
PK	Pyruvate kinase, ATP:pyruvate phosphotransferase (2.7.1.40)
LDH	Lactate dehydrogenase, L-lactate:NAD oxidoreductase (1.1.1.27)
G-6-P DH	Glucose-6-phosphate dehydrogenase, D-glucose-6-phosphate: NADP oxidoreductase (1.1.1.49)
6-PGA DH	6-Phosphogluconate dehydrogenase, 6-phospho-D-gluconate: NADP oxidoreductase (1.1.1.44)
Aldolase	Ketose-1-phosphate aldehyde-lyase (4.1.2.7)
TK	Transketolase, D-sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate glycolaldehydetransferase (2.2.1.1)
TA	Transaldolase, D-sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate dihydroxyacetonetransferase (2.2.1.2)
PRI	Phosphoribose isomerase, D-ribulose-5-phosphate ketol-isomerase (5.3.1.6)
PKPE	Phosphoketopentose epimerase, D-ribulose-5-phosphate-epimerase (5.1.3.1)
FDPase	Fructose-1,6-diphosphatase, D-fructose-1,6-diphosphate 1-phosphohydrolase (3.1.3.11)
ATPase	Adenosine triphosphatase, ATP phosphohydrolase (3.6.1.3)
SDPase	Sedoheptulose-1,7-diphosphatase

LIST OF TABLES

TABLE | Page

LIST OF FIGURES

FIGURES Page

FIGURE Page

PREFACE

Investigations on the carbohydrate metabolism in rat skeletal muscle was initiated in this laboratory by Dr. T.Wood in 1965. The works reported here constituted part of this program.

As a first step, the glycolytic properties of muscle homogenates were studied by Mrs. Garcia-Arocha, which provided the basis for this investigation into the factors controlling the rate of glycolysis.

In addition, investigations have been initiated into the operation and control of the pentose phosphate pathway in this tissue.

INTRODUCTION

A. Glycolysis in muscle

Historical

The phenomenon of carbohydrate metabolism was first observed by Claude Bernard (1877) (1), who stated that animal tissues were able to transform sugars to lactic acid.

The term glycolysis was introduced by Lepine (1909) (2) to indicate the disappearance of carbohydrate during the metabolic activities of a tissue. This rather broad meaning was later restricted by Warburg (1923) (3) to the fission of the carbohydrate molecule by a fermentation reaction to yield an acidic product, which is usually lactic acid in animal tissues. The overall equation of this type of transformation is represented as follows:

$$C_6H_{12}O_6 = 2\ C_3H_6O_3$$

Glucose Lactic acid

The pioneer research of Fletcher and Hopkins in 1906 (4) showing that glycolysis was greater in working than in resting muscle led to the realization by Hill (5) and Meyerhof (6) of the close relationship between glycolysis and energy requirement for muscular work.

In 1912 Embden and his coworkers (7,8,9,10) began their investigation of lactic acid production in muscle. They found that lactic acid production in muscle tissues bears many resemblances to fermentation in yeast (11), for example, the requirement for inorganic phosphate, the presence of Harden-Young ester (hexose diphosphate) and Robison ester (hexose monophosphate). They also found that muscle juice contains lactic acid precursors, a "Lact-acidogen" which when depleted by the formation of lactic acid, could be replenished by muscle glycogen.

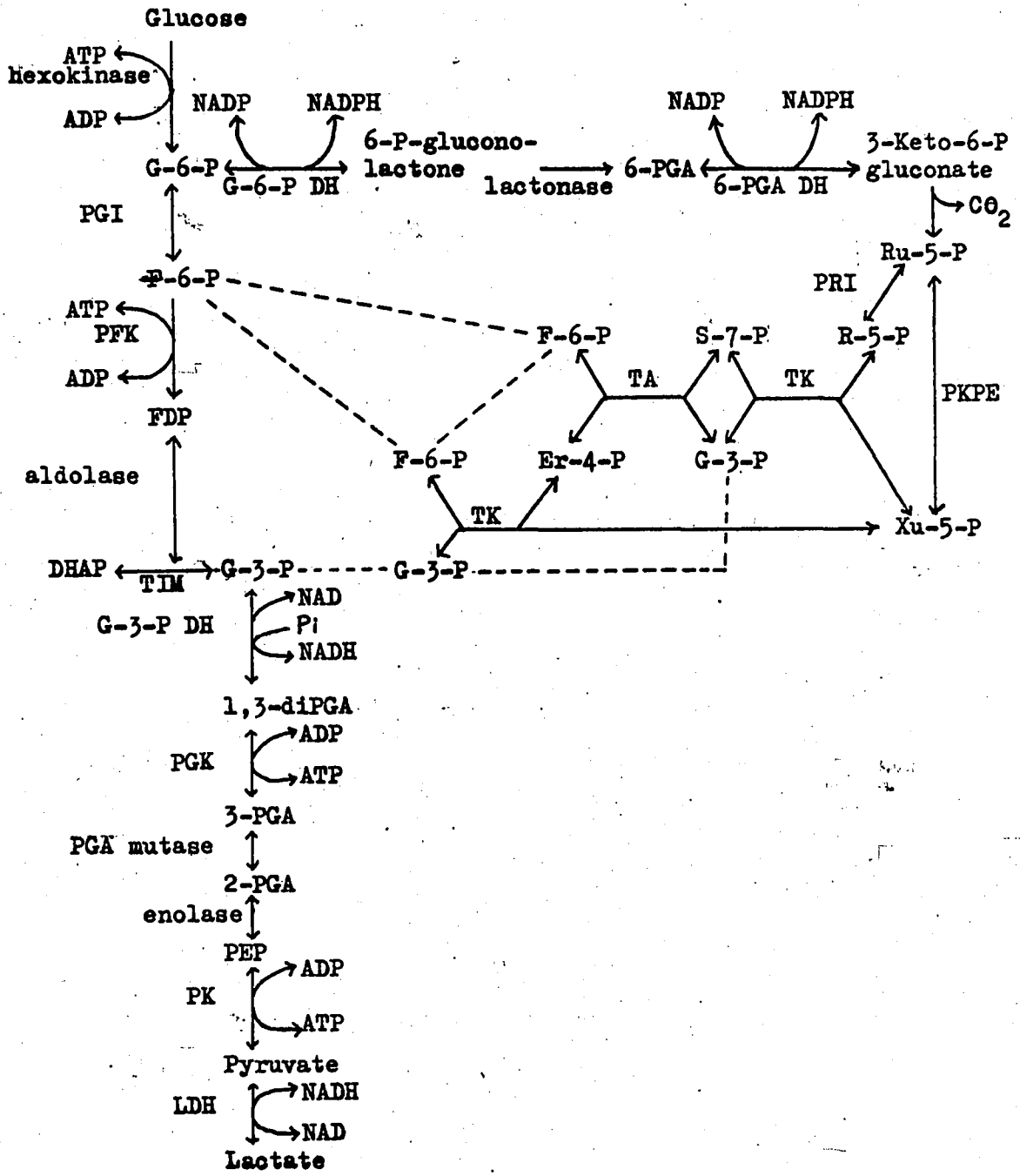
A series of investigations on muscle extract by Meherhof and his coworkers in the 1920's (12,13,14,15) revealed that, as in yeast, a heat labile, dialysable coenzyme was present in the extract and was required for glycolysis. The coenzyme (coenzyme 1 or NAD) was later identified and studied by von Euler and Myrback (16). Meyerhof also found a heat labile activator, or enzyme, the addition of which to muscle extract accelerated the rate of conversion of hexose to lactic acid; he named this substance hexokinase.

The role of adenylic acid in glycolysis was clarified by the work of Fiske and Subbarow (17) and Lohmann (18). They reported that adenylic acid was not itself a normal constituent of muscle but a break-down product of a compound, isolated by them as a crystalline salt, which proved to be ATP. The important role of ATP as well as magnesium in glycolysing muscle extracts was subsequently elucidated (19,20).

Following the discovery of the essential components of glycolysis, work began on the individual steps or intermediary metabolism of glycolysis. This involved a large amount of research by many investigators including Embden, Meyerhof, Warburg, Cori, Parnas and many others. The glycolytic pathway or Embden-Meyerhof pathway, named in honour of their pioneer work, is shown in Fig. 1 in conjunction with the pentose phosphate pathway.

An understanding of the energy relationships involved in the glycolytic pathway, especially the concept of high energy phosphate compounds was largely due to the efforts of Lipmann (21) and Kalckar (22). Burk (23) has calculated that there was a free energy change of -58,000 cal in the degradation of one glucose to two lactic acids. Lipmann (24) calculated that the energy obtained can be accounted for as high energy phosphate bonds in "phosphopyruvic

Fig.1. The Embden Meyerhof Pathway and the Pentose Phosphate Pathway.



acid" and "phosphoglyceryl phosphate".

Lohmann (25) found that in muscle the ATP diminished rapidly on mincing the muscle tissue and an approximately equivalent amount of inorganic phosphate appeared. Engelhardt (26) showed that highly purified preparation of myosin retained ATPase activity and suggested that myosin itself, the contractile protein of muscle, was actually the enzyme liberating the energy stored in the terminal phosphate bond of ATP, which was thus immediately available for conversion into mechanical work.

Fiske and Subbarow (27) and Eggleton and Eggleton (28) showed that creatine phosphate, when largely decomposed during a long series of muscle contractions, was reconstituted quite rapidly during recovery in oxygen. Similarly, anaerobically, creatine phosphate was reconstituted very effectively at the expense of glycolysis (29). Lundsgaard (30,31) showed that muscle contraction proceeded after complete blocking of glycolytic processes by iodoacetate and was accompanied by creatine phosphate breakdown.

Glycolysis in muscle homogenate and extract

Earlier work on glycolysis was mainly concerned with the conditions favouring a high rate of hexose consumption and lactic acid production and many of these studies were done in tissue extracts or homogenates.

Meyerhof (32) showed that cutting the muscle and suspending the tissue "brei" in phosphate solution increased the resting rate of glycolysis about ten times; and if the muscle extract was obtained and fortified with coenzyme from boiled extract, the rate of glycolysis was further increased (33).

Working with dialysed muscle extract, Kendall and Stickland (34,35) confirmed the requirement for inorganic phosphate, magnesium, ATP and coenzymes for supporting glycolysis. They showed that although the coenzyme was necessary for lactic acid production from glycogen, the conversion of glycogen to hexose mono- and di-phosphate did not require coenzyme.

Neifakh and Mel'Nikova (36) were able to obtain high rates of glycolysis from dialysed muscle extract fortified with glycogen, fructose-1, 6-diphosphate, ATP, magnesium, inorganic phosphate, cysteine and nicotinamide.

Lepage (37) and Wenner and his coworkers (38) suggested that whole unfractionated homogenate could be a more suitable preparation for studying glycolysis. The advantage of using homogenate, besides its accessibility to introduction and removal of various intermediates and effectors, was that it contained all of the enzymes, ions and other factors of the intact cell. Both the Potter type "water homogenate" (39) and the "isotonic homogenate" described by Elliot and his coworkers (40) have been widely used. The later investigators suggested that the isotonic homogenate retained more of the cellular organization of an intact cell than a hypotonic homogenate.

The disadvantage of using a homogenate was that it contained several degradative enzymes (NADase, ATPase and adenylic deaminase) which were released during the preparation.

Adenylic deaminase was first described by Schmidt (41) and its properties studied by Kalckar (42) and Nikiforuk and Colowick (43), the latter investigators showed the sensitivity of the enzyme towards fluoride. Most of the enzyme activity was found to be associated with the myofibrillar fraction in muscle tissue (44).

Myrback (45) was probably the first to observe that the disintegration of animal tissue released a heat labile system that rapidly destroyed the biological activity of pyridine nucleotides. Handler and Klein (46) were able to show NADase activity in rat skeletal muscle and confirmed the inhibitory effect of nicotinamide on the enzyme first reported by Mann and Quastel (47). Utter and his coworkers (48) assumed that the "inhibitor" of glycolysis associated with the structural elements was mainly the nucleotidase which functioned by decomposing the coenzyme.

The presence of ATPase activity in muscle was first observed by Lohmann (25). Meyerhof and Geliazkowa (49) showed that, in brain homogenate, ATPase could inhibit glycolysis by breaking down the ATP. Meyerhof and Wilson (50) showed that over ninety percent of the ATPase in brain homogenate was in the particulate fraction.

Using fluoride to inhibit phosphatase and adenylic deaminase and nicotinamide to inhibit NADase, Lepage (51) showed that the glycolytic rate of the whole unfractionated homogenate was higher than the corresponding extract obtained by fractional ultracentrifugation and that by adding the various fractions back to the extract, a glycolytic rate equal to that of the homogenate was obtained.

More recently, homogenate systems have been used in the investigations of glycolysis in muscle in muscular dystrophy (52,53) and of glycogenolysis in muscle (54,55).

Garcia-Arocha (56) has shown that rat muscle homogenates prepared in nicotinamide and fortified with ATP, magnesium, NAD, potassium and dithiothreitol were able to glycolyse glucose-6-phosphate and fructose-1,

6-diphosphate at a high rate under anaerobic conditions. The highest rate obtained was 1620 μmoles lactic acid/hour/gram muscle which was comparable with the maximum rate in muscle in situ of 1500 μmoles/hour/gram muscle reported by Neifakh and Mel'Nikova (36) and of 1800 μmoles/hour/gram muscle reported by Pedersen and Sacks (57) in muscle tetanised for 30 seconds.

Regulation of glycolysis in muscle.

Morgan and his coworkers (58,59), working with perfused rat diaphragm and heart, showed that membrane transport was the major rate limiting factor for glucose uptake and that transport can be stimulated by anoxia or insulin, causing the intracellular glucose and its phosphorylated intermediates to increase. They suggested that hexokinase was the controlling step under these conditions.

Further studies (61,62) revealed that under aerobic conditions the inhibition of hexokinase was a consequence of the rate limiting effect of phosphofructokinase which caused an accumulation of glucose-6-phosphate which in turn inhibited hexokinase. The inhibition of hexokinase by its product glucose-6-phosphate was first reported by Crane and Sols (60).

Hohorst and his coworkers (63) showed that in rat abdominal wall muscle, the mass action ratios of phosphohexose isomerase, phosphoglucose mutase, triose phosphate isomerase, phosphoglycerate mutase and enolase were close to the thermodynamic equilibrium constants in both resting and working state. Hence these enzymes were not likely to be rate limiting.

Pedersen and Sacks (57) measured the distribution of intermediates in resting cat gastrocnemius and found a high level of glucose-6-phosphate,while the fructose-6-phosphate level was below the limit of measurement and they suggested that phosphohexose isomerase was rate limiting. Wilson and his coworkers (64) working with the same muscle found the ratio of glucose-6-phosphate and fructose-6-phosphate was at equilibrium and suggested that phosphohexose isomerase was not rate limiting, in agreement with the findings of other investigators on frog leg muscle (65) and rabbit abdominal muscle (63).

In studies in vitro, Lardy and Parks(66) found that ATP was inhibitory to phosphofructokinase. Bucher (67) reported that in insect wing muscle, phosphofructokinase was activated when the metabolism increased during muscular activity. He stated that phosphofructokinase could be activated in vitro by changes in the concentrations of ATP, magnesium and fructose-6-phosphate. Passoneau and Lowry (68) in their work with muscle phosphofructokinase showed that the inhibition may be overcome by either ADP, AMP, inorganic phosphate, 3'5' cyclic AMP, fructose diphosphate, or more effectively, by a combination of these effectors. Lowry and his coworkers (69) postulated that whenever the formation of ATP did not keep up with its utilization, the inorganic phosphate, ADP and, in particular, AMP levels will increase and that this combination enhanced phosphofructokinase activity autocatalytically.

Danforth and Helmreich (70) and Karpatkin and coworkers (71) have shown that in frog sartorius, glycolytic rate increased several hundred fold with muscle contraction and that the increase in phosphorylase a activity

during contraction could account for the increase in glycolytic flux from glycogen. Ozand and Narahara (65) showed that, like phosphorylase a, phosphofructokinase activity was also highly stimulated during muscle contraction. Helmreich and Cori (72) elucidated the following characteristics of the frog sartorius system: (1) There was an on and off effect of enzymatic activation related to the contraction-relaxation cycle of muscle. (2) The rate of lactate formation was proportional within wide limits to the rate of stimulation. (3) The glycolytic system functioned as a well integrated unit, permitting large increases in flow rate without appreciable accumulation of intermediates. They further added that the changes in ATP, AMP and inorganic phosphate in their system during contraction was too small to account for the greatly stimulated phosphofructokinase activity and postulated that the stimulation was somehow related to the contraction process.

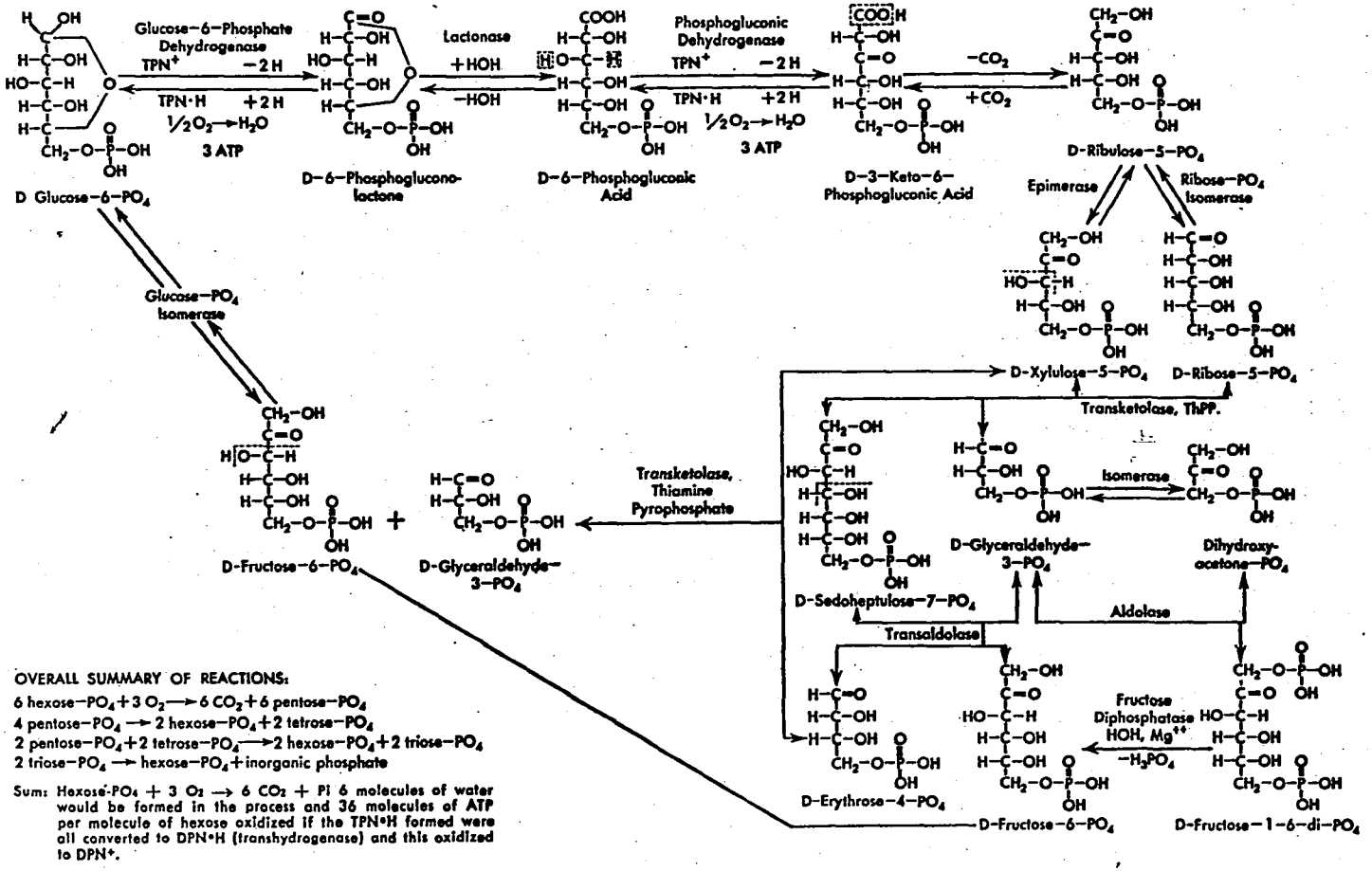
Other investigations of insect flight muscle (73), tetanized cat skeletal muscle (64), and post mortem ox skeletal muscle (74) also provide evidence that phosphorylase and phosphofructokinase are the regulatory steps in glycolysis.

In a thorough study of the level of intermediates of the electric organ of Electrophorus (75), which is considered analogous to muscle tissue, phosphorylase and phosphofructokinase were also identified as the principle sites of control of glycolysis. It was suggested that the activated glycolytic flux was subsequently switched off by the inhibition of phosphofructokinase due to the rise in the ATP/ADP x P_i potential.

Working with rat muscle homogenate, Garcia-Arocha (56) found that when glycogen or glucose-6-phosphate were used as substrates for anaerobic glycolysis, accumulation of fructose diphosphate and triose phosphate occurred. This pointed to a rate limiting effect at the glyceraldehyde phosphate dehydrogenase step. She was able to show that the availability of the enzyme, oxidation of the essential sulfhydryl groups of the enzyme and the availability of inorganic phosphate and NAD were not responsible for the rate limiting effect and concluded that the cause was the pronounced substrate inhibition effect of 1,3-diphosphoglyceric acid on glyceraldehyde phosphate dehydrogenase. She further postulated that the accumulation of 1,3-diphosphoglyceric acid was a consequence of the rate limiting step at the phosphoglycerate kinase step due to a lack of ADP.

It is clear that the phosphorylase reaction is the only rate-limiting step in muscle glycolysis in which the factors controlling the activity of the enzyme are well understood. The switching on and off of phosphofructokinase activity seems to be controlled by other factors in addition to the levels of ATP, AMP and inorganic phosphate. The steps subsequent to phosphofructokinase have not been adequately studied although they were found to be well integrated with the phosphorylase and phosphofructokinase steps.

Part of the work presented here is an attempt to investigate some of the factors regulating glycolytic rate in rat muscle homogenate and extract with emphasis on phosphofructokinase and the rate of ADP turnover.



OVERALL SUMMARY OF REACTIONS:

6 hexose−PO_4 + 3 O_2 → 6 CO_2 + 6 pentose−PO_4

4 pentose−PO_4 → 2 hexose−PO_4 + 2 tetrose−PO_4

2 pentose−PO_4 + 2 tetrose−PO_4 → 2 hexose−PO_4 + 2 triose−PO_4

2 triose−PO_4 → hexose−PO_4 + inorganic phosphate

Sum: Hexose-PO_4 + 3 O_2 → 6 CO_2 + Pi 6 molecules of water would be formed in the process and 36 molecules of ATP per molecule of hexose oxidized if the TPN·H formed were all converted to DPN·H (transhydrogenase) and this oxidized to DPN+.

Fig. 2. The pentose phosphate pathway.

B. Pentose phosphate pathway in muscle

Historical and elucidation of the pentose phosphate pathway

Warburg and his coworkers (76,77) were the first to demonstrate nonglycolytic catabolism of glucose-6-phosphate. When glucose-6-phosphate was oxidized by the enzyme complex which they designated "zwischenferment", in the presence of NADP and oxygen, 6-phosphogluconate was produced. They also showed that further oxidation of 6-phosphogluconate was mediated by NADP (78). It was only much later that Cori and Lipmann (79) found that 6-phosphogluconolactone was the primary product of glucose-6-phosphate oxidation. The slow hydrolysis of 6-phosphogluconolactone was shown to be accelerated by the enzyme gluconolactonase (80).

Lipmann (81) showed that when fermentation by yeast juice was inhibited by bromoacetate, the oxidation of 6-phosphogluconate by NADP consumed one atom of oxygen per molecule of substrate. He suggested that the product was arabinose-5-phosphate and postulated a 2-keto-5-phosphogluconate to be the transient intermediate in the reaction. In a careful reinvestigation of the problem, Dickens (82) confirmed the presence of a pentose phosphoric acid as a result of oxidation and decarboxylation of hexose phosphoric acid. He excluded arabinose-5-phosphate as an intermediate and implicated ribose-5-phosphate as the physiological intermediate.

Among the products of 6-phosphogluconate oxidation by yeast, ribose-5-phosphate and glyceraldehyde phosphate were demonstrated paper chromatographically by Scott and Cohen (83). These authors (84) also provided chromatographic evidence for the presence of a 1,2-ene-diol pentose product which was the first hint of the presence of an isomerization intermediate. Horecker

and his coworkers (85,86,87) finally succeeded in isolating and identifying ribose-5-phosphate after incubation of yeast or liver 6-phosphogluconate dehydrogenase with 6-phosphogluconate and NADP. They also observed that at short incubation time the primary product was a ketopentose phosphoric acid ester which rearranged into ribose-5-phosphate at equilibrium. The primary product was separated from ribose-5-phosphate by column chromatography and identified as D-ribulose-5-phosphate (87). The same ester was obtained as the first product of 6-phosphogluconate oxidation by a purified dehydrogenase from Escherichia coli (88). The formation of ribose-5-phosphate from the isomerization of ribulose-5-phosphate explained the inversion of C-3 of the glucose chain and led necessarily to the assumption that the dehydrogenation of phosphogluconate occurred at C-3, with the formation of 3-keto-6-phosphogluconate (86) and not as earlier postulated as a 2-keto-5-phosphogluconate (81).

The sequence of reactions described above encompasses the oxidative portion of the pentose phosphate pathway and is shown in Fig. 2 together with the nonoxidative portion of the pathway.

Dickens (89) showed that the pentose phosphate was further metabolized by yeast juice to CO_2, ethanol, inorganic phosphate and an unidentified product. As early as 1938, Dische and coworkers (90,91) had provided evidence that the ribose from adenosine may be converted into triose and hexose phosphates by incubation with hemolysates, they explained their finding as due to the formation of ribose-5-phosphate and its cleavage into triose phosphate and glycolaldehyde. Later in 1951, Dickens and Glock showed that liver and kidney extracts were also able to metabolise ribose-5-phosphate (92). A splitting of the ribose moiety into triose phosphate and glycolaldehyde and the

recombination of the triose phosphates was also postulated by Waldvogel and Schlenk (93,94) who had isolated hexose monophosphate in high yield from the action of liver enzyme preparations on ribose-5-phosphate. Thus an enzyme with aldolase-like properties capable of splitting ribose-5-phosphate into triose phosphate and glycolaldehyde fragments was strongly implicated, especially in the light that deoxy-ribose-5-phosphate was indeed cleaved to triose phosphate and acetaldehyde by a spleen aldolase (95) and that aldolase would catalyse the formation of ketopentose phosphate from glycolaldehyde and dihydroxyacetone phosphate (96). However, the "aldolase" concept had to be discarded mainly due to the work of Glock (97,98). He showed that most of the ribose-5-phosphate disappeared under the influence of a liver fraction was recovered as hexose monophosphate and that hexose diphosphate was not found as either an intermediate or a product. He also showed that glycolaldehyde was not acted upon by the liver extract. These facts together with the demonstration that the hexose monophosphate recovery (75%) was greater than could be expected from the condensation of triose phosphates (50%) made it clear that the C-2 residue of the pentose was also incorporated into hexoses without the formation of glycolaldehyde.

Ashwell and Hickman (99) found that a D-xylulose phosphate was formed by the action of a fraction from mouse spleen on ribose-5-phosphate. Before this finding it was generally assumed that ribulose-5-phosphate was the only ketopentose phosphate concerned. They also observed the presence of a substance reacting as an erythro-3-pentulose among the reaction products and suggested that the interconversion of these two ketopentose phosphates may have occurred by way of a 2:3-ene-diol intermediate compound (100). The

name phosphoketopentose epimerase was introduced by Stumpf and Horecker (101) for the enzyme catalysing the interconversion of ribulose-5-phosphate and xylulose-5-phosphate. Dickens and Williamson (102) obtained ketopentose phosphates as the reaction product of rabbit muscle extract acting on ribose-5-phosphate and confirmed that the dephosphorylated ketopentose phosphates were ribulose and xylulose. Tabachnick and his coworkers (103) purified the isomerase from spinach leaves and epimerase from rabbit muscle and showed that the equilibrium mixture for the isomerase catalysed reaction is R5P/Ru5P : 75/25 and for the epimerase catalysed reaction is Xu5P/Ru5P : 75/25.

The enzyme responsible for the formation of heptose phosphate was first purified from rat liver (104) and then from spinach leaves (105) and was later obtained in the crystalline form from bakers' yeast (106,107). Since this enzyme in the presence of an acceptor aldehyde split a keto group from certain donor substrates and transfered the liberated "active glycolaldehyde" to the acceptor aldehyde, it was assigned the name transketolase (107). Views on the nature of the donor substrate for the transketolase reaction were at first erroneous, since the early preparations of the enzyme also contained phosphoribose isomerase and phosphoketopentose epimerase. Only after transketolase preparations free from isomerase and epimerase were obrained, was it recognized that D-xylulose-5-phosphate was the donor substrate and D-ribose-5-phosphate the acceptor substrate in the formation of the heptose phosphate, D-sedoheptulose-7-phosphate.

Transketolase purified from different sources always contained thiamine pyrophosphate as the prosthetic group and required magnesium for activity (107,108,109,110). The wide substrate specificity of the transketolase reaction could be demonstrated by the fact that, besides D-xylulose-5-phosphate, D-fructose-6-phosphate, D-sedoheptulose-7-phosphate, L-erythrulose and hydroxypyruvate could also serve as the active glycolaldehyde donor (111). Some of the more important reactions catalysed by transketolase are:

$$\text{D-R5P} + \text{D-Xu5P} \longleftrightarrow \text{D-S7P} + \text{D-G3P} \qquad 1$$

$$\text{D-F6P} + \text{D-G3P} \longleftrightarrow \text{D-Xu5P} + \text{D-Er4P} \qquad 2$$

$$\text{D-F6P} + \text{D-R5P} \longleftrightarrow \text{D-S7P} + \text{D-Er4P} \qquad 3$$

Since no free glycolaldehyde could be detected during the transketolase reaction, it was assumed that there occured the intermediary formation of a glycolaldehyde-thiamine pyrophosphate-enzyme complex (105). The intermediate, which was assumed to be a α, ß-dihydroxy-1-2-thiamine-pyrophosphate was synthesized and studied by Krampitz (112) and the entire enzyme complex was isolated by Datta and Racker (113) from a transketolase catalysed reaction.

Sedoheptulose-7-phosphate has been shown to be a normal constituent of animal tissue by its isolation from calf liver (114).

With enzyme preparations from liver or yeast (115,116) fructose-6-phosphate was formed from sedoheptulose-7-phosphate in a stoichiometric reaction with glyceraldehyde-3-phosphate. With the employment of C^{14} label at a specific position it was found the enzyme catalysed the transfer of a dihydroxyacetone moiety from sedoheptulose-7-phosphate to an acceptor substrate

(glyceraldehyde-3-phosphate) to form fructose-6-phosphate (reaction 4).

$$\text{D-S7P} + \text{D-G3P} \longleftrightarrow \text{D-F6P} + \text{D-Er4P} \qquad 4$$

The enzyme was named transaldolase by Horecker and Smyrniotis (115). The purest transaldolase obtained from yeast contains no prosthetic group and does not require a cofactor or metal ion for activity (117). Venkataraman and Racker (118) were able to isolate the intermediate dihydroxyacetone-enzyme complex from the reaction mixture.

The other product of the transaldolase-catalysed reaction (reaction 4) is erythrose-4-phosphate, which serves as an acceptor aldehyde for the active glycolaldehyde from the xylulose-5-phosphate donor in a transketolase mediated reaction (reaction 2).

Fructose-6-phosphate formed in reactions 2 and 4 could be converted by phosphoglucoisomerase to glucose-6-phosphate, and glyceraldehyde-3-phosphate formed in reactions 1 and 2 could be converted to fructose-1, 6-diphosphate by the action of triose phosphate isomerase and aldolase and then converted to fructose-6-phosphate and glucose-6-phosphate by the action of fructose diphosphatase and phosphohexose isomerase.

The overall reaction, starting with glucose-6-phosphate, yields products which could convert to glucose-6-phosphate, hence the completion of the cycle of the pentose phosphate pathway.

Using a reconstructed system containing all the enzymes and cofactors necessary for the pathway in highly purified form, and glucose-6-phosphate as substrate, Couri and Racker (119) have demonstrated the overall correctness of the pentose phosphate pathway. The accepted scheme of the

pathway is shown in conjunction with the glycolytic pathway in Fig. 1.

Alternative sequence of reactions in the pentose phosphate pathway

Although the scheme represented in Fig. 2 has been generally accepted as a working model for the pentose phosphate pathway, there is some evidence that alternative or additional sequence of reactions may be in operation, especially in the nonoxidative portion.

Horecker (120) and Klybas (121) and coworkers have shown that sedoheptulose-1,7-diphosphate can be prepared enzymically by the condensation of erythrose-4-phosphate and dihydroxyacetone phosphate mediated by aldolase and that sedoheptulose-1,7-diphosphate is split by muscle aldolase at one half the rate of fructose-1,6-diphosphate.

Racker and Shroeder (122) reported a high sedoheptulose diphosphatase activity in bakers' yeast. Couri and Racker (119) suggested that the following sequence of reactions may be operating as an alternative to a direct conversion of fructose diphosphate to fructose-6-phosphate.

G3P + S7P $\xleftarrow{\text{transaldolase}}$	Er4P + F6P	5
Er4P + DHAP $\xleftarrow{\text{aldolase}}$	SDP	6
SDP $\xrightarrow{\text{SDPase}}$	S7P + P_i	7
sum: 2 triose P $\longrightarrow$	F6P + P_i	

Sedoheptulose diphosphatase was also purified from rat liver by Bonsignore and coworkers (123). They showed that with a reconstructed system with added sedoheptulose diphosphatase, a sequence of reactions

similar to the one proposed by Couri and Racker (119) was operating.

Bonsignore and his coworkers (125) found that a thoroughly dialysed liver extract was able to actively convert fructose-6-phosphate to sedoheptulose-7-phosphate and xylulose-5-phosphate without the addition of triose phosphate. Later it was found that a combination of highly purified transketolase and transaldolase was also able to convert fructose-6-phosphate to sedoheptulose-7-phosphate without the addition of glyceraldehyde-3-phosphate (126). The proposed mechanism involved a coupled transketolase-transaldolase reaction where fructose-6-phosphate serves both as a glycolaldehyde donor for the transketolase reaction and dihydroxyacetone donor for the transaldolase reaction (reaction 8,9,10,11).

F-6-P + transketolase ⟶ (glycolaldehyde-transketolase) + Er-4-P (8)

F-6-P + transaldolase ⟶ (dihydroxyacetone-transaldolase) + G-3-P (9)

Er-4-P + (dihydroxyacetone-transaldolase) ⟶ S-7-P + transaldolase (10)

G-3-P + (glycolaldehyde-transketolase) ⟶ Xu-5-P + transketolase (11)

Regulation of pentose phosphate pathway

The availability of NADP as well as the rate of reoxidation of NADPH are two of the major factors in controlling glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activity and hence the pentose phosphate pathway activity. McLean (128) has shown that the addition of phenazine methosulfate as an electron acceptor stimulates the formation of $C^{14}O_2$ from 1-C^{14}-glucose by mammary gland adipose tissue. The addition of other artificial electron acceptors active with NADPH (methylene blue, pyocyanine) (129,130), the coupling with NADPH utilizing enzyme systems (malic enzyme) (131), or simply adding NADP (132) have all been shown to

have a similar effect in increasing the degradation of glucose through the pentose phosphate pathway. The content of NADP and NADPH in rat tissues has been reported by Glock and McLean (133) who found comparatively high levels of NADP and NADPH in liver, adrenal, kidney, lactating mammary gland and ovary. Recently, Gumaa and McLean (134) have demonstrated changes in the level and distribution of the pentose phosphate pathway intermediates according to the state of oxidation of the NADP-NADPH system, which was altered by the addition of oxamate and pyruvate. Herke and coworkers (135) showed that, in the brain 6-aminonicotinamide adenine dinucleotide phosphate, which is an inhibitor of NADP dependent dehydrogenases, induced a blockage in glucose degradation through the pentose phosphate pathway.

Kinetic analysis of purified human erythrocyte glucose-6-phosphate dehydrogenase revealed the existence of two states of the enzyme (136), with high and low affinity for NADP, respectively. The conversion from the low affinity state to the high affinity state is promoted by increasing the concentration of NADP or its competitive inhibitor NADPH (137). A similar transition has been reported for erythrocyte type B enzyme (138) and mammary gland enzyme (139). The physiological significance of this transition and its effect on the pentose phosphate pathway activity has yet to be evaluated.

It has been suggested that the high pentose phosphate pathway activity in certain organs, (such as adrenals and ovary, is related to the requirement of NADPH in the biosynthesis of steroids including the conversion of squalene to cholesterol, in the conversion of cholesterol to pregnenolone,

conversion of androgen to estrogen and for various steroid hydroxylation reactions (140,141,142,143,144).

NADPH is also involved in the biosynthesis of fatty acids for the reduction of crotonyl-CoA to butyryl-CoA (145,146) or more generally, for the reduction of α,ß-unsaturated acyl-CoA derivatives. The metabolic interrelationship between the pentose phosphate pathway and fatty acid synthesis was clearly demonstrated by the striking increase in glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities in rat mammary gland during lactation and the decline of these activities after involution of the mammary gland (147,148). It is interesting to note that long chain acyl-CoA derivatives are potent inhibitors of glucose-6-phosphate dehydrogenase (149).

One of the major roles of the pentose phosphate pathway is the formation of pentose phosphate which is required for the formation of phosphoribosyl pyrophosphate (150,151,152), the formation of the ribose moiety of nucleic acid (153,154,155), the synthesis of purine nucleotides (156,157) and of cytosine and uracil nucleotides (150,158,159).

Horecker (160) pointed out that the pentose phosphate cycle operates as two parallel pathways (oxidative and nonoxidative) for the conversion of hexose monophosphates into pentose phosphates. By studying the labelling pattern of ribose in nucleic acid, Hiatt (161) concluded that both the oxidative and nonoxidative routes participated in the formation of pentose phosphate in HeLa cells. In ascites tumor cells, as much as 80% of the ribose-5-phosphate was produced via the nonoxidative route (134). In Escherichia coli (162) and Torula utilis (163) the nonoxidative route

appeared to be the exclusive pathway. In animal tissues generally, the nonoxidative route played a major role accounting for more than fifty percent of the ribose of the RNA and the acid soluble nucleotides (153,154,164). However, the relative contribution of the two routes is liable to alteration by changes in physiological conditions. A relative decrease in the contribution of the nonoxidative route in rat was found during thiamine deficiency as a result of the decreased transketolase activity (165). In animals with actively regenerating liver or with tumor, the relative contribution of the oxidative route was increased since in these animals, there is an increased NADPH utilization for synthetic purposes(166).

Katz and coworkers (167) using tracer methods to determine the contribution of pentose phosphate pathway to the overall glucose metabolism reported that the contribution was 16% in normal rat fed ad libitum, 25% in insulin-treated rats, 14% in rats given growth hormone and 7% in adrenalin-treated rats.

McLean and his coworkers studied the changes in enzyme activities of the pentose phosphate pathway in rat liver (168) and rat adipose tissue (169) during various nutritional and hormonal conditions (starvation, high carbohydrate diet, high fat diet, adrenalectomy, thyroidectomy, hypophysectomy, alloxan diabetes etc.). They found that although all the enzymes of the pathway were influenced by these conditions, glucose-6-phosphate dehydrogenase and transketolase showed marked responses, thus pointing to their possible regulatory role. Gumaa and McLean (170) studied the transient and steady state distribution of intermediates of the pathway in ascites tumor cell and showed that glucose-6-phosphate dehydrogenase and

transketolase were the two enzymes most displaced from equilibrium and hence were most likely to have a rate limiting role. Kauffman and coworkers (171) studied the change in level of intermediates in rat brain and suggested that regulatory mechanisms were operating at both dehydrogenase steps.

The existence of pentose phosphate pathway in muscle

There are not many detailed investigations related to the function and regulation of the pentose phosphate pathway in muscle, probably due to the minimal participation of this pathway in the overall glucose metabolism in muscle.

Green and Landau (172) have estimated from tracer studies that about 2% of the glucose metabolized in abdominal muscle and diaphragm of mouse entered the pentose phosphate pathway. Hostetter and Landau (173) estimated that in rat muscle in vivo the pathway contributes no more than a few percent (0.401.3%) to the overall glucose metabolism.

The existence of a very weak oxidative route of the pentose phosphate pathway was first reported by Dickens and Glock (92) while Glock and McLean (174) showed the presence of a low level of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in rat skeletal muscle.

The first indication of the presence of a nonoxidative route of the pentose phosphate pathway in muscle was provided by Sable (175) who reported the conversion of ribose-5-phosphate to ketopentose phosphates as well as a very slow metabolism beyond the pentose phosphates. Glock and McLean (174) reported a fairly active utilization of ribose-5-phosphate and

resynthesis of hexose monophosphate from it in extracts of rat and mouse muscle. Dickens and Williamson (102) studied in greater detail the incubation product of ribose-5-phosphate with rabbit muscle extract or crude enzyme preparations and reported a very weak metabolism beyond ketopentose phosphates.

Srere and coworkers (108) purified phosphoketopentose epimerase from rabbit muscle. Scrivastava and Hubscher (176) reported transketolase and transaldolase activity in intestinal mucosa of rat. Recently, Tan and Wood (177) have established the presence of all the enzymes of the nonoxidative route in muscles in a number of species including the rat.

Investigations of pentose phosphate pathway activity in brain (171) liver (168), adipose tissue (169) and ascites tumor cell (170) have revealed that the function and regulation of the pathway are characteristic of the particular tissue. Therefore, with the estab lishment of the complete pathway in muscle, the next step would be the investigation of its characteristic function and regulation. Part of the work reported here is concerned with the establishment of methods for assaying the intermediates in the pathway and some preliminary investigations on the activity of the nonoxidative portion of the pathway in rat skeletal muscle.

C. The interrelationship between the glycolytic and pentose phosphate pathway.

Although the glycolytic and the pentose phosphate pathways have generally been considered as two separate pathways and studied independently, they are also closely interrelated by their requirement for

some common substrates. They may be considered as two multienzyme systems with three substrates in common; these are glucose-6-phosphate fructose-6-phosphate and glyceraldehyde-3-phosphate (see Fig. 1).

Couri and Racker (119) showed that with a reconstructed system the oxidation of glucose via the pentose phosphate pathway was inhibited in the presence of a glycolytic system. With limiting concentrations of both hexokinase and phosphofructokinase, the extent of inhibition was dependent on the amount of phosphofructokinase added which diverted more glucose to the glycolytic pathway.

Potter and Neimeyer (178) using an enriched cell free brain preparation, showed that the addition of NADP and NADPH decreases glycolysis, the result was explained by an accumulation of 6-phosphogluconate which inhibits phosphohexoisomerase (179). This block would permit an accumulation of glucose-6-phosphate which would in turn inhibit hexokinase (60) and hence the rate of glycolysis.

Phosphohexose isomerase was also known to be strongly inhibited by erythrose-4-phosphate (180,181) and sedoheptulose-7-phosphate (180). Venkataraman and Racker (180) suggested that these substances (erythrose-4-phosphate, sedoheptulose-7-phosphate and 6-phosphogluconate) acting singly or in combination may serve as regulators of glucose-6-phosphate metabolism.

Erythrose-4-phosphate was also reported to be inhibitory towards phosphoribose isomerase and triose phosphate isomerase (182). In ascites tumor cells, ribose-5-phosphate at 5 mM concentration inhibits 25% of the hexokinase activity (170). Fructose diphosphate was reported to be inhibitory towards 6-phosphogluconate dehydrogenase (183). The significance of these inhibitors of the glycolytic and pentose phosphate pathways has yet to be

studied in detail.

Unlike the glycolytic pathway, the pentose phosphate pathway does not require orthophosphate. However, an inhibitory effect of inorganic phosphate on the pentose phosphate pathway and its enzymes has been reported. As early as 1935, Theorell (184) reported inhibition of glucose-6-phosphate dehydrogenase by inorganic phosphate. Kravitz and Guarino (185) showed that in a 0.02 M phosphate medium, the pentose phosphate pathway activity was inhibited by 50% compared to a phosphate free medium. They attributed this phenomenon to the inhibition of glucose-6-phosphate dehydrogenase by inorganic phosphate but they did not show whether there was an increase of glycolysis in the phosphate medium. In addition, inorganic phosphate has been reported to inhibit transaldolase and transketolase (186,187,188).

MATERIALS AND METHODS

1. Materials.

Bakers'yeast lactate dehydrogenase, rabbit muscle fructose-6-phosphate kinase (phosphofructokinase) and spinach phosphoribose isomerase were obtained from the Sigma Chemical Company, St. Louis, Mo. Transketolase from Candida utilis was kindly provided by Miss M. E. Kiely. Phosphoketopentose epimerase from rat muscle was a gift of Mr. E. L. Tan. All other enzymes were obtained from Boehringer Mannheim Corp., New York, N.Y. Transaldolase was partially purified from Candida utilis (obtained from P. L. Biochemicals Inc., Milwaukee, Wis.) as described in RESULTS.

Glucose-6-phosphate, fructose-6-phosphate, 6-phospho-gluconate, fructose-1,6-diphosphate, phosphoenolpyruvate, lithium lactate, ribose-5-phosphate, sedoheptulosan hydrate, rabbit muscle glycogen, dextrose(α-D-glucose), AMP, ADP, ATP, NAD, NADH, NADP were obtained from the Sigma Chemical Company. Erythrose-4-phosphate dimethylacetal dicyclohexylammonium salt was obtained from Calbiochem, Los Angeles, Calif., and was converted to the free form according to the supplier's instruction. Ribulose-5-phosphate (as the ribose-5-phosphate/ribulose-5-phosphate mixture), xylulose-5-phosphate (as the ribose-5-phosphate/ribulose-5-phosphate/xylulose-5-phosphate mixture), and sedoheptulose-7-phosphate were prepared as described in Section 6 below.

Imidazole and glycylglycine were obtained from the Sigma Chemical Company. Tris-hydroxymethylamino methane, triethanolamine hydrochloride and

nicotinamide were obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio. Cysteine hydrochloride and carbazole were obtained from the Fisher Scientific Company, Fairlawn, N.Y. The carbazole was further purified by resublimation. Phloroglucinol was obtained from Matheson, Coleman and Bell, East Rutherford, N.J. Concentrated sulfuric acid (95-98%) used in colorimetric determinations was purchased from Shawinigan (The McArthur Chemical Company Ltd., Montreal, Que.). DEAE-Sephadex was obtained from Pharmacia, Uppsala, Sweden. Myanesin (Mephenesin, 3-0-toloxy-propane-1,2-diol) was donated by Squibb and Sons Ltd., Montreal, Que. Pentothal was obtained from Abbott Laboratories Ltd., Montreal, Que. All other chemicals were purchased from Fisher Scientific Company and from J. T. Baker Chemical Company, Phillipsburg, N.J.

For enzymatic assays, a "Calbiometer" fixed wavelength photometer (Calbiochem) or a Beckman DB spectrophotometer (Beckman Instruments Inc., New York, N.Y.) coupled to a Varicord model 43 recorder (Photovolt Corp., New York, N.Y.) were used. For colorimetric determinations, a SP.600 spectrophotometer from Unicam Instruments Ltd., Cambridge, England, was used for absorbance measurements. A radiometer (Type PHM 22r (Copenhagen), Bach-Simpson Ltd., London, Ontario), standardized with sodium borate standard buffer, pH 9.22, was used for all pH measurements.

2. Preparation of rat muscle homogenates and extracts.

Male Wistar rats were used in all experiments. For glycolysis experiments, rats weighing 250-350 gram (2-1/2 - 3 months old) were used. The rat was starved overnight and anaesthetized by intraperitoneal injection of sodium pentothal (5mg/100g rat weight). After the rat was anaesthetized, a muscle relaxant, myanesin (15mg/100g rat weight) was injected intraperitoneally. The effect of the drug was apparent within 2-3 minutes when the limbs became completely relaxed and flaccid. The rat was killed by cervical dislocation. The limbs of the animal did not show any sign of convulsion. This group of rats was termed nonstimulated. In one set of experiments where stimulated rats were required, treatment of the animal was the same as the nonstimulated rats except that the myanesin injection was omitted. The limbs of the animal convulsed violently after killing.

For pentose phosphate pathway experiments, bigger rats were used (350-400 grams, 3-4 months old). They were fed ad libitum, anaesthetized with ether, and then killed by cervical dislocation.

Homogenization media: For the pentose phosphate pathway experiments, 0.05 M triethanolamine buffer, pH 7.5, was used for the preparation of homogenates and extracts. For glycolytic experiments, 0.04 M nicotinamide, pH 6.8 was used for the preparation of slightly acidic homogenates and extracts, 0.04 M nicotinamide-0.05 M imidazole buffer, pH 7.5, was used for the preparation of slightly alkaline homogenates and extracts. Other homogenization media

employed in the preparation of homogenates and extracts for the phospho-fructokinase assays were described in p. 60.

Homogenates: After killing, the body of the rat was removed to a cold room at 4°C where all subsequent procedures were performed. Muscle was quickly removed from the leg and thigh regions of the hind limb, clear of fat and connective tissue and cut into small pieces. A measured volume of cold homogenization medium was added and the mixture was homogenized in a Lourdes Multi-Mixer (Lourdes Instruments Corp., Old Bethpage, N.Y.) at top speed for 2 minutes. In general, 25 ml of homogenization medium was added to 6 gram of muscle yielding 30 ml of homogenate, i.e. 0.2 gram muscle per ml of homogenate.

Extracts: Muscle extracts were prepared from the homogenates by millipore filtration or by ultracentrifugation. In the first method, the homogenate was centrifuged at 3600 g for 15 minutes and the supernatant was filtered successively through a 220 mμ and a 100 mμ millipore filter (Millipore Filter Corp., Bedford, Mass.). The final filtrate was stored in ice and was used as the muscle extract. The advantage of the filtration method was that a small amount of extract could be obtained quickly, eg. 2 ml of extract could be obtained from the homogenate in 20 minutes. One disadvantage was that filtration through the 100 mμ filter was slow (2 ml filtrate/5 minutes) and for every 2 ml filtrate collected the old filter which had become clogged had to be replaced by a new one. Each change of filter was accompanied by considerable loss of filtrate.

When a large amount of muscle extract was required, it was prepared by ultracentrifugation. The 3600 g supernatant was quickly filtered through the 220 mμ millipore filter and the filtrate was centrifuged in a Beckman Model L Ultracentrifuge at 105,000 g for 30 minutes at 6°C.

3. Incubation procedures.

The desired substrate, cofactor, buffer, etc., were placed in a 15 ml round bottom pyrex centrifuge tube and the volume was made up to 1.0 ml with distilled water. This mixture and the homogenate or extract were allowed to equilibrate in a water bath at the desired temperature. At zero time, 2.0 ml of homogenate or extract was added to the mixture and the contents mixed thoroughly. After incubation for the desired period of time the tube containing the incubation mixture was quickly transferred to ice and 3.0 ml of ice cold 3% (w/v) perchloric acid was added followed by vigorous stirring. The perchloric acid mixture was allowed to stand in ice for 10 minutes with regular stirring and was then centrifuged at 3600 g for 15 minutes at 4°C. The supernatant was transferred to a 50 ml beaker resting in a tray of ice and small portions of powdered potassium bicarbonate were added followed by vigorous stirring. The pH of the supernatant was tested with narrow range pH paper. The addition of potassium bicarbonate was continued until the pH reached 6.8-7.0. The neutralized supernatant was allowed to stand in the cold for 2 hours for the precipitation of potassium perchlorate, and the supernatant was collected after centrifugation at 3600 g for 15 minutes.

Two ml of the deproteinized, neutralized supernatant was concentrated tenfold by freeze-drying and was subjected to paper chromatography. The remaining supernatant (about 4.0 ml) was used for enzymatic and colorimetric assays.

4. Paper chromatography.

Glycolytic intermediates: The phosphorylated compounds in the freeze-dried supernatants were separated and identified by the paper chromatographic technique described by Wood for glycolytic intermediates (189) and for phosphoenolpyruvate and pyruvate specifically (190).

Pentose phosphate pathway intermediates: The method for paper chromatographic separation and detection of intermediates of the pentose phosphate pathway described by Wood (191,192) was used.

5. Column chromatography.

A 14 cm x 1 cm column of DEAE-Sephadex A25 was packed by gravity and washed with 25 ml of 0.05 M NaCl. The sample (3 ml of a mixture of pentose phosphates) was applied to the column and washed in with 20 ml of distilled water. The column was eluted with a linear gradient of 250 ml of 0.1 M NaCl running into 250 ml of water. The flow rate was adjusted by a polystaltic pump (Buchler Instruments, Fort Lee, N.J.) to 0.5 ml per minute and 5 ml fractions were collected. The positions of ribose-5-phosphate, ribulose-5-phosphate and xylulose-5-phosphate were detected by specific colorimetric methods and the presence of ribulose-5-

phosphate and xylulose-5-phosphate was further confirmed by enzymatic test.

6. Preparation of substrates.

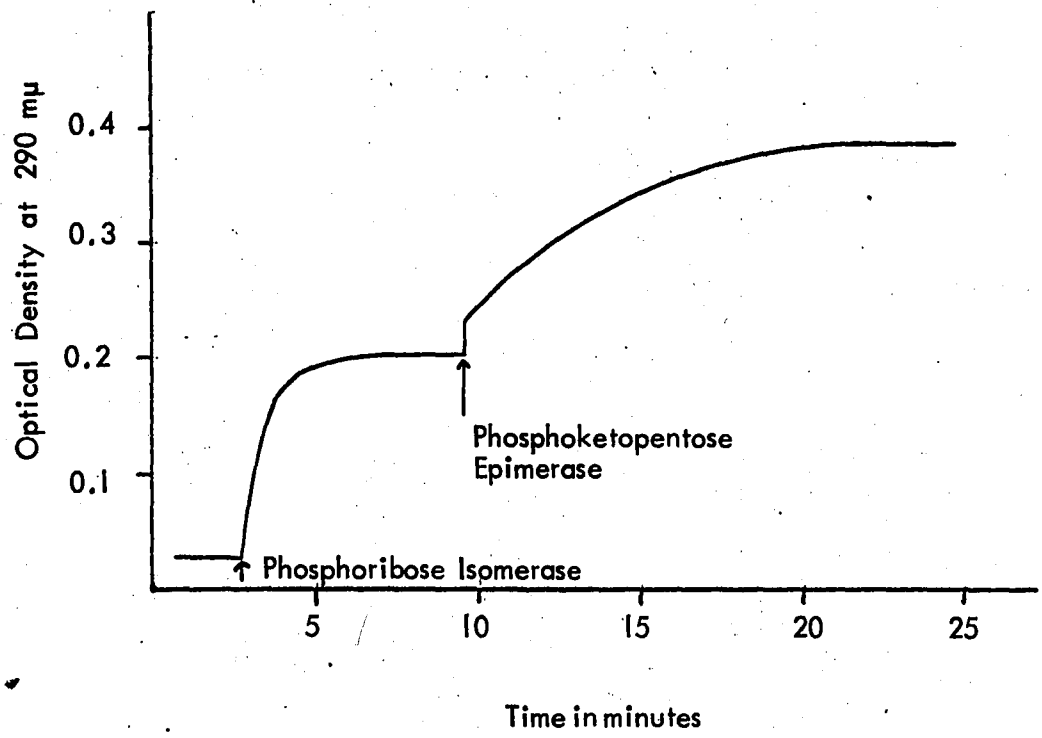
Ribose-5-phosphate/ribulose-5-phosphate mixture: The mixture was prepared by incubating ribose-5-phosphate with phosphoribose isomerase until equilibrium was reached. To follow the course of reaction, the method described by Wood (193) was used. This assay depends on the observation that ketopentose phosphate (ribulose-5-phosphate and xylulose-5-phosphate) has an absorption peak at 290 mμ whereas aldopentose phosphate (ribose-5-phosphate) has not (cf. assay method of Knowles et al. (194)).

The reaction mixture in a volume of 3.0 ml contained (final concentrations); 18 mM glycylglycine buffer, pH 7.4, 10 mM ribose-5-phosphate, and 4.0 I.U. of spinach phosphoribose isomerase. This was incubated at 37°C and equilibrium was reached when the absorbance at 290 mμ had increased to a maximum and remained stationary (Fig. 3).

Ribose-5-phosphate/ribulose-5-phosphate/xylulose-5-phosphate mixture: An equilibrium mixture of ribose-5-phosphate/ribulose-5-phosphate was first prepared as described. When equilibrium had been reached, 0.5 I.U. of phosphoketopentose epimerase was added to the mixture. There was a further increase in absorbance at 290 mμ due to the formation of xylulose-5-phosphate and the reaction at 37°C was followed at 290 mμ to the new equilibrium (Fig. 3).

Deproteinization could be carried out with perchloric acid treatment

Fig. 3. Preparation of an equilibrium mixture of ribose-5-phosphate/ ribulose-5-phosphate/xylulose-5-phosphate.



as described under the incubation procedures section or by ultrafiltration.

Ultrafiltration: The Diaflo apparatus (10 ml cell) obtained from Amicon Corp., Lexington, Mass. was used. The samples were filtered through a PM-30 membrane at a pressure of 50 lb per sq. in. The filtration of a 3.0 ml reaction mixture was complete in 3 to 5 minutes.

The ultrafiltration procedure had several advantages over the perchloric acid treatment: (a) it was faster, (b) the sample was not exposed to acid conditions, (c) there was no increase in volume after deproteinization, (d) no addition of cation or anion (H^+, K^+, ClO_4^-, CO_3^{2-}) was necessary. However, when the sample was viscous or contained a high concentration of protein and particles, the ultrafiltration procedure was not applicable due to blockage of the filter.

Sedeheptulose-7-phosphate: An equilibrium mixture of ribose-5-phosphate/ribulose-5-phosphate/xylulose-5-phosphate was first prepared. The reaction mixture in a final volume of 2.0 ml contained: 80 mM glycylglycine buffer, pH 7.4 and 15 mM ribose-5-phosphate. Two international units of spinach phosphoribose isomerase were added and the reaction at 37°C was followed at 290 mμ as described above. After equilibrium was reached, 0.5 I.U. of phosphoketopentose epimerase was added and the reaction was again followed to equilibrium. To this mixture, still containing the isomerase and epimerase, were added 0.3 I.U. transketolase, 2.0 I.U. glyceraldehyde phosphate dehydrogenase, 30 μmoles NAD, 250 μmoles potassium phosphate, pH 7.4, and

enough 50 mM glycylglycine buffer, pH 7.4, to make a final volume of 3.0 ml. The reaction mixture was incubated at 37°C for 2 hours and was then deproteinized with 3.0 ml 3% perchloric acid.

The resulting mixture was assayed enzymatically and colorimetrically for the content of sedoheptulose-7-phosphate.

7. Assay of enzyme activity.

Phosphofructokinase: The assay method of Shonk and Boxer (195) was adopted with slight modifications.

$$\text{F-6-P} + \text{ATP} \xrightarrow{\text{PFK}} \text{FDP} + \text{ADP}$$

$$\text{FDP} \xleftrightarrow{\text{aldolase}} \text{DHAP} + \text{G-3-P}$$

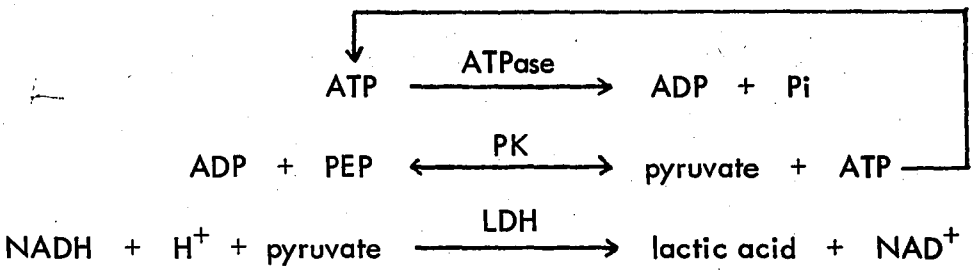
$$\text{G-3-P} \xleftrightarrow{\text{TIM}} \text{DHAP}$$

$$\text{NADH} + \text{H}^+ + \text{DHAP} \xrightarrow{\text{Gl-1-P DH}} \text{Gl-1-P} + \text{NAD}^+$$

The assay mixture in a volume of 2.0 ml contained the following concentrations: 100 mM imidazole buffer, pH 7.5, 10 mM EDTA, 13 mM magnesium chloride, 130 mM potassium chloride, 2.5 mM ATP, 3.5 mM fructose-6-phosphate, 0.13 mM NADH, 0.9 I.U. aldolase, 0.7 I.U. glycerol-1-phosphate dehydrogenase, and 3.9 I.U. triose phosphate dehydrogenase. The reaction was started by the addition of 0.01 to 0.05 ml of the sample (muscle homogenate or extract) diluted 5 to 25 times with 1 mg/ml bovine serum albumin. The reaction rate at room temperature was followed at 340 mμ.

The reaction was linear for at least 10 minutes, during which the initial rate was recorded.

Adenosine triphosphatase*:



The assay mixture in a final volume of 2.0 ml contained the following final concentrations: 100 mM imidazole buffer, pH 7.5, 7.5 mM magnesium chloride, 75 mM potassium chloride, 1 mM ATP, 0.38 mM phosphoenolpyruvate, 0.13 mM NADH, 0.5 I.U. pyruvate kinase and 0.5 I.U. lactate dehydrogenase. The reaction at room temperature was started by the addition of 0.01 to 0.05 ml of the homogenate or extract diluted 5 to 25 times with 1 mg/ml bovine serum albumin, and was followed at 340 mμ. A lag period of 1 to 2 minutes was usually observed after which a linear reaction rate was maintained for at least 5 minutes. This rate was recorded.

Adenylic deaminase: The assay method described by Kalckar (42) was used.

$$AMP + H_2O \xrightarrow{\text{adenylic deaminase}} IMP + NH_3$$

The assay mixture consisted of 3.0 ml of a 50 mM sodium citrate buffer,

* T. Wood, unpublished method.

pH 6.5, containing 0.033 mM adenosine monophosphate. The reaction was started by adding the sample (homogenate or extract) diluted in N KCl and the change in absorbance at 265 mμ was followed at room temperature. The enzyme activity was calculated from the initial linear rate. The molar extinction coefficient of 7.0 $cm^3 \mu moles^{-1} cm^{-1}$ was used for the conversion of absorbance into μmoles of adenosine monophosphate deaminated to inosinic acid.

Transketolase: The assay method of dela Haba et al. (107) as described by Kiely et al. (196) was used.

$$\text{R-5-P} + \text{Xu-5-P} \xleftrightarrow{\text{TK}} \text{S-7-P} + \text{G-3-P}$$

$$\text{G-3-P} \xleftrightarrow{\text{TIM}} \text{DHAP}$$

$$\text{NADH} + \text{H}^+ + \text{DHAP} \xrightarrow{\text{Gl-1-P DH}} \text{Gl-1-P} + \text{NAD}^+$$

The assay mixture in a final volume of 2.0 ml contained the following final concentrations: 100 mM glycylglycine buffer, pH 7.4, 2.5 mM magnesium chloride, 0.1 mM thiamine pyrophosphate, 1 mM dithiothreitol, 0.13 mM NADH, 2 mM ribose-5-phosphate, 0.05 mM xylulose-5-phosphate, 0.26 I.U. glycerol-1-phosphate dehydrogenase, 1.6 I.U. triose phosphate isomerase. The reaction at room temperature was started by the addition of 0.01 to 0.05 ml of the sample containing transketolase activity, and followed at 340 mμ.

Transaldolase: The assay method of Tchola and Horecker (197) was adopted.

$$\text{F-6-P} + \text{Er-4-P} \xleftrightarrow{\text{TA}} \text{S-7-P} + \text{G-3-P}$$

$$\text{G-3-P} \xleftrightarrow{\text{TIM}} \text{DHAP}$$

$$\text{NADH} + \text{H}^+ + \text{DHAP} \xrightarrow{\text{Gl-1-P DH}} \text{Gl-1-P} + \text{NAD}^+$$

The assay mixture contained in a final volume of 2.0 ml, 40 mM triethanolamine buffer, pH 7.5, 10 mM EDTA, 3.5 mM fructose-6-phosphate, 0.8 mM erythrose-4-phosphate, 0.13 mM NADH, 0.7 I.U. glycerol-1-phosphate dehydrogenase, 3.9 I.U. triose phosphate isomerase. The reaction at room temperature was started by the addition of 0.01 to 0.05 ml of sample containing transaldolase and the rate of the reaction was followed at 340 mμ. Enzyme activity was calculated from the initial linear rate.

Glucose-6-phosphate dehydrogenase: The assay method of Kornberg and Horecker (198) was used.

6-Phosphogluconate dehydrogenase: The assay method of Horecker and Smyrniotis (199) was used.

8. Enzymatic assay of metabolic intermediates.

Pyruvate, phosphoenolpyruvate, ADP and ATP were assayed enzymatically according to methods described in Bergmeyer (200). The enzymatic assay of lactic acid using yeast lactate dehydrogenase and potassium ferricyanide described in Bergmeyer was also used.

Serial assay of triose phosphate, xylulose-5-phosphate and ribulose-5-phosphate:

$$\text{Ru-5-P} \xleftrightarrow{\text{PKPE}} \text{Xu-5-P}$$

$$\text{R-5-P} + \text{Xu-5-P} \xleftrightarrow{\text{TK}} \text{S-7-P} + \text{G-3-P}$$

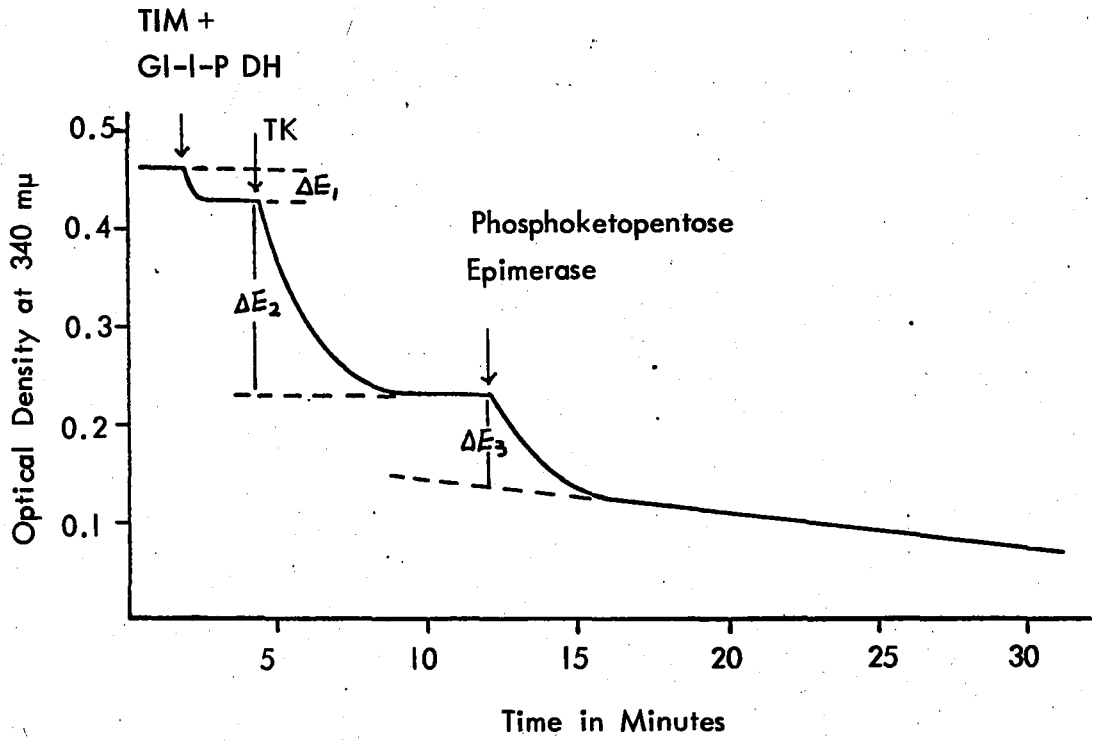
$$\text{G-3-P} \xleftrightarrow{\text{TIM}} \text{DHAP}$$

$$\text{NADH} + \text{H}^+ + \text{DHAP} \xrightarrow{\text{Gl-1-P-DH}} \text{Gl-1-P} + \text{NAD}^+$$

Two ml of assay mixture contained the following in final concentrations: 100 mM glycylglycine buffer, pH 7.4, 2.5 mM magnesium chloride, 0.1 mM thiamine pyrophosphate, 0.13 mM NADH, sample (usually 0.01 to 0.05 ml of a deproteinized and neutralized incubation mixture), 3.9 I.U. triose phosphate isomerase, 0.7 I.U. glycerol-1-phosphate dehydrogenase. The change in absorbance, ΔE_1, at this stage represented the triose phosphate content in the assay mixture. The change in absorbance, ΔE_2, after the addition of 0.5 I.U. transketolase represented the xylulose-5-phosphate content. Finally 0.05 I.U. of phosphoketopentose epimerase was added and the change in absorbance, ΔE_3, corresponded to the content of ribulose-5-phosphate.

Figure 4 shows a tracing of a typical assay. Triose phosphate (ΔE_1) and xylulose-5-phosphate (ΔE_2) could be determined accurately due to the stable and horizontal baselines. Determination of ribulose-5-phosphate from ΔE_3 was more difficult because the final baseline was not horizontal. This was due to the presence of traces of phosphoribose isomerase in the epimerase preparation, which continued to convert small amounts of ribose-5-phosphate in the assay mixture to ribulose-5-phosphate, contributing to the residual baseline activity.

Fig.4. The enzymatic assay of triose phosphate, xylulose-5-phosphate and ribulose-5-phosphate.



ΔE_1 represents triose phosphate content, ΔE_2 represents xylulose-5-phosphate content, ΔE_3 represents ribulose-5-phosphate content.

Erythrose-4-phosphate assay: The enzymatic assay was essentially the same as the procedure for assaying transaldolase activity except that in this case purified transaldolase was used and the order of addition of the various reaction components was changed.

Two ml of assay mixture contained the following in final concentrations: 40 mM triethanolamine buffer, pH 7.5, 10 mM EDTA, 3.5 mM fructose-6-phosphate, 0.13 mM NADH, 3.9 I.U. triose phosphate isomerase, 0.7 I.U. glycerol-1-phosphate dehydrogenase, and the sample (0.01 to 0.05 ml of a deproteinized, neutralized, incubation mixture). The reaction was started by adding 0.2 I.U. of transaldolase and the decrease in absorbance at 340 mμ was recorded.

Fructose-6-phosphate contains a small amount of contaminant which results in a small decrease in absorbance after the addition of transaldolase in the absence of added erythrose-4-phosphate. This decrease in absorbance due to the contaminant has to be subtracted from the observed decrease in absorbance to yield the actual value accounting for erythrose-4-phosphate.

Sedoheptulose-7-phosphate assay: The assay method described by Cooper et al. (201) was adopted with modification.

$$\text{FDP} \xleftrightarrow{\text{aldolase}} \text{G-3-P} + \text{DHAP}$$

$$\text{G-3-P} + \text{S-7-P} \xleftrightarrow{\text{TA}} \text{Er-4-P} + \text{F-6-P}$$

$$\text{F-6-P} \xleftrightarrow{\text{PGI}} \text{G-6-P}$$

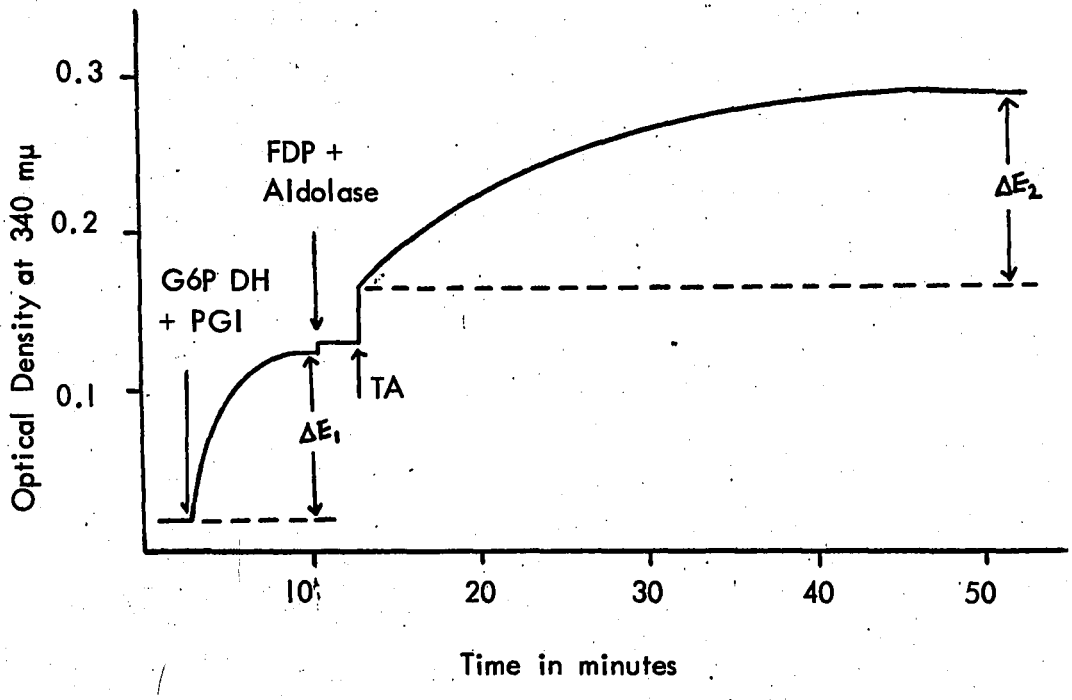
$$\text{NADP}^+ + \text{G-6-P} \xrightarrow{\text{G-6-P DH}} \text{6-PGA} + \text{NADPH} + \text{H}^+$$

The assay mixture in a volume of 2.0 ml contained (final concentrations): 100 mM glycylglycine buffer, pH 7.4, 0.39 mM NADP, 7.8 I.U. phosphoglucose isomerase, 1.4 I.U. glucose-6-phosphate dehydrogenase, and the sample (0.01 to 0.05 ml of concentrated, deproteinized incubation mixture). The increase in absorbance at 340 mμ, ΔE_1, represented the hexose monophosphates in the assay mixture. After the completion of this reaction, 5 μmoles fructose-1,6-diphosphate, 4.1 I.U. of aldolase and finally 4.0 I.U. of transaldolase were added. The increase in absorbance, ΔE_2, represented the concentration of sedoheptulose-7-phosphate (Fig. 5).

The enzymatic assay of sedoheptulose-7-phosphate is one of the more complicated assays. The sample usually contained a very small amount of sedoheptulose-7-phosphate and had to be concentrated before the assay. Both sedoheptulose-7-phosphate and erythrose-4-phosphate are competitive inhibitors of phosphoglucose isomerase (181,118), so to minimize the inhibitory effect, excess of this enzyme was added. Excess of aldolase was also added to insure the supply of a saturating level of glyceraldehyde-3-phosphate for the transaldolase reaction. As shown in Fig. 5, the reaction rate under these conditions is still very slow.

Another complication arose when the sample containing sedoheptulose-7-phosphate also contained a large amount of hexose monophosphates (G-6-P, F-6-P). In the initial stage of the reactions, the large amount of hexose monophosphates was converted to 6-phosphogluconic acid and a corresponding

Fig.5. The enzymatic assay of hexose monophosphate and sedo-heptulose-7-phosphate.



ΔE_1 represents hexose monophosphate content, ΔE_2 represents sedoheptulose-7-phosphate content.

amount of NADPH was formed which caused the absorbance at 340 mμ to reach the end of the density scale. The following procedure was designed to remove the NADP produced at the initial stage of the reactions:

To the sample containing sedoheptulose-7-phosphate and hexose monophosphate was added: 100 mM glycylglycine buffer, pH 7.4 (enough to make up a final volume of 2.0 ml), 7.8 I.U. phosphoglucose isomerase, 1.4 I.U. glucose-6-phosphate dehydrogenase and 2.0 μmoles NADP. The mixture was allowed to react at room temperature for 10 minutes. Twenty mg of activated charcoal was then added and mixed gently for 5 minutes. The mixture was filtered through a small piece of filter paper and the volume of filtrate measured. The charcoal treatment removed the pyridine nucleotides. The filtrate was transferred to a quartz cuvette and 100 mM glycylglycine buffer, pH 7.4 was added (final volume 2.0 ml), and the assay of sedoheptulose-7-phosphate was carried out as described above.

9. Colorimetric determinations.

Lactic acid: The method of Barker and Summerson (202) was used. Lithium lactate (0.02 to 1.2 μmoles) was used for the establishment of the standard curve. The colour developed was measured at 560 mμ.

Inorganic phosphate: The colorimetric procedure described by Wood (189) was used. Potassium phosphate (0.3 to 3.0 μg phosphorous) was used for establishing the standard curve. The developed colour was measured at 725 mμ.

Protein: The Biuret method (203) was used. Bovine serum albumin (1 to 10 mg) was used for establishing the standard curve. The colour developed was read at 550 mμ.

Ribose-5-phosphate: The "phloroglucinol" colorimetric procedure described by Dische and Borenfreund (204) was followed. Ribose-5-phosphate, 98% pure, obtained from Sigma was used to prepare a standard curve (Fig. 6).

The reagent consisted of a mixture of 55 ml glacial acetic acid, 1.0 ml concentrated HCl, 0.5 ml 0.8% glucose and 2.5 ml freshly prepared 5% phloroglucinol in ethanol.

To 0.4 ml of the sample (0.05 to 0.25 μmoles ribose-5-P) in a pyrex tube was added 5.0 ml of the reagent with mixing. The mixture was heated in a vigorously boiling water bath for 15 minutes and then allowed to cool at room temperature for 20 minutes. The colour developed was read at 552 mμ against a water blank. Prolonged standing at room temperature was accompanied by a gradual decrease in absorbance.

Ketopentose phosphates (ribulose-5-phosphate and xylulose-5-phosphate): The cysteine carbazole colour method of Dische and Borenfreund (205) was used.

The sample containing 0.02 to 0.3 μmoles ketopentose phosphates was placed in a pyrex tube and made up to 1.0 ml with distilled water. To this was added 0.2 ml of 1.5% cysteine hydrochloride followed by 6.0 ml of H_2SO_4 (concentrated H_2SO_4/H_2O : 7/3) with mixing. To this solution was added 0.2 ml of a freshly prepared solution of resublimed carbazole in ethanol (0.12%)

Fig.6. The standard curve of the phloroglucinol color reaction for ribose-5-phosphate.

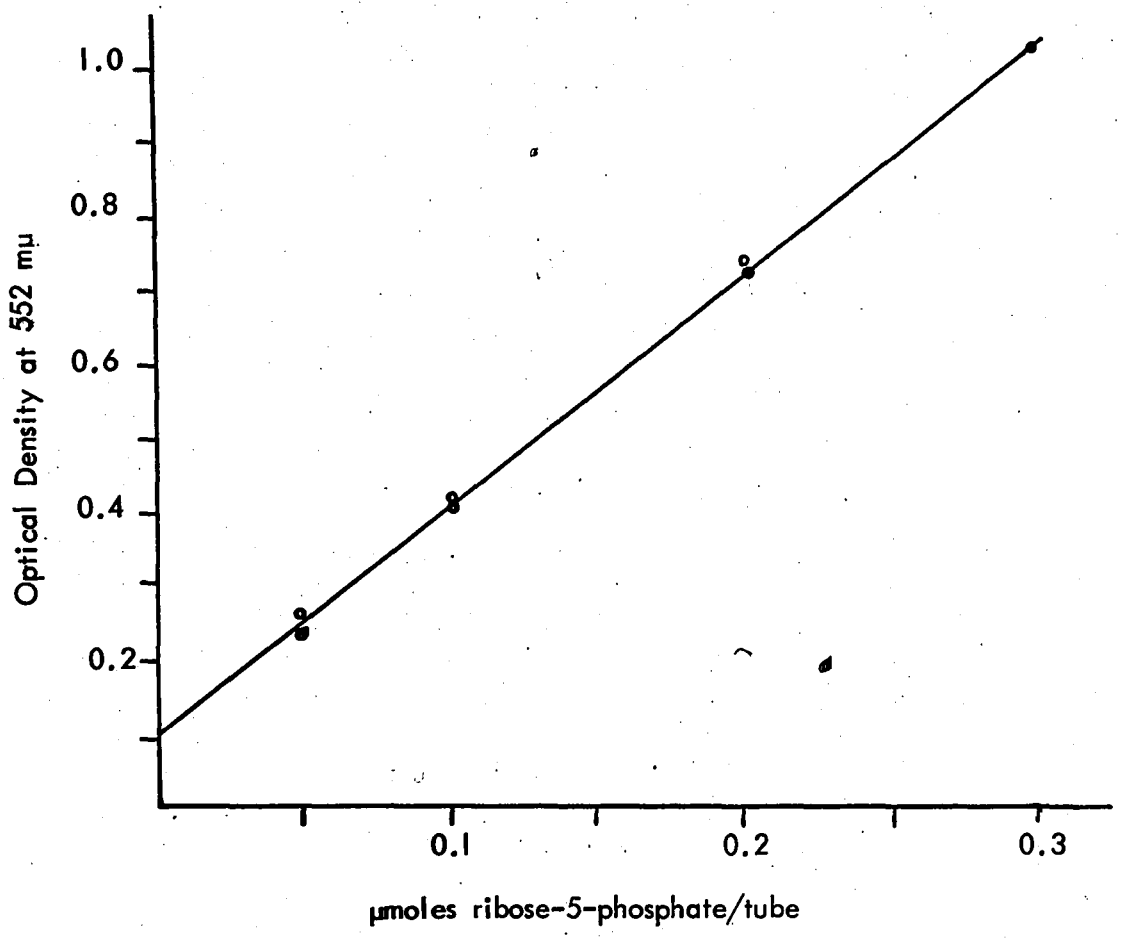
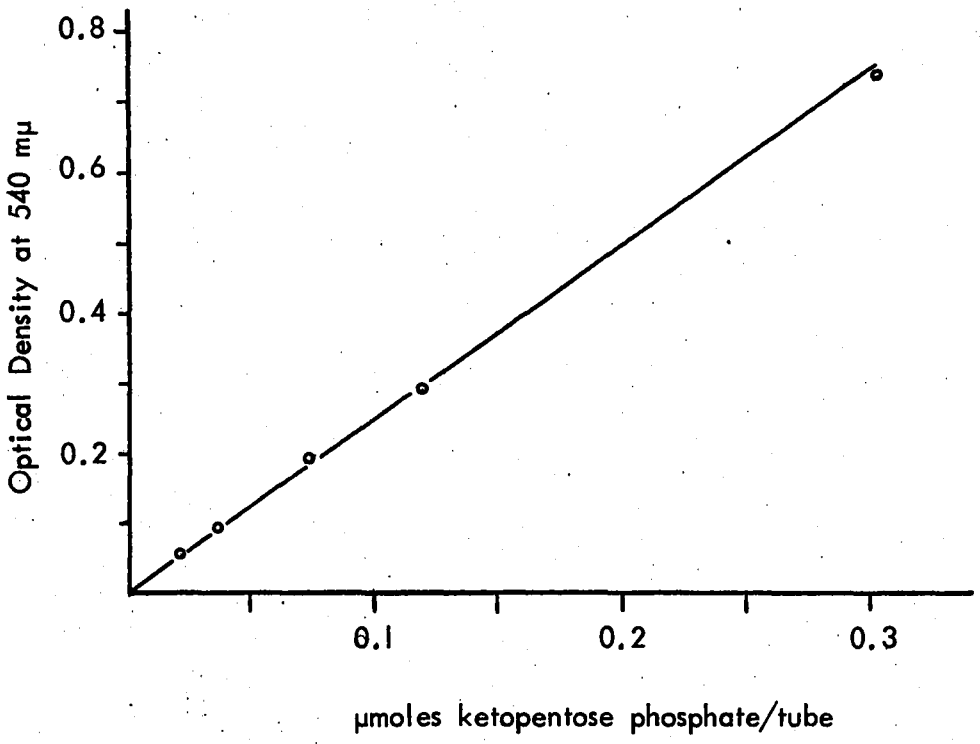


Fig.7. The standard curve of cysteine carbazole color reaction for ketopentose phosphate.



with thorough mixing. The mixture was incubated for 2 hours at 37°C and the purple colour developed was read immediately at 540 mμ against a water blank.

A preparation of ketopentose phosphates, containing only a small amount of ribose-5-phosphate, was obtained by column elution of a mixture of ribose-5-phosphate/ribulose-5-phosphate/xylulose-5-phosphate, in which partial separation of ribose-5-phosphate from the ketopentose phosphates was achieved (see RESULTS).

The standard curve (Fig. 7) was linear within the concentrations tested (0.02 to 0.33 μmoles) and the conversion factor under the present condition at 2 hours, 37°C, was 0.25 OD unit at 540 mμ for 0.1 μmoles ketopentose phosphates in the reaction tube.

Sedoheptulose-7-phosphate: Three types of colour test for heptulose were examined: the orcinol-trichloracetic acid method (206); the modified orcinol method (182); and the cysteine-H_2SO_4 method of Dische (207). When pure sedoheptulosan was used as the source of heptulose, it was found that all three methods gave reproducible results and linear standard curves. But the determination of heptulose (in the form of sedoheptulose-7-phosphate) had to be carried out in the presence of a number of substances which were expected to be present in the incubation mixture, eg. fructose-6-phosphate, ribose-5-phosphate, xylulose-5-phosphate, erythrose-4-phosphate, etc. A brief experiment was done to determine whether the presence of these substances, in concentrations expected to be found in the incubation mixtures, would interfere with each

colour reaction. It can be seen from the results in Table I that the cysteine-H_2SO_4 method showed the least interference from the presence of these substances. This reaction was also twice as sensitive as the modified orcinol method and six times as sensitive as the orcinol-trichloroacetic acid method. It was therefore used in our experiments.

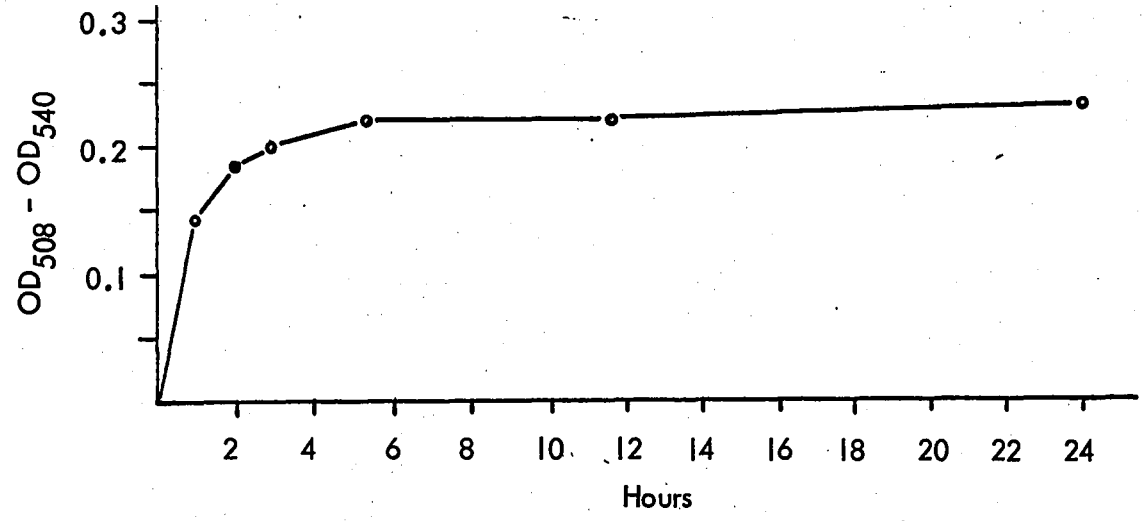
Cysteine-H_2SO_4 method. To one ml of sample was added with cooling 4.5 ml H_2SO_4 (concentrated H_2SO_4/H_2O : 6/1). The mixture was mixed thoroughly and was held successively for 3 minute intervals at 0°C, 20°-25°C, and 100°C in the appropriate water baths. The mixture was cooled at room temperature for 20 minutes and 0.1 ml of 3% cysteine hydrochloride (freshly prepared) was added with vigorous shaking. A pink colour slowly developed over a 10 hour period and remained stable for at least another 14 hours at room temperature (Fig. 8a). The colour was read at 15 hours at 508 and 540 mμ. The difference in absorbance at these two wavelengths ($OD_{508} - OD_{540}$) was proportional to the heptulose concentration.

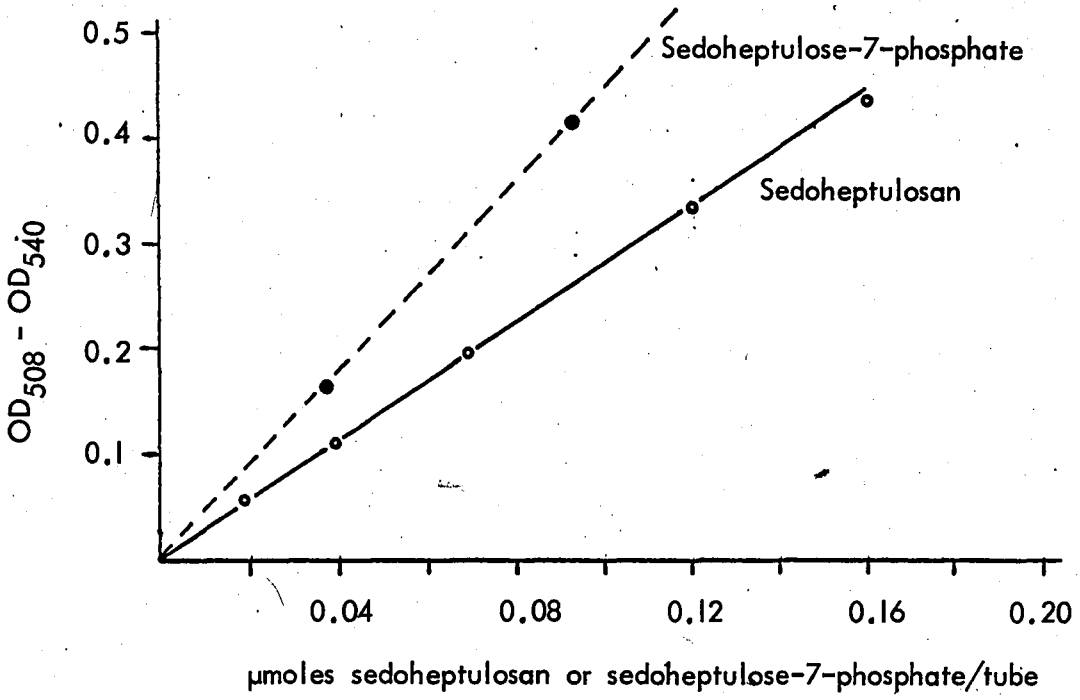
Due to the limited supply of sedoheptulose-7-phosphate, sedoheptulosan was used for establishing the standard curve (Fig. 8b). It was found in two separate determinations that sedoheptulose-7-phosphate gave 46% higher colour yield than an isomolar amount of sedoheptulosan. Thus, a "projected" sedoheptulose-7-phosphate standard curve was plotted in relation to the sedeheptulosan standard curve (Fig. 8b). This projected standard curve was used in our experiments when colorimetric determinations of sedoheptulose-7-phosphate were required.

Table I. The effect of ribose-5-phosphate, xylulose-5-phosphate, erythrose-4-phosphate, fructose-6-phosphate and dithiothreitol on the colour reactions of the orcinol-trichloroacetic acid method, modified orcinol method and cysteine-sulfuric acid method.

	Orcinol-TCA Method		Modified Orcinol Method		Cysteine-H_2SO_4 Method	
	Amount added (µmoles)	OD_{620}	Amount added (µmoles)	OD_{625}	Amount added (µmoles)	OD_{508}-OD_{540}
Sedoheptulosan	0.50	0.247	0.16	0.292	0.08	0.245
+ R5P	0.50	0.338	1.0	0.232	0.20	0.259
+ Xu5P	0.36	0.308	0.60	0.280	0.36	0.242
+ Er4P	0.20	0.246	0.20	0.170	0.20	0.235
+ F6P	0.35	0.236	0.35	0.193	0.35	0.232
+ DTT	0.50	0.231	0.50	0.025	0.50	0.252

Fig.8. Cysteine-sulfuric color reaction for sedoheptulose-7-phosphate and sedoheptulosan; (a) time course of color development (0.08 μmoles sedoheptulosan)., (b) standard curves.





RESULTS*

The ability of homogenates and extracts to support glycolysis.

Rat skeletal muscle homogenates and extracts were prepared with 0.04 M nicotinamide, pH 6.8. The resulting homogenates and extracts had a slightly acidic pH of 6.6.

Three ml of incubation mixture contained (final concentrations): 100 mM imidazole buffer, pH 7.5, 4 mM magnesium chloride, 33 mM potassium chloride, 2 mM dithiothreitol, 2.7 mM ATP, 0.5 mM NAD, 2.0 ml of homogenate or extract, and one of the following substrates: 50 mg glycogen, 54 µmoles glucose, 54 µmoles glucose-6-phosphate. The control contained all the above ingredients except the substrate. The mixtures were incubated at 30°C for 5 minutes and were then deproteinized as described in MATERIALS AND METHODS.

The lactic acid production in each incubation mixture is shown in Table II. Both the homogenate and the extract were slow in converting glucose to lactic acid. During 5 minutes of incubation with glucose, the net lactic acid production in the homogenate incubation mixture was 1 µmole and in the extract incubation mixture was 3.6 µmoles. When glycogen or glucose-6-phosphate were added as substrate, the homogenate showed a much higher ability to convert either of these two substrates into lactic acid than the extract. With glycogen as substrate, the homogenate showed a net lactic acid production of 12.6 µmoles after 5 minutes of incubation while the extract produced only 3.6 µmoles in 5

* Part of this work has been published as a short communication in The Biochemical Journal (208).

minutes. Similarly, with glucose-6-phosphate as substrate, the rate of lactic acid production in the homogenate incubation mixture (15 µmoles/5 minutes) was higher than that of the extract (2.4 µmoles/5 minutes).

The phosphate compounds in the incubation mixtures were analysed by paper chromatography (Fig. 9). In the homogenate and extract controls, no phosphorylated intermediates were detected on the chromatogram apart from Pi and dense spots due to the added nucleotides. The Pi, IMP, AMP and ADP spots were derived partly from the muscle but chiefly from the degradation of the added ATP.

The chromatograms of the incubation mixtures with glucose as substrate showed no detectable phosphorylated intermediates. The absence of a glucose-6-phosphate spot indicated that it had not accumulated. This, together with the very low rate of lactic acid production suggested that in both the homogenate and extract incubation mixtures, hexokinase was the initial limiting step when glucose was used as substrate.

When the extract was incubated with glycogen or glucose-6-phosphate, the chromatograms showed dense spots of glucose-6-phosphate and fructose-6-phosphate but no other glycolytic intermediates. The chromatogram of the incubation mixtures containing homogenate and either glycogen or glucose-6-phosphate as substrate revealed a different pattern compared to that of the extracts. The accumulation of fructose-1,6-diphosphate and triose phosphate was indicated by the presence of dense spots on the chromatograms; this was

Table II. Lactic acid, pyruvate and phosphoenolpyruvate production in rat muscle homogenate and extract incubation mixtures with glucose, glycogen or glucose-6-phosphate as substrates.

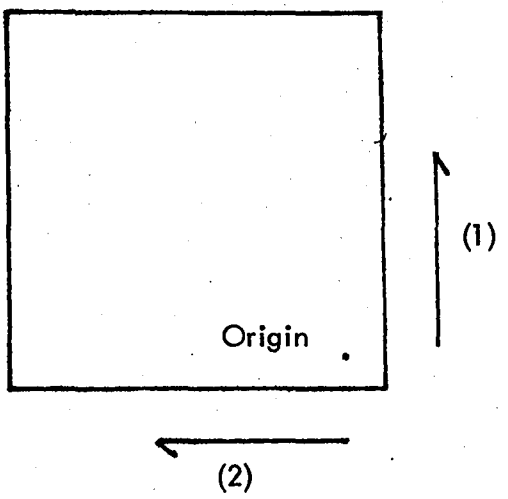
	Homogenate Incubation				Extract Incubation			
	Control	Glucose	Glycogen	G6P	Control	Glucose	Glycogen	G6P
Lactic acid (μmoles)	24	25	36.6	39	18.6	22.2	22.2	21.0
P-enolpyruvate (μmoles)	none	none	1.0	1.9	none	none	none	none
Pyruvate (μmoles)	n.d.	n.d.	0.5	0.2	n.d.	n.d.	n.d.	n.d.

"none" means not detected on paper chromatograms (see Fig. 10)

n.d., not determined

Fig.9. Paper chromatograms of the products of the homogenate and extract incubation mixtures with glucose, glycogen or glucose-6-phosphate as substrates.

Footnote :

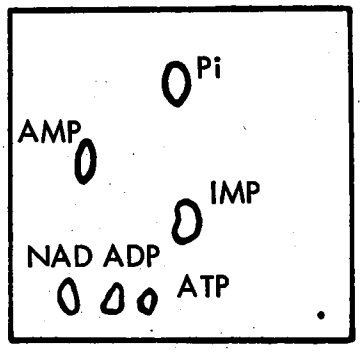


(1) : First run, run twice in GW_3 (5 hours + 4 hours).

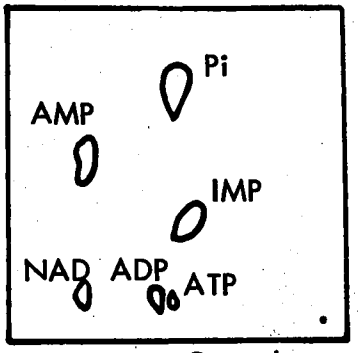
GW_3 = n-Butanol, n-propanol, acetone 80% (w/v), formic acid 30% (w/v), trichloroacetic acid (40:20:25:25:15 by volume).

(2) : Second run, run once in isobutyric acid/ N ammonium hydroxide solvent (100:60 by volume), 9 hours.

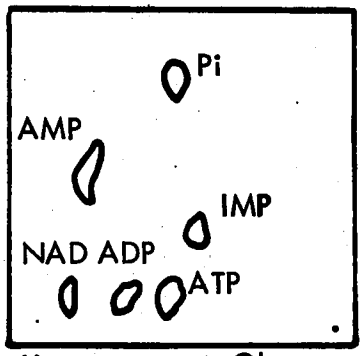
P.T.O.



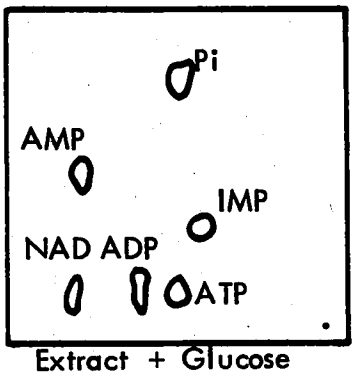
Homogenate Control



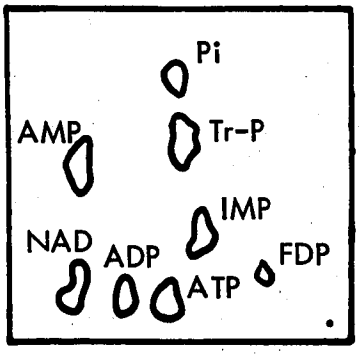
Extract Control



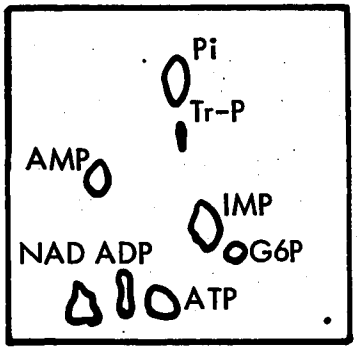
Homogenate + Glucose



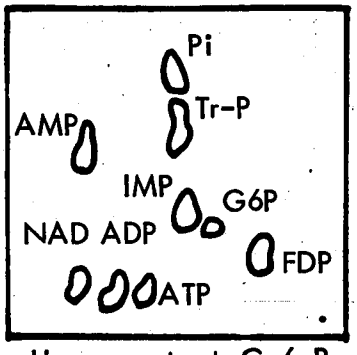
Extract + Glucose



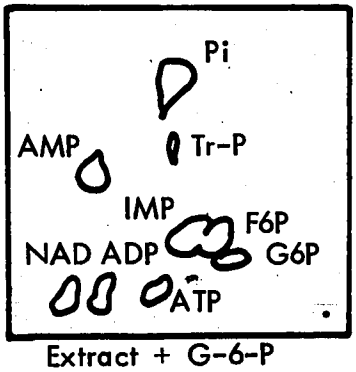
Homogenate + Glycogen



Extract + Glycogen



Homogenate + G-6-P

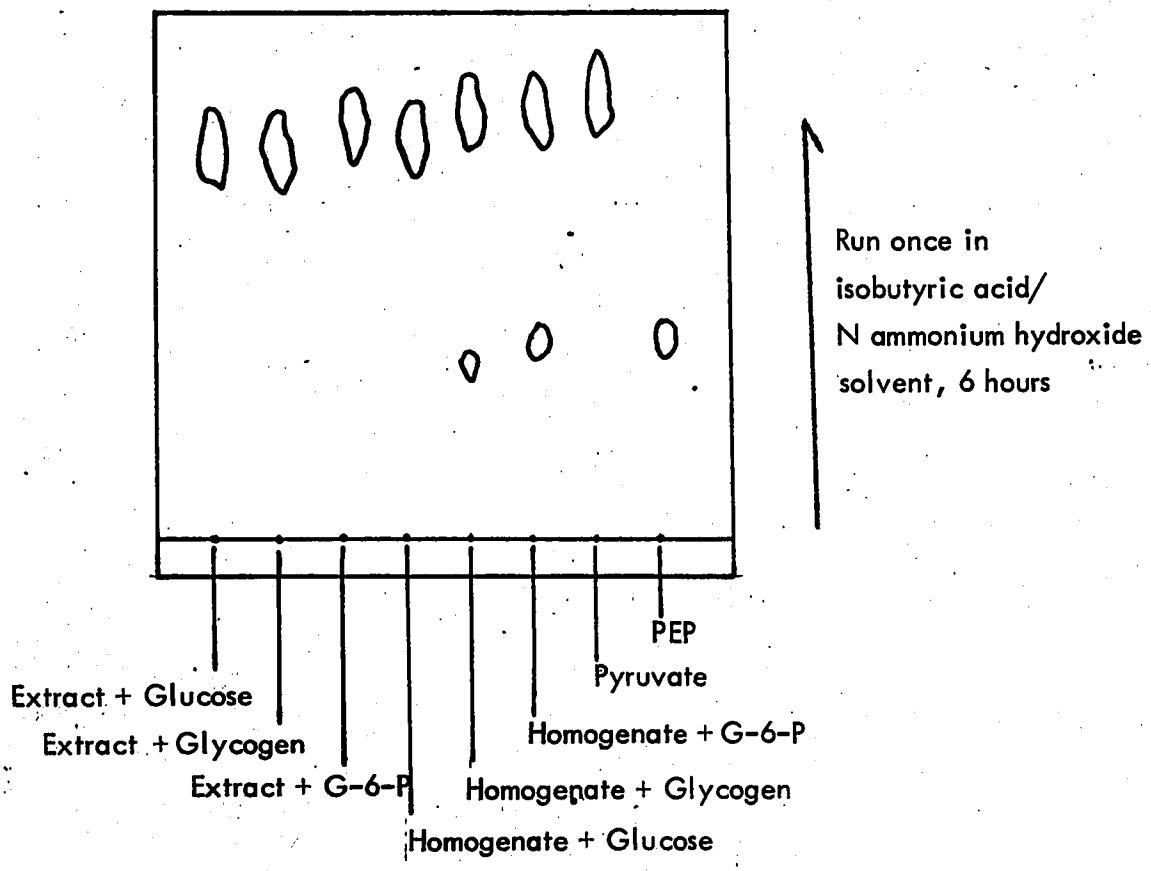


Extract + G-6-P

accompanied by the almost total disappearance of hexose monophosphate spots. The accumulation of hexose monophosphates in the extract was accompanied by a low rate of lactic acid production whereas the disappearance of the hexose monophosphate and the accumulation of fructose-1,6-diphosphate and triose phosphate was accompanied by a high rate of lactic acid production. This suggested that when glycogen or glucose-6-phosphate was used as substrate, phosphofructokinase was the rate limiting step in the extract incubation mixtures. Although phosphofructokinase was not rate limiting in the homogenate incubation mixtures, the accumulation of triose phosphates despite the high glycolytic rate suggested that the apparent rate limiting step was at glyceraldehyde phosphate dehydrogenase.

All the incubation mixtures were also analysed by paper chromatography for their phosphoenolpyruvate and pyruvate contents. It can be seen (Fig. 10) that all of the incubation mixtures contained pyruvate but only two of them contained phosphoenolpyruvate in high enough concentration to be detected by paper chromatography. These were the two homogenate mixtures with glucose-6-phosphate and glycogen as substrate, respectively. Their pyruvate and phosphoenolpyruvate contents were assayed enzymatically (Table II). It should be noted that phosphoenolpyruvate had accumulated despite the equilibrium constant of pyruvate kinase being highly in favour of pyruvate and ATP formation (209,210). The fact that the pyruvate kinase reaction was far from equilibrium pointed to a possible regulation at this step.

Fig.10. Paper chromatographic detection of pyruvate and phosphoenolpyruvate in the homogenate and extract incubation mixtures with glucose, glycogen or glucose-6-phosphate as substrates.



The effect of pH on rat muscle phosphofructokinase (208).

Stimulated and nonstimulated rats were used. Muscle homogenates and extracts were prepared with one of the following homogenization media:

(a) 0.04 M nicotinamide, pH 6.8;

(b) 0.05 M imidazole buffer, pH 6.6;

(c) 0.05 M imidazole buffer, pH 7.0;

(d) 0.05 M trishydroxymethylamino methane, pH 8.5.

The homogenates were centrifuged at 3600 g and the extracts were prepared from the 3600 g supernatants by millipore filtration. The pH of the homogenates, 3600 g supernatants and the extracts were measured immediately after preparation and the phosphofructokinase activities were measured as described in MATERIALS AND METHODS.

Table III lists the level and distribution of the enzyme activities in these three fractions. Although the pH of the homogenates varied within a narrow range of 6.6 to 8.2, the total enzyme activity in the homogenates was found to be markedly dependent on the pH. The enzyme activity in the slightly alkaline homogenates (pH 8.2) was 4 to 8 times higher than in the slightly acidic homogenates (pH 6.6). Along with the variation in enzyme activities there was a marked difference in the pattern of distribution of the enzyme activities in the homogenate, 3600 g supernatant and the extract. If the enzyme activity found in the extract (millipore filtrate) was considered as representing the soluble enzyme, then the percentage of soluble enzyme can be expressed as: enzyme

Table III. Phosphofructokinase activity in rat muscle homogenates, supernatants and extracts prepared at different pH.

Physiological state	Enzyme activity (μmoles/min/g of muscle)			Soluble enzyme (% of total)
	Homogenate (H)	3600g Supernatant (S)	Extract (E)	$\frac{E}{H} \times 100$
(a) Non-stimulated	10.1	4.0	1.1	10
Stimulated	11.8	3.2	1.0	8.5
Non-stimulated	25.8 (pH 6.6)	n.d.	1.7 (pH 6.7)	7.0
Stimulated	27.4 (pH 6.6)	n.d.	6.2 (pH 6.7)	23
Stimulated	29.2	n.d.	11.2	38
Non-stimulated	32.2 (pH 6.8)	40.3 (pH 6.5)	15.7 (pH 6.5)	49
(b) Stimulated	10.7 (pH 6.9)	6.9 (pH 6.8)	2.2 (pH 6.8)	21
Non-stimulated	25.2 (pH 6.9)	4.0 (pH 6.9)	5.0 (pH 6.8)	20
Non-stimulated	60.3 (pH 6.8)	13.3 (pH 6.5)	18.1 (pH 6.5)	30
(c) Stimulated*	40.4 (pH 7.1)	16.1 (pH 6.9)	16.1 (pH 6.9)	40
Non-stimulated	46.7 (pH 7.4)	35.0 (pH 7.0)	16.9 (pH 6.7)	36
Non-stimulated	49.0 (pH 7.2)	15.3 (pH 7.0)	23.7 (pH 6.9)	48
(d) Stimulated	62.5 (pH 8.2)	97.5	92.5 (pH 8.2)	148
Non-stimulated	80.1 (pH 8.2)	105.0	90.5 (pH 8.2)	110
Stimulated	84.0 (pH 8.2)	80.0	96.0 (pH 8.2)	112

* Rat exercised for 45 min. on a treadmill before death.

Muscle was homogenized in: (a) 40 mM-nicotinamide, pH 6.8; (b) 50 mM-imidazole buffer, pH 6.6; (c) 50 mM-imidazole buffer, pH 7.0; (d) 50 mM-tris-chloride, pH 8.5.

activity in the extract/enzyme activity in the homogenate x 100%. It could be seen that the percentage of soluble enzyme was lowest in the slightly acidic homogenates, which also possessed the lowest enzyme activity. The slightly alkaline homogenates, which possessed the highest enzyme activity had all of the enzyme in the soluble form. An examination of the enzyme acitvities in the 3600 g supernatants provided some indication as to the localization of the insoluble enzyme. It appeared that, with a few exceptions, most of the insoluble enzyme was associated with the 3600 g sedimentable fraction. Part of the insoluble enzyme was present in the 3600 g supernatants, and since it was excluded by the 100 mμ millipore filters, the enzyme was either larger than 100 mμ or associated with particles larger than 100 mμ.

The conditions of the animals just before death, i.e. stimulated or nonstimulated, did not appear to have any effect on the level and distribution of the enzyme.

The results of this experiment were compatible with the existence of a pH dependent soluble-insoluble phosphofructokinase with the soluble enzyme being more active than the insoluble form.

The glycolytic behaviour of a slightly alkaline muscle extract.

On account of the increased amount of soluble, active phosphofructokinase in the alkaline medium, it was thought that a slightly alkaline muscle extract might possess different, possibly higher glycolytic activity compared to the

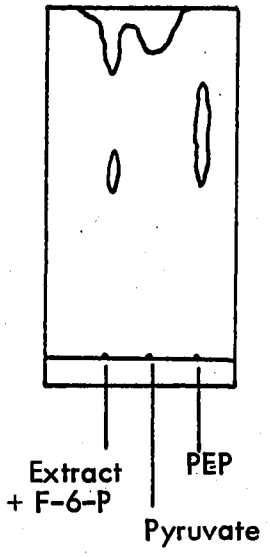
slightly acidic extract studied in the first incubation experiment (Table II, Fig. 9, 10).

Rat muscle extract was prepared with 0.04 M nicotinamide - 0.05 M imidazole buffer, pH 7.5. The incubation mixture in a final volume of 3.0 ml contained (final concentrations): 100 mM imidazole buffer, pH 7.5, 4 mM magnesium chloride, 2 mM dithiothreitol, 0.5 mM NAD, 2.7 mM ATP, 18 mM fructose-6-phosphate and 2.0 ml of muscle extract. The control contained all the above ingredients except fructose-6-phosphate. The mixtures were incubated at 30°C for 5 minutes and were then deproteinized and neutralized.

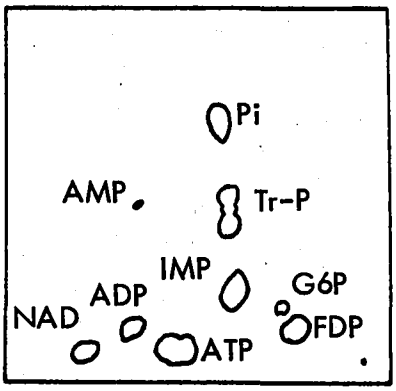
It was found that at the end of incubation, the levels of lactic acid in the fructose-6-phosphate incubation mixture was 28 µmoles and in the control was 20.4 µmoles; the net lactic acid production was therefore 7.6 µmoles.

As shown in Fig. 11a, the accumulation of phosphoenolpyruvate was detected by paper chromatography indicating a rate limiting effect operating at the pyruvate kinase step. The high concentration of fructose-1,6-diphosphate and triose phosphate in the incubation mixture was indicated by the dense spots on the chromatogram (Fig. 11b). Only a small amount of hexose monophosphate was left in the incubation mixture as indicated by the absence of fructose-6-phosphate spot and the barely detectable glucose-6-phosphate spot on the chromatogram. Thus, the slightly alkaline muscle extract possessed an active phosphofructokinase and was able to convert most of the fructose-6-phosphate to fructose-1,6-diphosphate. This was in contrast with the slightly

Fig.11. Paper chromatograms of the products of the incubation of a slightly alkaline muscle extract with fructose-6-phosphate; (a) phosphoenolpyruvate and pyruvate., (b) other glycolytic intermediates.



(a)



(b)

acidic muscle extract which showed a distinct block at the phosphofructokinase step.

The net lactic acid production (7.6 μmoles/5 minutes) was significantly higher than that of the slightly acidic extract (2.4 μmoles/5 minutes). This was in accord with the removal of the phosphofructokinase limiting effect in the slightly alkaline extract. The accumulation of triose phosphate in the incubation mixture indicated that in the slightly alkaline muscle extract, the glyceraldehyde phosphate dehydrogenase reaction had become rate limiting.

Adenosine triphosphatase and adenylic deaminase activities in muscle homogenates and extracts.

As mentioned in the INTRODUCTION the three major degradative enzymes in a muscle homogenate were NADase, adenosine triphosphatase and adenylic deaminase. With the presence of nicotinamide in the homogenate, NADase activity was inhibited. The activities of adenosine triphosphatase and adenylic deaminase in rat muscle homogenates and extracts were assayed.

Rat muscle was homogenized in 0.04 M nicotinamide - 0.05 M imidazole buffer, pH 7.5, and the enzymatic assays were carried out immediately.

Table IV shows a weak adenylic deaminase activity in rat muscle homogenate; the activities were only 1/10th to 1/27th of that of the adenosine triphosphatase, and almost all of the activity was associated with the particulate fraction of the homogenate. Similarly, adenosine triphosphatase activity was

Table IV. Adenylic deaminase and adenosine triphosphatase activities in rat muscle homogenates and extracts.

Experiment	Adenosine triphosphatase (μmoles/min/g muscle)	Adenylic deaminase (μmoles/min/g muscle)
1. Homogenate	96	9.5
Extract	11	0.0
2. Homogenate	136	8.0
Extract	5	0.7
3. Homogenate	137	5.0
Extract	28	0.4
4. Homogenate	145	7.0
Extract	6	0.1
5. Homogenate	32.2	n.d.
Extract	4.4	n.d.
6. Homogenate	43	n.d.
Extract	5	n.d.

n.d. means not determined

found predominantly in the particulate fraction, the ratio: extract ATPase/ homogenate ATPase ranged from 1/27 to 1/8.

The effect on glycolysis of varying the proportions of homogenate and extract.

Slightly alkaline homogenate and extract were prepared in 0.04 M nicotinamide - 0.05 M imidazole buffer, pH 7.5 and the adenosine triphosphatase activities were assayed.

Incubation mixtures in a final volume of 3.0 ml contained (final concentrations): 100 mM imidazole buffer, pH 7.5, 2 mM dithiothreitol, 4 mM magnesium chloride, 0.5 mM NAD, 2.7 mM ATP, 18 mM fructose-6-phosphate, and 2.0 ml of a mixture of homogenate and extract in various proportions. C_1 and C_2 are homogenate and extract control, respectively, which contained all the reaction ingredients except the substrate fructose-6-phosphate. The mixtures were incubated for 5 minutes at 30°C, and were then deproteinized and neutralized.

The levels of lactic acid, ATP and ADP in each incubation mixture are listed in Table V. It can be seen that with only extract present, the net lactic acid production in 5 minutes was 7 µmoles. With increasing proportions of homogenate in the mixture the lactic acid production increased until the amount of homogenate in the mixture reached 0.4 ml. With this quantity the lactic acid production approached that of the whole homogenate.

The adenosine triphosphatase activities in the homogenate and extract were found to be 140 µmoles/minute/gram muscle and 12 µmoles/minute/gram

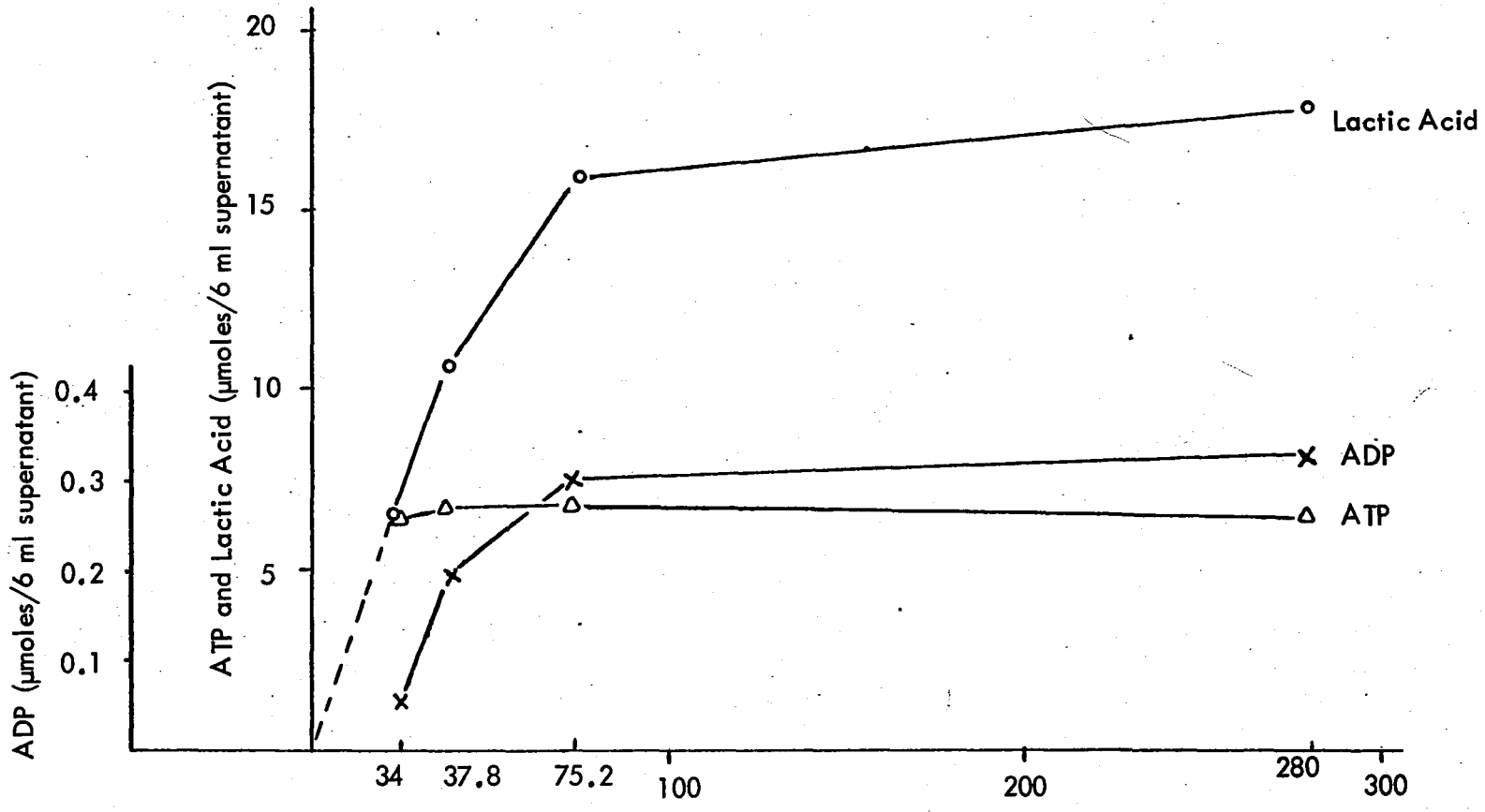
Table V. Lactic acid, ATP and ADP levels in the fructose-6-phosphate incubation mixtures with varying proportions of homogenate and extract.

	Homogenate (ml)	Extract (ml)	Total lactic acid (μmoles)	Net lactic acid (μmoles)	ADP (μmoles)	ATP (μmoles)
A	2.0	0.0	29	17	0.38	6.5
B	0.4	1.6	28	16	0.29	6.9
C	0.1	1.9	23	11	0.20	6.9
D	0.0	2.0	19	7	0.06	6.7
C_1*	2.0	0.0	12	0	n.d.	n.d.
C_2	0.0	2.0	12	0	n.d.	n.d.

* C_1 and C_2 are controls containing 2.0 ml of homogenate and extract, respectively, and all other ingredients except the substrate, fructose-6-phosphate.

n.d. means not determined

Fig. 12. The levels of lactic acid, ATP and ADP as a function of the ATPase activities in the incubation mixtures.



muscle, respectively. The amount of adenosine triphosphatase activity in the incubation mixture was calculated according to the proportion of homogenate and extract in the mixture (1 ml of homogenate or extract was derived from 0.2 gram muscle). The adenosine triphosphatase activity in each incubation mixture was plotted against the lactic acid, ATP and ADP levels in each incubation mixture (Fig. 12). The lactic acid production increased approximately linearly with the adenosine triphosphatase activity until the triphosphatase activity reached 75.2 μmoles/5 minutes when the lactic acid production approached that of the whole homogenate which contained an adenosine triphosphatase activity of 280 μmoles/5 minutes. Despite the difference in adenosine triphosphatase activity, the ATP level in each incubation mixture was maintained at a more or less constant level. On the other hand, the increase in ADP concentration reflected the effect of the adenosine triphosphatase activity. Also, as shown in Fig. 12, the ADP level was closely related to the net lactic acid production, a high level of ADP was associated with a high rate of lactic acid production in the incubation mixture. Thus, the relationship between adenosine triphosphatase activity and lactic acid production was clearly shown to be through the supply of ADP.

Partial purification of transaldolase from Candida utilis.

The purification method of Tchola and Horecker (197) was adopted with some modifications. The following procedures were performed in a cold room

at 4°C unless otherwise stated.

Initial extract: Thirty-eight grams of finely ground dried Candida utilis yeast and 110 ml of cold 0.17 M sodium bicarbonate were blended in a Waring blender at top speed for 6 minutes. The homogenate was gently stirred with 206 ml of water for 5 minutes and was then centrifuged at 10,000 g for 10 minutes at 2°C. The residue was extracted twice with 206 ml of water and the supernatants were pooled and made up to 1.0 litre with 0.17 M sodium bicarbonate (initial extract, 1.0 litre).

0-23% Acetone fractionation: To the extract (1 litre) was added 5 N acetic acid until the pH reached 4.8. The extract was then cooled to 1°C and 300 ml of acetone (precooled to -70°) was added with stirring over a period of 1.5 minutes. The suspension was immediately centrifuged at 10,000 g for 10 minutes at -10°C. The precipitate was extracted twice with two portions of 80 ml water and the extract was collected by centrifugation, pooled and adjusted to pH 7.0 with N NaOH. (0-23% acetone extract, 150 ml.)

23-35% Acetone fractionation: To the supernatant of the 0-23% acetone fractionation was added 250 ml of -70°C acetone over a period of 2.0 minutes. The suspension was immediately centrifuged at 10,000 g for 10 minutes at -10°C and the precipitate was dissolved in 80 ml of water and adjusted to pH 7.0 with N NaOH. (23-35% acetone fraction, 80 ml.)

35-50% Acetone fractionation: To the supernatant of the 23-35% acetone fractionation was added 500 ml of -70°C acetone over a period of 3 minutes.

Stirring was continued for another 12 minutes at -10° and the precipitate was collected by centrifugation at 10,000 g for 10 minutes at -10°C and dissolved in 80 ml of water and adjusted to pH 7.0 with N NaOH. (35-50% acetone fraction, 85 ml.)

Calcium phosphate gel adsorption: The 35-50% acetone fraction was dialysed against 1 litre of 5 mM phosphate buffer, pH 6.5. The buffer was changed once during the overnight dialysis. The dialysed fraction was mixed with an equal volume of calcium phosphate gel (30 mg/ml) prepared according to the procedure of Tchola and Horecker (197). The suspension was stirred for 10 minutes and the gel was collected by centrifugation at 3600 g for 20 minutes. To the gel was added 80 ml of 0.05 M phosphate buffer, pH 9.0 and the mixture was stirred in the cold for 15 minutes. The supernatant was collected by centrifugation at 3600 g for 20 minutes. The gel was re-extracted once with 80 ml of the same buffer and the supernatants were pooled. (Calcium phosphate eluate, 167 ml.)

Acid ammonium sulfate fractionation: To the calcium phosphate gel eluate (167 ml) was added 63 grams powdered ammonium sulfate over a period of 10 minutes. The solution was adjusted to pH 5.0 with acetic acid and was then stirred for 10 minutes. The precipitate was collected by centrifugation at 3600 g for 20 minutes and dissolved in a small amount of cold 0.04 M triethanolamine - 0.01 M EDTA buffer, pH 7.6. (0-60% ammonium sulfate fraction, 7.8 ml.)

To the supernatant of the 0-60% ammonium sulfate fraction was added

Table VI. Purification of transaldolase from Candida utilis.

	Vol. (ml)	Total Transaldolase (I.U.)	Total Transketolase (I.U.)	Total Protein (mg)	S.A. of Transaldolase (I.U./mg protein)
Initial extract	1000	2060	1400	7320	0.28
0-23% Acetone	150	53	0.0	90	0.59
23-35% Acetone	80	80	51	340	0.24
35-50% Acetone	85	1200	0.0	850	1.41
Dialysed 35-50% acetone	85	700	0.0	850	0.82
Calcium phosphate gel	167	430	0.0	255	1.69
0-60% Ammonium sulfate	7.8	120	0.8	78	1.54
60-90% Ammonium sulfate	5.1	108	0.0	37.4	2.97

33.4 grams ammonium sulfate. After stirring for 10 minutes, the precipitate was collected by centrifugation and dissolved in a small amount of 0.04 M triethanolamine-0.01 M EDTA buffer, pH 7.6. (60-90% ammonium sulfate fraction, 5.1 ml.)

As shown in Table V I, most of the transaldolase activity was found in the 35-50% acetone fraction; no transketolase activity was detected in this fraction but a strong "NADH-oxidase" activity was observed. Only 58% of the transaldolase activity in the 35-50% acetone fraction was recovered after dialysis. Forty percent of the transaldolase activity in the dialysed fraction was lost at the subsequent calcium phosphate adsorption and elution steps. A small amount of transketolase activity reappeared in the 0-60% ammonium sulfate fraction. The 60-90% ammonium sulfate fraction had the highest specific activity for transaldolase (2.97 I.U/mg protein) representing a tenfold purification over the initial extract. The overall recovery in this fraction was 5% with respect to the initial extract. The transaldolase activity in the 60-90% ammonium sulfate fraction was high enough to be used for the enzymatic assays of sedoheptulose-7-phosphate and erythrose-4-phosphate. More important, this fraction was free from contaminating enzymes (transketolase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and "NADH-oxidase") which might interfere with the enzymatic assays.

Column chromatographic separation of ribose-5-phosphate from ribulose-5-phosphate and xylulose-5-phosphate.

The elution patterns of the ribose-5-phosphate/ribulose-5-phosphate mixture and the ribose-5-phosphate/ribulose-5-phosphate/xylulose-5-phosphate mixture are shown in Fig. 13 and 14, respectively. Ribose-5-phosphate came off first and was followed by ribulose-5-phosphate 5 tubes later (Fig. 13). This showed that the two phosphates possessed properties sufficiently different to be separated on an anionic exchange column. The elution pattern of the ribose-5-phosphate/ribulose-5-phosphate/xylulose-5-phosphate mixture was similar but only one ketopentose phosphate peak was detected colorimetrically (Fig. 14). This indicated that ribulose-5-phosphate and xylulose-5-phosphate were eluted together at the same ionic strength.

Fraction 56, 57, 58 and 59, which contained only small amounts of ribose-5-phosphate, were pooled and assayed enzymatically for xylulose-5-phosphate and ribulose-5-phosphate content. These pooled fractions, containing known amounts of ketopentose phosphate (ribulose-5-phosphate and xylulose-5-phosphate) were used for the determination of a standard curve for the cysteine carbazole colorimetric assay (see MATERIALS AND METHODS).

Fig.13. Column elution of a ribose-5-phosphate/ribulose-5-phosphate mixture.

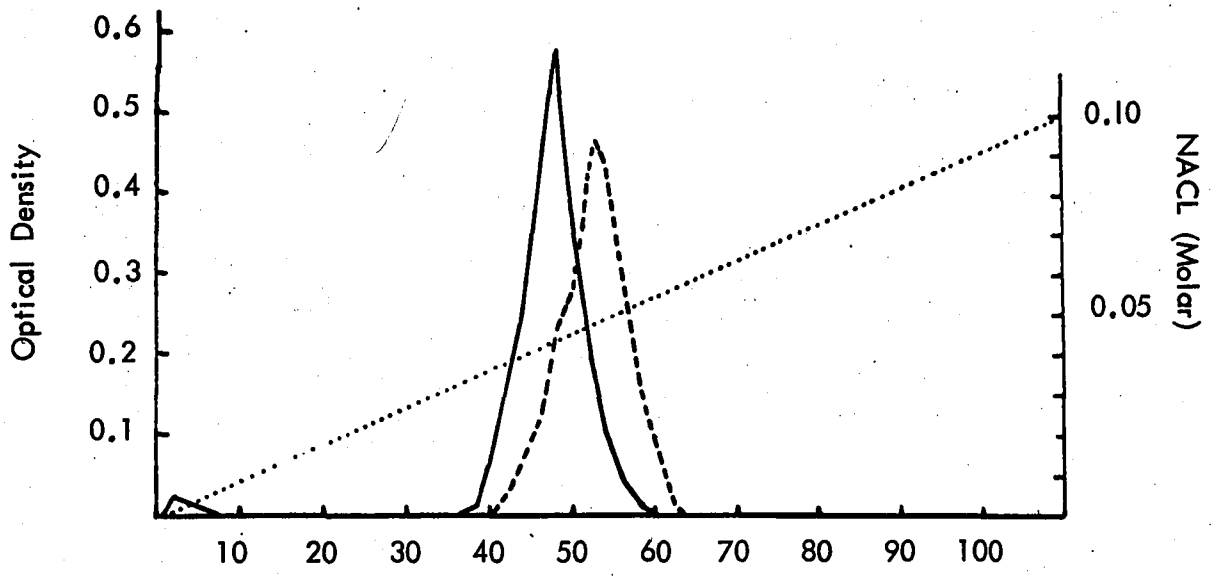
Fig.14. Column elution of a ribose-5-phosphate/ribulose-5-phosphate/xylulose-5-phosphate mixture.

——— OD_{552} (the phloroglucinol color reaction for ribose-5-phosphate).

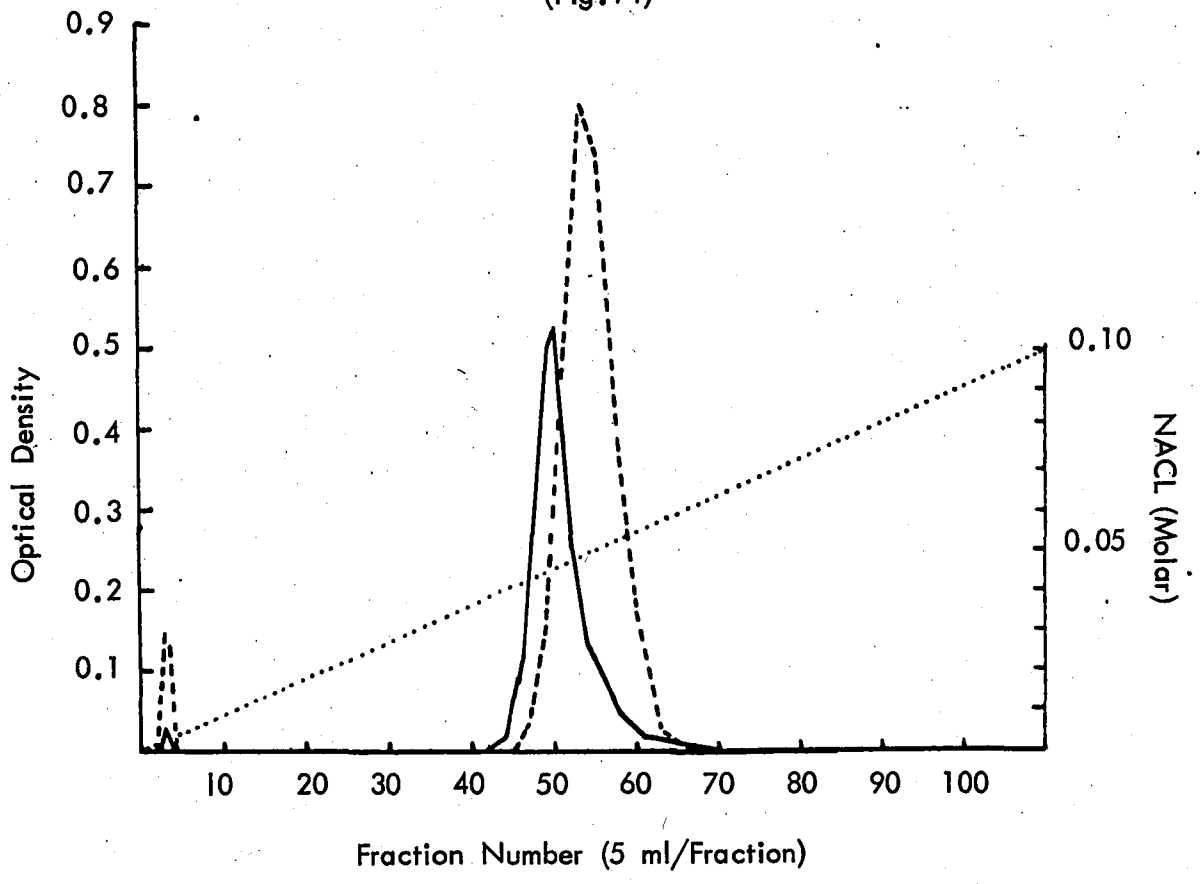
------ OD_{540} (the cysteine-sulfuric color reaction for ketopentose phosphate).

..... NaCl gradient.

(Fig.13)


Optical Density
0.6
0.5
0.4
0.3
0.2
0.1
0.10
0.05
NACL (Molar)
10
20
30
40
50
60
70
80
90
100

(Fig.14)


Optical Density
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.10
0.05
NACL (Molar)
10
20
30
40
50
60
70
80
90
100
Fraction Number (5 ml/Fraction)

Incubation of muscle extract with ribose-5-phosphate.

Rat muscle homogenates were prepared in 0.05 M triethanolamine buffer, pH 7.5 and the extracts were obtained as the 105,000 g supernatants.

Three ml of incubation mixture contained (final concentrations): 100 mM imidazole buffer, pH 7.5, 17 mM magnesium chloride, 8.3 mM ribose-5-phosphate and 2.0 ml muscle extract. The mixtures were incubated at 37°C for the desired period of time and then deproteinized and neutralized.

Since NADP and NADPH were not added, the oxidative route of the pentose phosphate pathway was not operative and the ribose-5-phosphate was expected to be metabolized via the nonoxidative route of the pathway.

Paper chromatographic analysis was done on all samples to detect the presence and relative amounts of ribose-5-phosphate, ribulose-5-phosphate, xylulose-5-phosphate, erythrose-4-phosphate and sedoheptulose-7-phosphate. These phosphates, together with triose phosphate and hexose monophosphate were analysed by specific enzymatic and/or colorimetric methods.

Experiment-I: Table VII shows the result of a preliminary experiment with the inclusion of two controls. The inorganic phosphate content in the extract control (C_1) was 8.7 µmoles while no inorganic phosphate was present in the ribose-5-phosphate control (C_2). There was only a small increase in inorganic phosphate during the incubation. The amount liberated after 60 minutes of incubation with ribose-5-phosphate was 1.6 µmoles. This showed that no appreciable amounts of phosphatase acting on pentose phosphate were present.

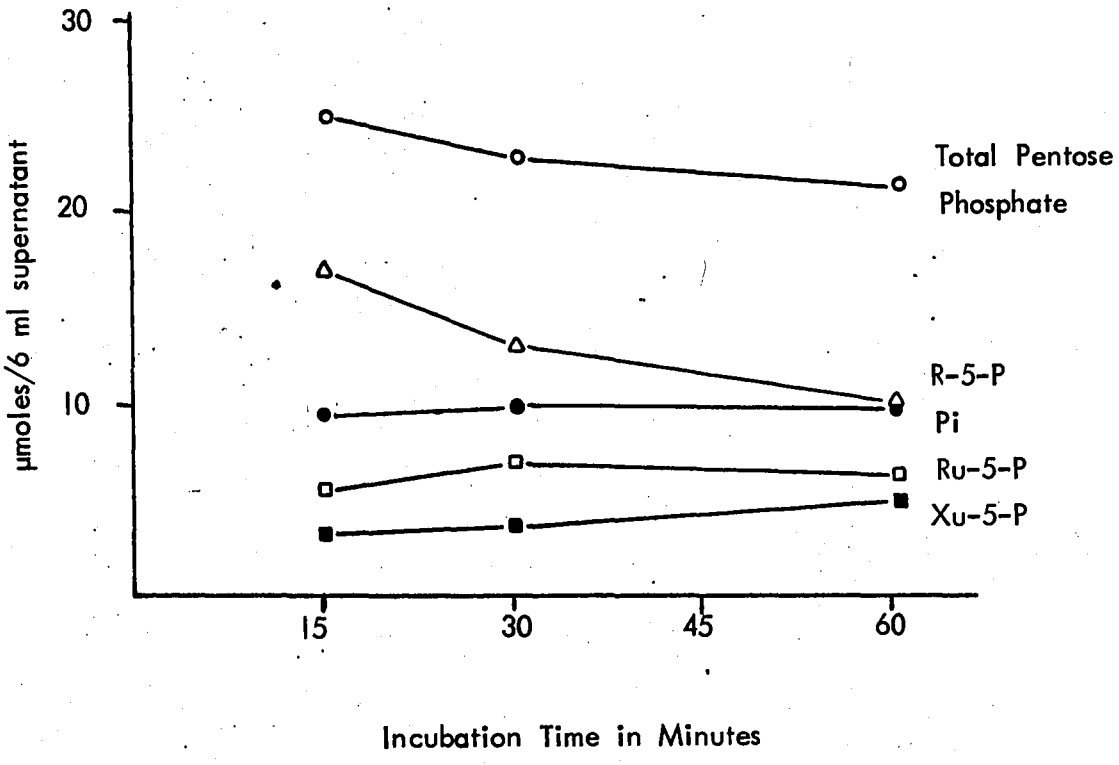
The extract control (C_1) showed no detectable amount of ribose-5-phosphate

Table VII. Incubation of ribose-5-phosphate with rat muscle extract - Experiment 1.

	Methods of determination	Amount in μmoles/6 ml supernatant				
		C_1*	C_2	15'	30'	60'
Ribose-5-P	colorimetric	0.0	25	17	13	10
Total ketopentose phosphate	colorimetric	0.5	0.4	8.5	10.4	11.5
Xylulose-5-P	enzymatic	0.0	0.0	3.1	3.5	5.0
Ribulose-5-P	total ketopentose phosphate - xylulose-5-P	0.5	0.4	5.4	6.9	6.5
Total pentose phosphate	ribose-5-P + total ketopentose phosphate	0.5	25.4	25.5	23.4	21.5
Sedoheptulose-7-P	colorimetric	n.d.	n.d.	0.0	0.0	0.0
Erythrose-4-P	paper chrom.	none detected				
Triose-P	enzymatic	0.0	0.0	0.0	0.0	0.0
Pi	colorimetric	8.7	0.0	9.3	10.2	10.3

* C_1 and C_2 are homogenate and ribose-5-phosphate control, respectively, and were incubated at 37°C for 60 minutes.

Fig.15. Incubation of ribose-5-phosphate with rat muscle extract, Experiment-I.



or xylulose-5-phosphate present endogenously but about 0.5 μmoles of cysteine carbazole positive materials were present. The ribose-5-phosphate control (C_2) contained 0.4 μmoles of cysteine carbazole positive materials. This could be due to the presence of impurities in the commercial ribose-5-phosphate used which was 98% pure.

As shown in Fig. 15, the disappearance of ribose-5-phosphate with time was accompanied by an increase in ketopentose phosphates. At the end of 60 minutes incubation, the total pentose phosphates were 21.5 μmoles compared with 25.5 μmoles at 15 minutes. The difference of 4.0 μmoles could be interpreted as the amount of pentose phosphates that had reacted beyond ketopentose phosphates to form the other intermediates of the nonoxidative pentose phosphate pathway, namely, sedoheptulose-7-phosphate, glyceraldehyde-3-phosphate, erythrose-4-phosphate and fructose-6-phosphate. However, the validity of such an interpretation was limited by the fact that since each total pentose phosphate value was a summation of values from two separate determinations, the inherent error might be quite large.

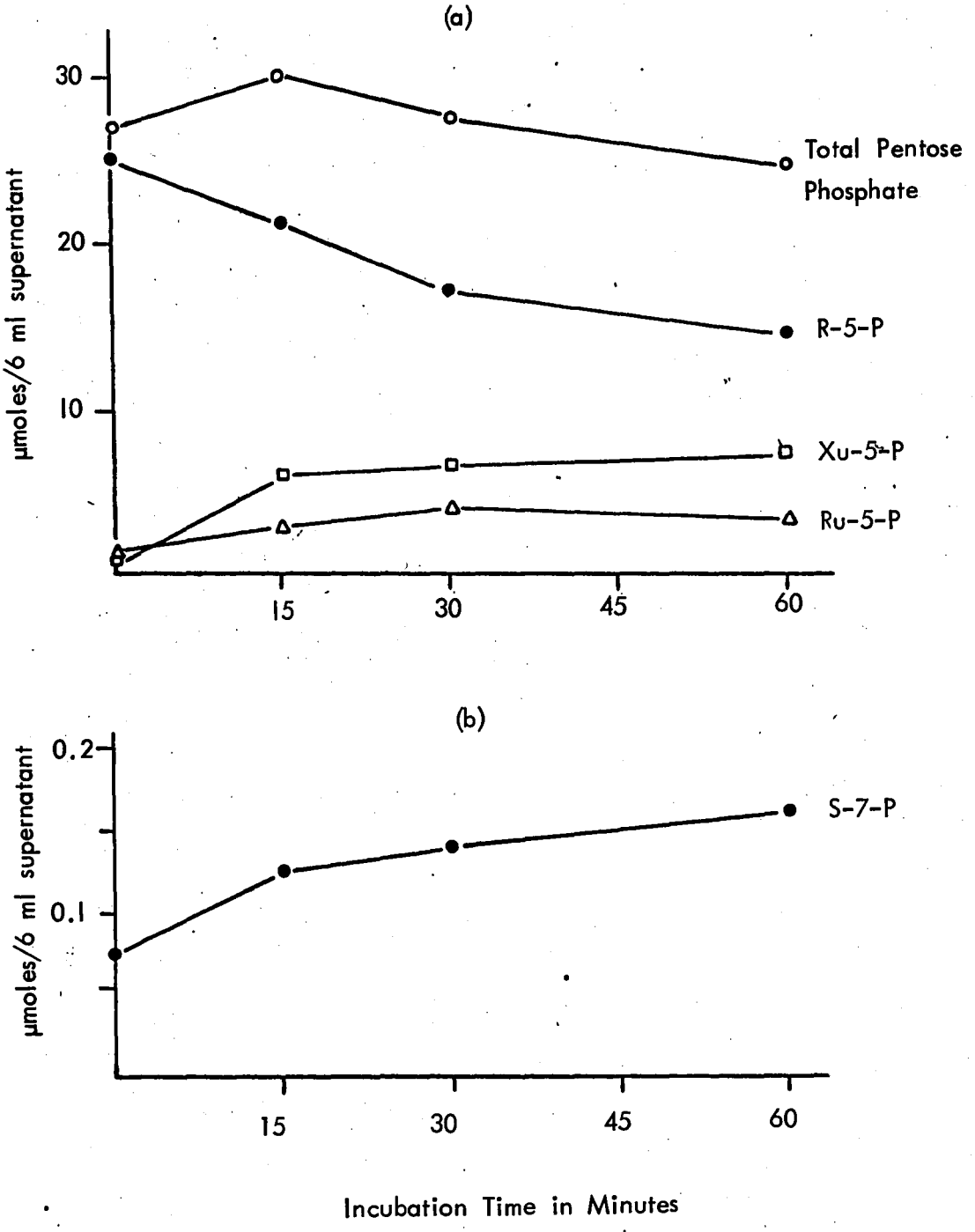
Experiment-2: All the intermediates of the nonoxidative pentose phosphate pathway were determined (Table VIII, Fig. 16a,b). As in Experiment-1, the disappearance of ribose-5-phosphate was accompanied by increases in both ribulose-5-phosphate and xylulose-5-phosphate (Fig. 16a). The xylulose-5-phosphate concentration in the 15, 30 and 60 minutes incubation mixtures was higher than the ribulose-5-phosphate concentrations. This is in contract with

Table VIII. Incubation of ribose-5-phosphate with rat muscle extract - Experiment 2.

	Methods of determination	Amount in μmoles/6 ml supernatant			
		0'	15'	30'	60'
Ribose-5-P	colorimetric	25	21	17	14.4
Total ketopentose phosphate	colorimetric	2.1	8.8	10.4	10.3
Xylulose-5-P	enzymatic	0.8	5.8	6.5	7.1
Ribulose-5-P	total ketopentose phosphate - xylulose-5-P	1.3	3.0	3.9	3.2
Total pentose phosphate	total ketopentose phosphate + ribose-5-P	27.1	29.8	27.4	24.7
Sedoheptulose-7-P	colorimetric	0.08	0.13	0.14	0.16
Erythrose-4-P	enzymatic	0.00	0.04	0.02	0.02
Triose phosphate	enzymatic	0.00	0.00	0.00	0.00
Hexose monophosphate	enzymatic	0.02	n.d.	0.03	0.03

n.d. = not determined

Fig.16. Incubation of ribose-5-phosphate with rat muscle extract, Experiment-2; (a) change in the levels of pentose phosphates with time of incubation., (b) time course of sedoheptulose-7-phosphate formation.



the result of Experiment-1 in which the opposite was observed.

The high value of total pentose phosphate observed at 15 minutes incubation was probably due to experimental error as the total pentose phosphate cannot be higher than the initial value at 0 minute. The total pentose phosphate at 60 minutes incubation showed a drop of 2.4 µmoles from the 0 minute value. This could mean that 2.4 µmoles of pentose phosphate were converted to intermediates beyond the pentose phosphate. However, the only other compound that was produced in high enough concentration to be measured and showed any tendency of increasing concentration with time was sedoheptulose-7-phosphate (Fig. 16b). The total increase after 60 minutes incubation was 0.08 µmoles. The presence of sedoheptulose-7-phosphate was also detected on paper chromatogram. Triose phosphates were not detected enzymatically while the hexose monophosphates and erythrose-4-phosphate levels were between 0.02 and 0.04 µmoles in all the mixtures.

Experiment-3: Incubation of ribose-5-phosphate was carried out with both dialysed and nondialysed portions of the same extract. The dialysed extract was obtained by dialysing 11 ml of a muscle extract against 1 litre of 0.001 M triehanolamine buffer, pH 7.5 in the cold. T he buffer was changed once during the overnight dialysis.

The time course of ribose-5-phosphate disappearance and ribulose-5-phosphate and xylulose-5-phosphate formation in both the dialysed and non-dialysed extract incubation mixtures (Table IX, Fig. 17a, 18a) were comparable

Table IX. Incubation of ribose-5-phosphate with dialysed and nondialysed muscle extract-Experiment-3.

		Methods of determination	Amounts in µmoles/6 ml supernatant			
			0'	15'	30'	60'
Ribose-5-P	D	colorimetric	20.4	10.8	9.0	7.8
	ND		21.0	12.6	12.0	10.2
Total ketopentose phosphate	D	colorimetric	1.0	9.0	11.7	11.9
	ND		1.4	9.8	11.2	11.9
Xylulose-5-P	D	enzymatic	0.0	5.3	7.3	7.0
	ND		0.0	5.8	7.0	8.0
Ribulose-5-P	D	total ketopentose	1.0	3.7	4.4	4.9
	ND	phosphate - xylulose-5-P	1.4	4.0	4.2	3.9
Total pentose phosphate	D	total ketopentose	21.4	19.8	20.7	19.7
	ND	phosphate + ribose-5-P	22.4	22.4	23.2	22.1
Sedoheptulose-7-P	D	colorimetric	0.04	0.08	0.17	0.29
		enzymatic	0.00	0.08	0.13	0.16
	ND	colorimetric	0.05	0.12	0.15	0.16
		enzymatic	0.04	0.16	0.16	0.10
Erythrose-4-P	D	enzymatic	0.02	0.00	n.d.	0.04
	ND		n.d.	0.00	n.d.	0.00
Triose phosphate	D	enzymatic	0.04	0.04	n.d.	0.02
	ND		0.00	n.d.	0.00	0.00
Hexose monophosphate	D	enzymatic	0.04	0.08	0.05	0.04
	ND		0.02	0.05	0.05	0.04

D = dialysed extract; ND = nondialysed extract; n.d. = not determined.

Fig.17. Incubation of ribose-5-phosphate with muscle extract (nondialysed), Experiment-3; (a) changes in the levels of pentose phosphates with time of incubation., (b) time course of sedoheptulose-7-phosphate formation.

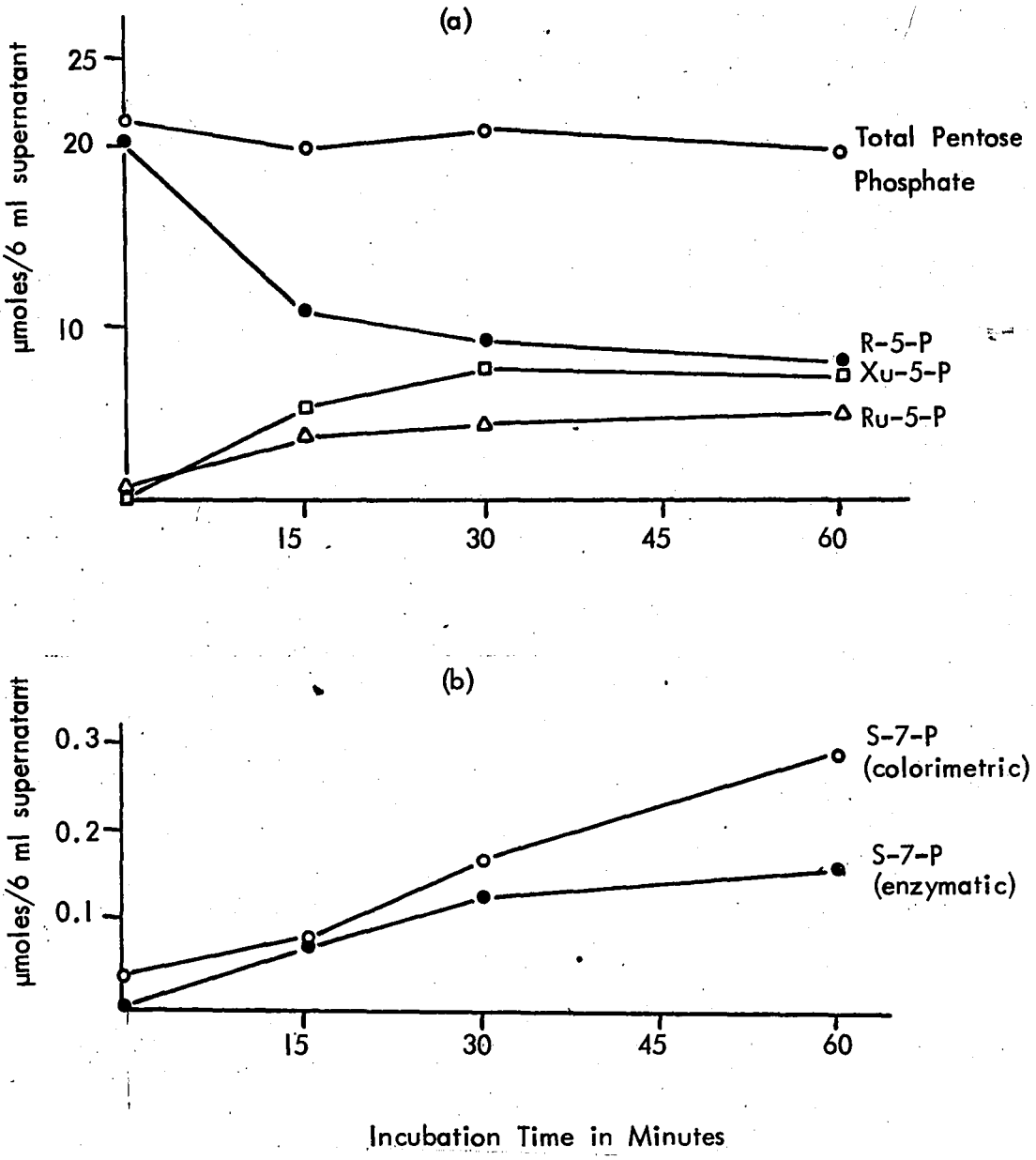
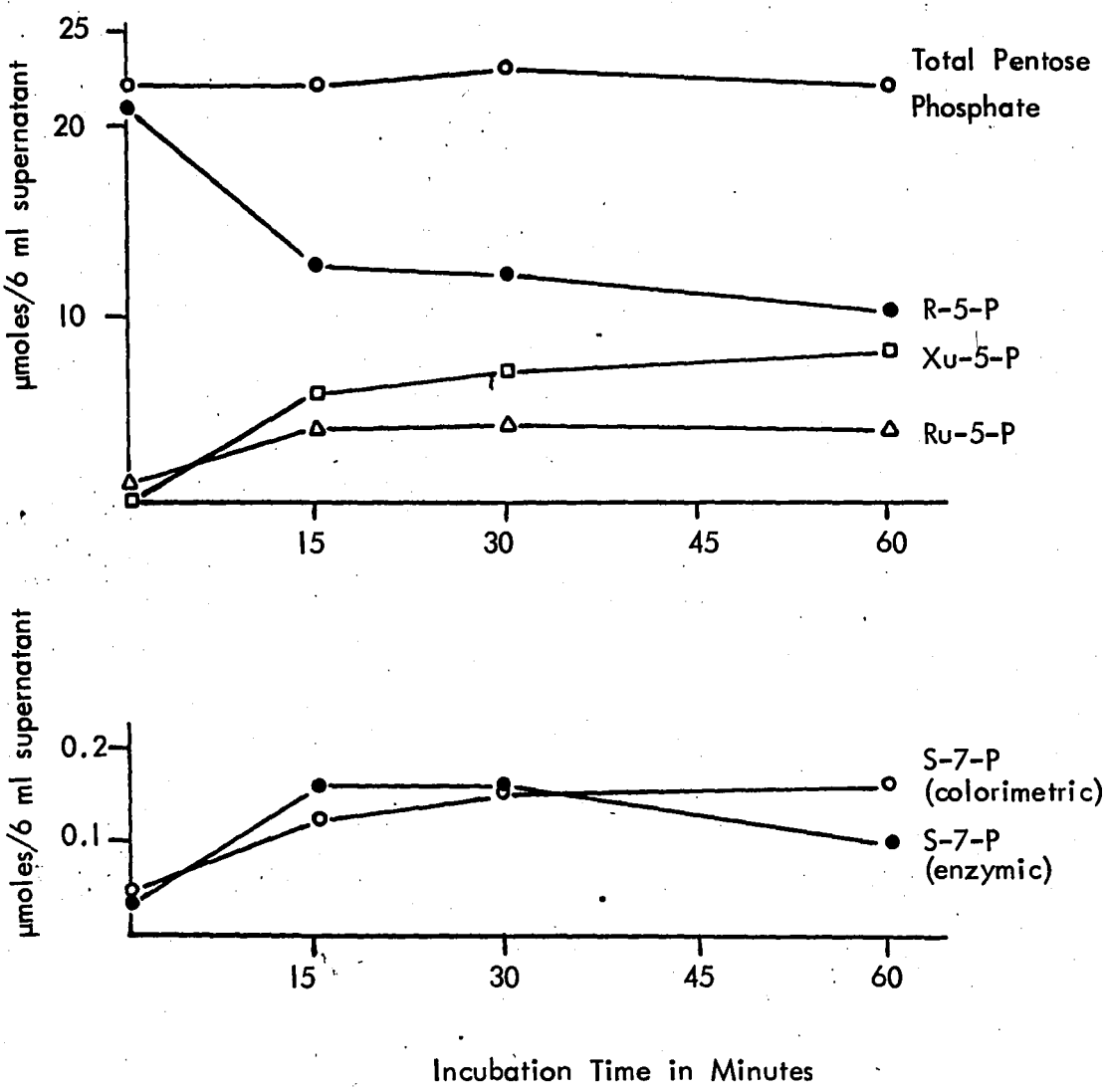


Fig.18. Incubation of ribose-5-phosphate with rat muscle extract (dialysed), Experiment-3; (a) changes in the levels of pentose phosphates with time of incubation., (b) time course of sedoheptulose-7-phosphate formation.



with the time course of Experiment-2 (Fig. 16a).

The amount of ribose-5-phosphate which disappeared within the first 15 minutes in the dialysed extract (9.6 µmoles) was 1.2 µmoles more than in the nondialysed extract (8.4 µmoles). Between 15 minutes and 60 minutes incubation the time course and concentration of ribose-5-phosphate, ribulose-5-phosphate and xylulose-5-phosphate were not significantly different in the dialysed and nondialysed extracts.

As in Experiment-2, sedoheptulose-7-phosphate was the only intermediate in addition to the pentose phosphates which was present in sufficient amounts to be assayed and to be detected on paper chromatograms. Due to the low concentrations of sedoheptulose-7-phosphate, both the enzymic and colorimetric methods were of low sensitivity and the two sets of values showed poor agreement (Table IX). From the time course of sedoheptulose-7-phosphate formation (17b, 18b), the main difference between the dialysed and nondialysed extract was that in the former the production of sedoheptulose-7-phosphate was linear over the first 30 minutes incubation while in the nondialysed extract sedoheptulose-7-phosphate level increased within the first 15 minutes of incubation and then remained unchanged during the following 45 minutes of incubation.

Erythrose-4-phosphate assayed enzymatically in both the dialysed and nondialysed extract incubation mixtures was between 0.02 and 0.04 µmoles. Paper chromatography did not detect its presence. Triose phosphate and hexose

monophosphate levels were between 0.02 and 0.08 μmoles and did not show any pattern of dependence on the time of incubation.

Experiment-4: A shorter interval of incubation (0, 5, 10, 15, 30 minutes) was used in order to obtain a more accurate picture of the rapid disappearance of ribose-5-phosphate and formation of ribulose-5-phosphate and xylulose-5-phosphate.

In this experiment, ribulose-5-phosphate was assayed both colorimetrically and enzymatically. As shown in Table X, there was close agreement between the values obtained enzymatically and colorimetrically. The enzymatic assay was used as it was considered more accurate.

The time course of the change in ribose-5-phosphate, rubulose-5-phosphate and xylulose-5-phosphate (Fig. 19) was essentially similar to those in Experiment-2 and -3. It can be seen that the level of xylulose-5-phosphate was initially lower than that of ribulose-5-phosphate. This was in accordance with the fact that ribulose-5-phosphate is the substrate for phosphoketopentose epimerase in the formation of xylulose-5-phosphate. After 5 minutes incubation, the level of xylulose-5-phosphate began to overtake that of ribulose-5-phosphate and for the remaining 25 minutes of incubation it was higher than the ribulose-5-phosphate level.

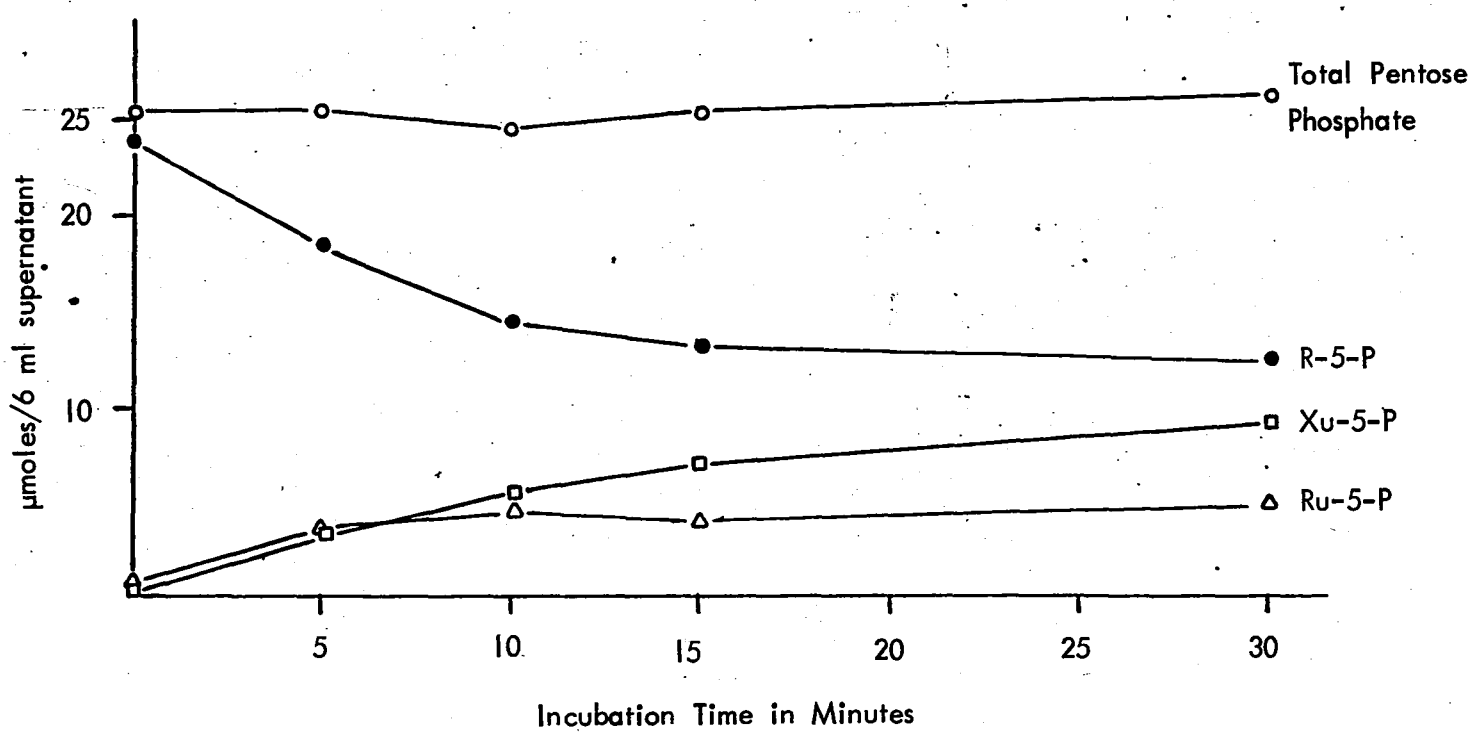
Contrary to the first three incubation experiments, the total pentose phosphates level did not show any decrease with time of incubation. In fact, the level showed a slight increase of 0.5 μmoles at 60 minutes over the 0 minute

Table X. Incubation of ribose-5-phosphate with rat muscle extract - Experiment 4.

	Methods of determination	Amounts in µmoles/6 ml supernatant				
		0'	5'	10'	15'	30'
Ribose-5-P	colorimetric	24.0	18.5	14.0	13.5	12.3
Total ketopentose phosphate	colorimetric	1.6	6.5	9.0	10.8	12.8
Xylulose-5-P	enzymatic	0.6	3.5	5.6	7.0	8.9
Ribulose-5-P	total ketopentose phosphate - xylulose-5-P	1.0	3.0	3.4	3.8	3.9
	enzymatic	0.8	3.6	4.4	3.9	4.9
Total pentose phosphate	ribose-5-P + ribulose-5-P (enzymatic) + xylulose-5-P	25.6	25.6	24.0	24.4	26.1
Sedoheptulose-7-P	enzymatic	0.00	0.00	0.00	0.00	0.00
Erythrose-4-P	enzymatic	n.d.	0.00	n.d.	0.00	0.00
Triose phosphate	enzymatic	0.00	0.00	0.00	0.00	0.00
Hexose monophosphate	enzymatic	0.04	n.d.	n.d.	n.d.	0.04

n.d. = not determined

Fig.19. Short interval incubation of ribose-5-phosphate with rat muscle extract, Experiment-4.



incubation (Table X). This indicated that no pentose phosphate had reacted further. This was confirmed by the absence of sedoheptulose-7-phosphate, erythrose-4-phosphate and triose phosphate in all the incubation mixtures.

The short interval incubation permitted the calculation of the initial rate of ribose-5-phosphate disappearance which was an indication of the muscle phosphoribose isomerase activity. A value of 2.75 μmoles/minute/gram muscle was obtained.

In all four ribose-5-phosphate incubation experiments, the changes in the ribose-5-phosphate, ribulose-5-phosphate and xylulose-5-phosphate levels were rapid in the first 15 minutes, slowed down between 15 and 30 minutes, and after 30 minutes there were very little changes in their levels. The ratios of R-5-P/Ru-5-P/Xu-5-P at the end of incubations are shown in Table XI. The ratio of ribose-5-phosphate was set at 3 so that comparisons with the equilibrium ratio for R-5-P/Ru-5-P/Xu-5-P of 3/1/3 (103) could be made.

Table XI. The ratios of ribose-5-phosphate/ribulose-5-phosphate/xylulose-5-phosphate after 60 minutes incubation of ribose-5-phosphate with muscle extracts.

	R-5-P	Ru-5-P	Xu-5-P
Experiment-1	3.0	2.0	1.6
Experiment-2	3.0	0.7	1.5
Experiment-3 dialysed	3.0	1.9	2.8
nondialysed	3.0	1.1	2.3
Experiment-4*	3.0	1.2	2.2

* 30 minute values.

Incubation of muscle extract with fructose-6-phosphate and fructose-1,6-diphosphate.

A rat muscle homogenate was prepared with 0.05 M triethanolamine buffer, pH 7.5, and the extract was prepared as the 105,000 g supernatant.

Three ml of incubation mixture contained (final concentrations): 100 mM imidazole buffer, pH 7.5, 17 mM magnesium chloride, 8.3 mM fructose-6-phosphate, 8.3 mM fructose-1,6-diphosphate, and 2.0 ml muscle extract. The mixtures were incubated at 37° for the desired period of time (0, 15, 30, 60 minutes).

In one incubation mixture, an extract of mouse ascites tumor cells was used in place of rat muscle extract. The extract was obtained by extracting 25 mg of the acetone powder of mouse ascites tumor cells with two volumes of 1.0 ml 50 mM triethanolamine buffer, pH 7.5.

In another incubation tube, the stability of erythrose-4-phosphate to perchloric acid treatment was tested. To an incubation mixture containing all the components was added 3.0 ml 3% perchloric acid followed immediately by the addition of 3.3 μmoles erythrose-4-phosphate. The remaining deproteinization and neutralization procedure was then carried out. Subsequent enzymatic assay on this neutralized and deproteinized mixture revealed that all 3.3 μmoles of erythrose-4-phosphate was recovered indicating the stability of this compound towards the perchloric acid treatment and neutralization procedure.

Table XII shows the results of the enzymatic and colorimetric assays. Triose phosphates were formed by the action of aldolase in the muscle extract on the added fructose-1,6-diphosphate. The amount was the highest at 15 minutes incubation, at 60 minutes only 0.3 μmoles were left. The amount of hexose monophosphate did not decrease with time of incubation. Instead, it started to increase at 15 minutes (when the triose phosphate level was beginning to decline) with a net gain of 6 μmoles at 60 minutes incubation (Fig. 20a). Erythrose-4-phosphate, ribose-5-phosphate, ribulose-5-phosphate and xylulose-5-phosphate were not detected on paper chromatograms, and neither erythrose-4-phosphate nor xylulose-5-phosphate could be detected enzymatically.

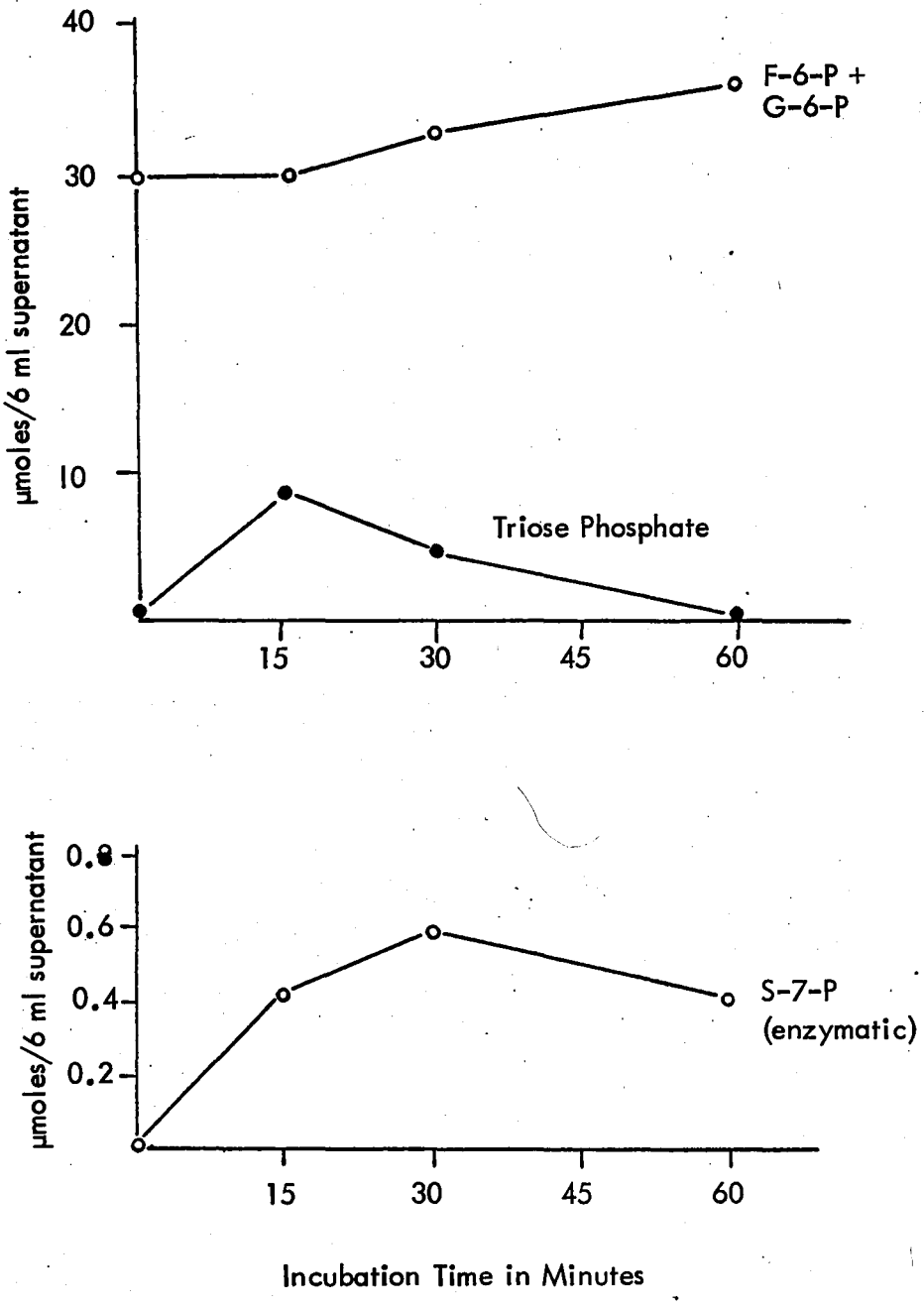
Sedoheptulose-7-phosphate was the only incubation product other than the triose phosphate which was present in sufficient concentration to be detected by paper chromatogram and by enzymatic and colorimetric assays. The enzymatic assay was complicated by the presence of high concentrations of hexose monophosphate in the incubation mixtures and the sedoheptulose-7-phosphate had to be assayed by a specially designed method (see MATERIALS AND METHODS). The colorimetric assays yielded values which were much lower than the enzymatic assay (Table XII), possibly due to fructose-6-phosphate interfering with the cysteine-sulfuric color reaction. As observed in Fig. 20b, the level of sedoheptulose-7-phosphate (assayed enzymatically) increased during the first 30 minutes of incubation and then decreased slightly in the 60 minutes incubation mixture.

Table XII. Incubation of fructose-6-phosphate and fructose-1,6-diphosphate with rat muscle extract and rat ascites tumor cell extract.

	Methods of determination	Rat muscle extract (Amount in µmoles/6 ml supernatant)				Ascites tumor cell extract
		0'	15'	30'	60'	60'
Hexose monophosphate	enzymatic	30	30	33	36	20
Triose phosphate	enzymatic	1.1	8.8	4.7	0.3	11
Sedoheptulose-7-P	colorimetric	0.00	0.12	0.15	0.16	0.37
	enzymatic	0.00	0.42	0.58	0.42	0.43
Erythrose-4-P	enzymatic	n.d.	0.00	0.00	0.00	0.00
Xylulose-5-P	enzymatic	n.d.	0.00	0.00	0.00	0.00

n.d. = not determined

Fig.20. Incubation of fructose-6-phosphate and fructose-1,6-diphosphate with rat muscle extract., (a) changes in hexose monophosphate and triose phosphate levels with time of incubation., (b) time course of sedoheptulose-7-phosphate formation.



The extract of ascites tumor cells is known to be rich in the enzymes of the pentose phosphate pathway (177) and was used as a basis of comparison for the results obtained with the muscle extract. After 60 minutes incubations (Table XII), a high level of triose phosphate was present and the sedoheptulose-7-phosphate level (assayed enzymatically) was the same as in the 60 minutes incubation of rat muscle extract. Like the muscle extract, no erythrose-4-phosphate or xylulose-5-phosphate were found enzymatically or by paper chromatography.

Enzymatic synthesis of sedoheptulose-1,7-diphosphate.

Ling and co-workers (211) have reported the ability of rabbit muscle phosphofructokinase to phosphorylate sedoheptulose-7-phosphate in the presence of ATP, forming sedoheptulose-1,7-diphosphate. The following is an attempt to carry out this synthesis:

$$\text{S-7-P} + \text{ATP} \xrightarrow{\text{PFK}} \text{SDP} + \text{ADP}$$

The reaction mixture, in a final volume of 4.0 ml, contained the followings in final concentrations: 75 mM glycylglycine buffer, pH 7.5, 7.5 mM EDTA, 7.5 mM magnesium chloride, 37.5 mM potassium chloride, 3.8 mM ATP, 9.1 I.U. rabbit muscle phosphofructokinase (obtained from Sigma) and 0.65 μmoles sedoheptulose-7-phosphate as substrate. The mixture was incubated at 37°C and at specific times, 0.4 ml of sample was withdrawn,

deproteinized and assayed for sedoheptulose-1,7-diphosphate as described in the following.

Enzymatic assay of sedoheptulose-1,7-diphosphate.

Aldolase is known to split sedoheptulose-1,7-diphosphate into dihydroxyacetone phosphate and erythrose-4-phosphate (120, 121). Based on this aldolase function, Horecker et al (120) described an enzymatic method of determining sedoheptulose-1,7-diphosphate in which dihydroxyacetone phosphate was assayed. Our assay method is an improvement on this method; in addition to dihydroxyacetone phosphate, erythrose-4-phosphate was also assayed in the same mixture by means of transaldolase. In this way the compound assayed can be identified specifically as sedoheptulose-1,7-diphosphate.

$$\text{SDP} \xleftarrow{\text{aldolase}} \text{DHAP} + \text{Er-4-P}$$

$$\text{NADH} + \text{H}^+ + \text{DHAP} \xrightarrow{\text{Gl-1-P DH}} \text{Gl-1-P} + \text{NAD}^+$$

$$\text{Er-4-P} + \text{F-6-P} \xleftarrow{\text{TA}} \text{S-7-P} + \text{G-3-P}$$

$$\text{G-3-P} \xleftarrow{\text{TIM}} \text{DHAP}$$

$$\text{NADH} + \text{H}^+ + \text{DHAP} \xrightarrow{\text{Gl-1-P DH}} \text{Gl-1-P} + \text{NAD}^+$$

The assay mixture in a final volume of 2.0 ml contained (final concentrations): 40 mM triethanolamine buffer, pH 7.5, 5 mM EDTA, 0.13 mM NADH, sample (0.4 ml deproteinized, neutralized reaction mixture), 1.2 I.U. glycerol-1-phosphate dehydrogenase, and 1.1 I.U. aldolase. As shown in Fig. 21, the decrease in absorbance at 340 mμ, ΔE_1, represented dihydroxy-

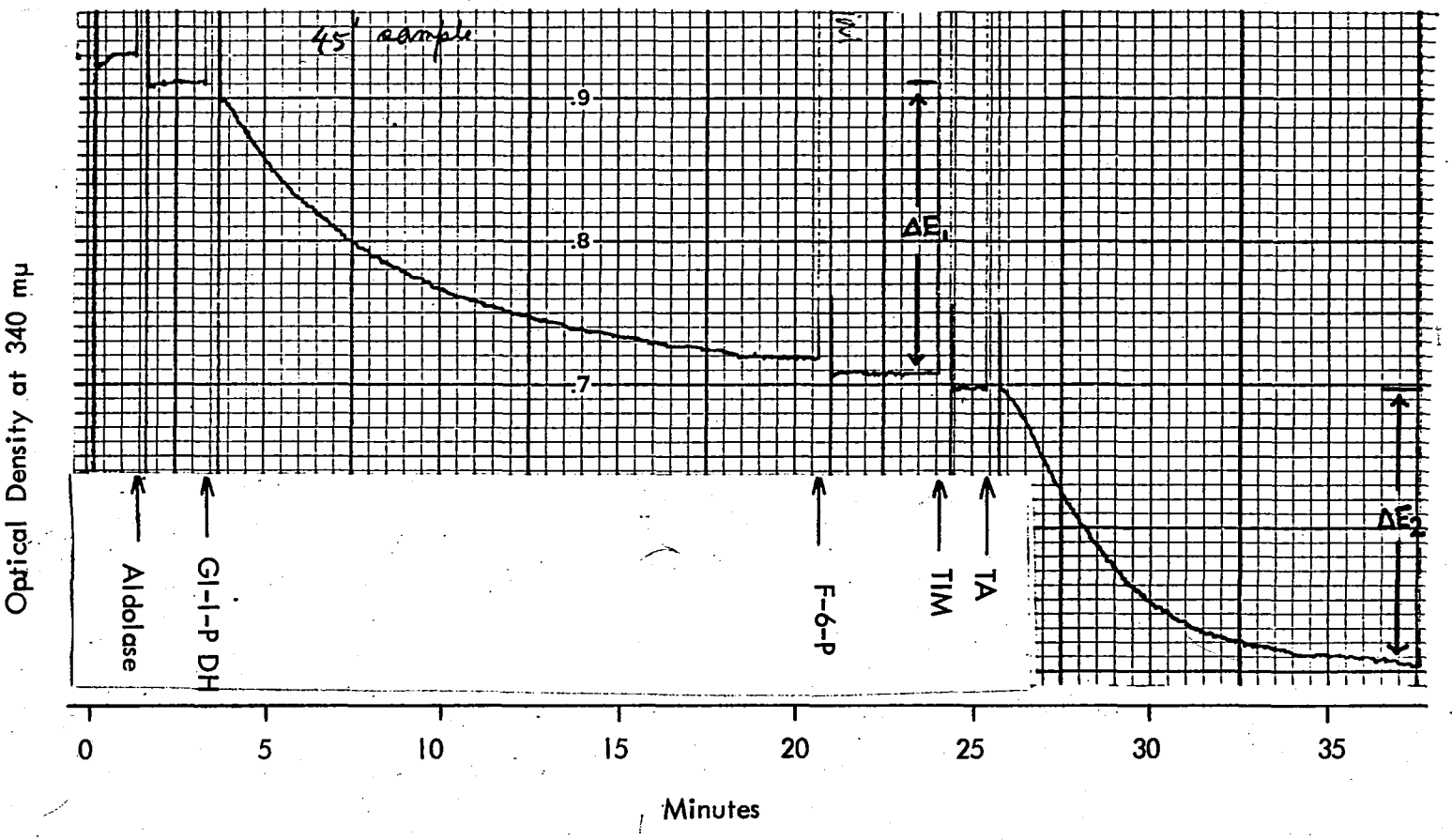


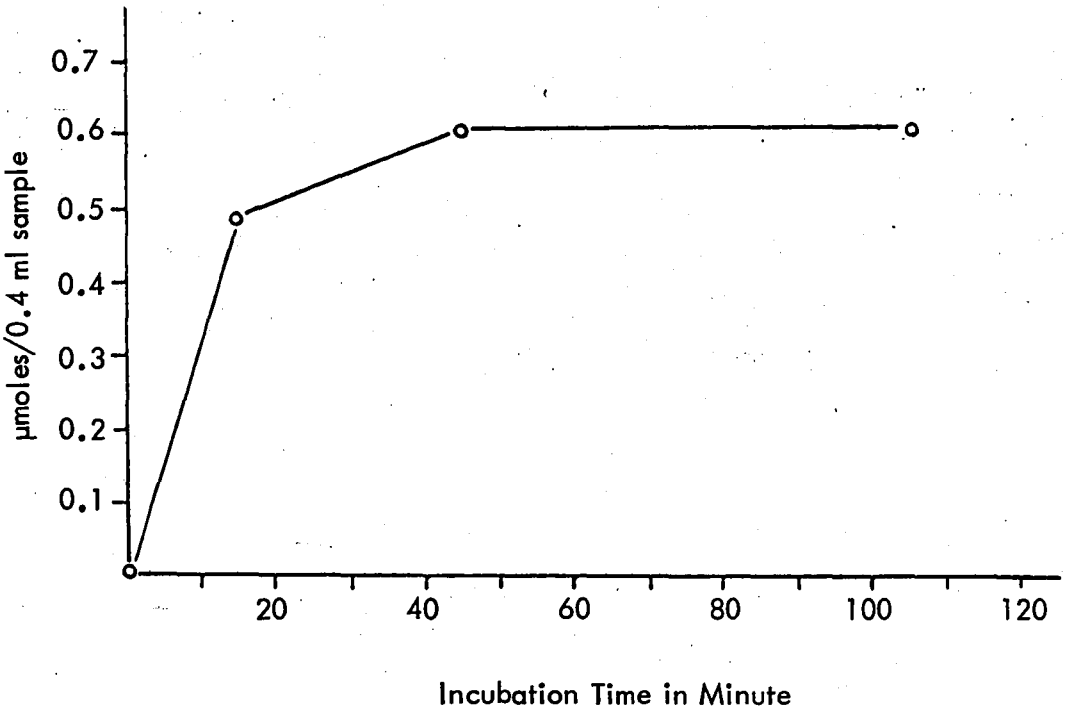
Fig. 21. Enzymatic assay of sedoheptulose-1,7-diphosphate using aldolase.

ΔE_1 represents dihydroxyacetone phosphate content, ΔE_2 represents erythrose-4-phosphate content.

Table XIII. Enzymatic assay of sedoheptulose-1,7-diphosphate.

	µmoles/0.4 ml sample			
	0'	15'	45'	105'
Dihydroxyacetone phosphate	0.00	0.048	0.060	0.060
Erythrose-4-phosphate	0.00	0.048	0.063	0.064

Fig. 22. Time course of sedoheptulose-1,7-diphosphate formation.



acetone phosphate in the assay mixture. After this reaction was completed, 2.0 I.U. of triose phosphate isomerase, 7 μmoles of fructose-6-phosphate and finally, 0.5 I.U. of transaldolase were added. The decrease in absorbance at this stage, ΔE_2, represented the content of erythrose-4-phosphate.

The stoichiometric correspondence of dihydroxyacetone phosphate and erythrose-4-phosphate assayed (Fig. 21) by this method confirmed the presence of sedoheptulose-1,7-diphosphate in the sample. Table XIII shows the same stoichiometry for dihydroxyacetone phosphate and erythrose-4-phosphate in the other samples withdrawn at different time intervals. The time course of sedoheptulose-1,7-diphosphate formation (Fig. 22) shows that, under these conditions, the phosphorylation of sedoheptulose-7-phosphate was 80% complete at 15 minutes, and at 50 minutes 98% of the sedoheptulose-7-phosphate in the reaction mixture had been converted to the diphosphate.

A new enzymatic method for the assay of sedoheptulose-7-phosphate*

The basis for this assay was essentially a combination of the phosphorylation of sedoheptulose-7-phsophate and the enzymatic assay of the resultant sedoheptulose-1,7-diphosphate. As the erythrose-4-phosphate and dihydroxyacetone phosphate was formed stoichiometrically, the assay of dihydroxyacetone phosphate alone was sufficient to establish the amount of sedoheptulose-1,7-diphosphate and hence of sedoheptulose-7-phosphate.

* T.Wood and W.M.Poon., Arch. Biochem. Biophys., accepted for publication.

$$\text{S-7-P} + \text{ATP} \xrightarrow{\text{PFK}} \text{SDP} + \text{ADP}$$

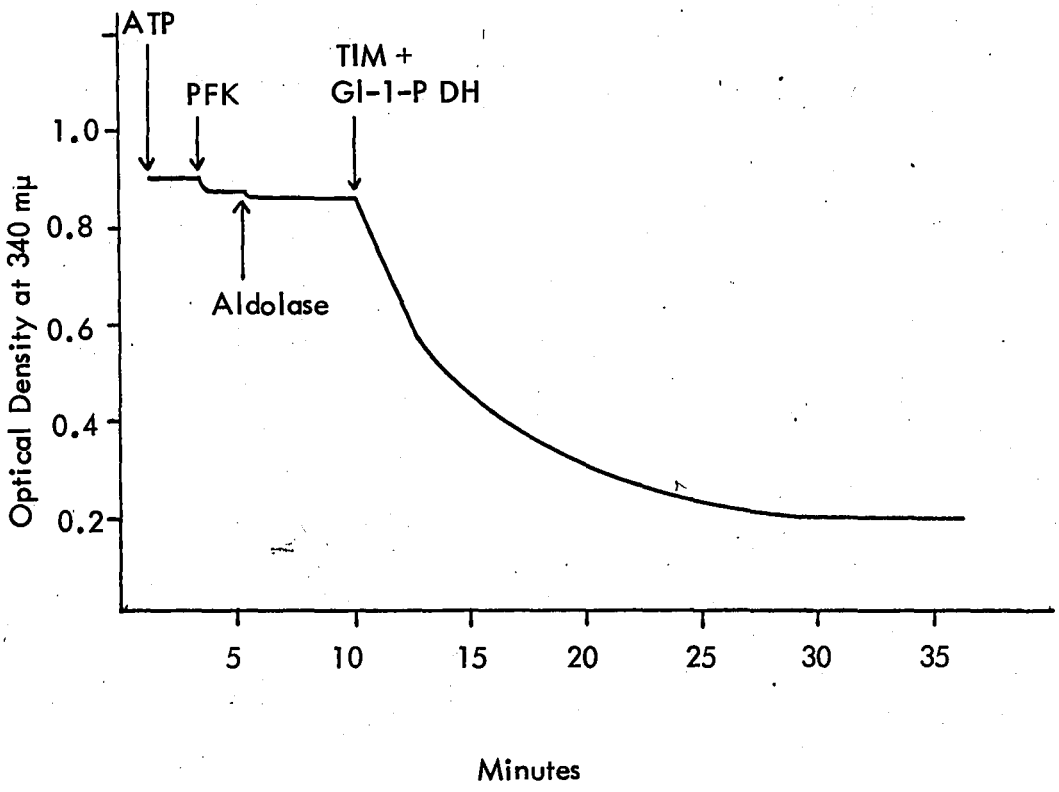
$$\text{SDP} \xleftrightarrow{\text{aldolase}} \text{Er-4-P} + \text{DHAP}$$

$$\text{NADH} + \text{H}^+ + \text{DHAP} \xrightarrow{\text{Gl-1-P DH}} \text{Gl-1-P} + \text{NAD}^+$$

Two ml of assay mixture contained (final concentrations): 100 mM triethanolamine buffer, pH 7.5, 10 mM EDTA, 12 mM magnesium chloride, 20 mM potassium chloride, 0.25 mM ATP, 0.13 mM NADH, sample containing sedoheptulose-7-phosphate (0.02 to 0.2 µmoles), 4.5 I.U. phosphofructokinase, 1.2 I.U. glycerol-1-phosphate dehydrogenase. The mixture was allowed to react for 2 to 4 minutes and then 0.9 I.U. of aldolase was added, the decrease in absorbance at 340 mµ represents the sedoheptulose-7-phosphate content in the assay mixture.

Fig. 23 is a tracing of the enzymic assay in which 0.2 µmoles of sedoheptulose-7-phosphate were added to the assay mixture. It can be seen that the reaction was rapid and was completed within 20 minutes. The initial and final baselines were horizontal. The total change in absorbance at 340 mµ was calculated to be equal to the amount of sedoheptulose-7-phosphate initially added to the assay mixture.

Fig. 23. The enzymatic assay of sedoheptulose-7-phosphate using aldolase.



DISCUSSION

The slow rate of conversion of glucose to lactic acid in both the homogenate and extract incubation mixtures has been shown to be due to the limiting effect of hexokinase. One possible explanation is the high sensitivity of hexokinase to inhibition by glucose-6-phosphate (60,212,213). However, the glucose-6-phosphate concentration in the incubation mixtures was very low judging from the absence of a glucose-6-phosphate spot on the chromatogram (Fig.9). In the incubation mixtures, the ATP and glucose concentrations were saturating and the endogenous concentration of inorganic phosphate was 3 to 4 mM; both ATP (214) and Pi (215) have been reported to be able to overcome the inhibition of hexokinase by glucose-6-phosphate. It seems that other factor(s) are contributing to the rate limiting effect at the hexokinase step. Hexokinase in muscle appears to be the lowest in activity in comparison with the other glycolytic enzymes (Table XIV). Values as low as 0.9 μmoles/minute/gram muscle have been reported in rat skeletal muscle (195). If such a low level of hexokinase occurred in our homogenate systems, it would be quite sufficient to account for the low lactic acid formation with glucose as substrate.

In most tissues, hexokinase is found both in the soluble and the particulate fractions, eg. tumor cells (216), rat brain (217) and adipose tissue (218) as well as the microsomal fraction of skeletal muscle (219,220). In pig heart homogenate, only 10 to 12% of the hexokinase activity was found in the supernatant (221)

It follows that hexokinase in the rat muscle extract would be even lower than in the homogenate and thus the rate limiting effect would be similar.

When glucose-6-phosphate or glycogen was used as substrate, the hexokinase limiting effect was bypassed. The homogenate was able to glycolyse glucose-6-phosphate and glycogen at a high rate whereas the extract glycolysed these substances at 1/4th to 1/5th the rate of the homogenate (Table II). Under this condition, the rate limiting step in the extract system was identified to be at phosphofructokinase because of the accumulation of glucose-6-phosphate and fructose-6-phosphate which was observed on paper chromatograms (Fig. 9). In the homogenate phosphofructokinase was not rate limiting, and the accumulation of fructose diphosphate and triose phosphates indicated the apparent rate limiting reaction to be at the glyceraldehyde phosphate dehydrogenase step.

The possibility that phosphofructokinase was inhibited in the extract was first considered in view of the well established regulatory effect of ATP, ADP, AMP, Pi and magnesium (68,69). The concentration of ADP, AMP and Pi present endogenously will be the same in the homogenate and extract since they were derived from the same preparation. ATP and magnesium were added to both the homogenate and extract incubation mixtures in the same concentrations. For these reasons, it is unlikely that these compounds would exert different regulatory effects on the phosphofructokinase in the homogenate and extract. In view of the discovery that mammalian heart phosphofructokinase

(222,223) and rat muscle phosphofructokinase (224) existed in both a soluble and insoluble sedimentable form, the possibility of this phenomenon occurring in our homogenate and extract system was investigated. It was found that the phosphofructokinase in the rat muscle homogenate consisted of a soluble active form and a less active insoluble form depending on the pH of the homogenate (Table III). In a slightly acidic homogenate (pH 6.6) the enzyme was less active and a considerable portion of the enzyme was in the insoluble form. However, in a slightly alkaline homogenate (pH 8.2), the enzyme activity was 4 to 8 times higher and all of the enzyme was in the soluble form.

Since the homogenate and extract used in the first incubation experiment were prepared in a slightly acidic medium only a small portion of the enzyme, representing the soluble form, was present in the extract. This could explain the poor phosphofructokinase activity in the extract and hence its rate limiting effect. This result explains the finding of Margreth and coworkers (225) that frog muscle extract cannot glycolyse glucose-6-phosphate without the addition of a sarcotubular fraction (which according to these results would contain a high percentage of the total phosphofructokinase). The addition of this fraction enabled the extract to utilize glucose-6-phosphate with the production of lactic acid.

According to the results in Table III, if a muscle homogenate was prepared with a slightly alkaline medium, not only would the phosphofructokinase activity be higher than in a slightly acidic preparation but it would also have

a higher percentage of the enzyme in the soluble form which would appear in the extract. The result of the incubation experiment with such an extract revealed that phosphofructokinase was no longer rate limiting and the lactic acid production was much higher than with a slightly acidic muscle extract. Judging from the accumulation of fructose diphosphate and triose phosphate in the mixture (Fig. 11), the rate limiting step in the alkaline muscle extract, as in the homogenate, was apparently the glyceraldehyde phosphate dehydrogenase step.

The purified rabbit muscle phosphofructokinase has been shown to be easily inactivated by dilution at pH 6.7 (226). The reversibility of the active-inactive transition of mammalian heart phosphofructokinase was first reported by Mansour (222) who was able to reactivate the inactive sedimentable form of the enzyme by incubating at pH 8.0 with ATP and magnesium. Similar reactivation of insoluble phosphofructokinase of rat muscle has been reported (224). Whether this reversible interconversion of soluble active and insoluble less active forms occurs in vivo and participates in the regulation of muscle glycolysis is not clear. Mansour (223) observed that the activation and solubilization of the enzyme occurred only in contractile tissues such as heart, diaphragm and skeletal muscle but not in brain, kidney or liver, and suggested that this phenomenon may be peculiar to the structure and metabolic activity of these contractile cells. In an attempt to investigate this possibility it was found that, under our experimental conditions, the physiological state

(stimulated or nonstimulated) of the animal just before death did not appear to have any effect on the level and distribution of the soluble and insoluble enzymes (Table III). Probably, if such a reversible conversion did occur, it would be a transient phenomenon and might last only as long as the stimulation was maintained. In order to detect this change, an elaborate procedure involving induced muscle contraction followed immediately by quick freezing and then deproteinization at low temperature would be required. Our method which involved dissection of the muscle, homogenization and filtration at 4°C over a period of 1-1/2 hours would not have detected such change.

Another effect of pH on phosphofructokinase was pointed out by Trivedi and Danforth (227). They showed that frog and mouse muscle phosphofructokinase were extremely sensitive to changes in pH within the physiological range; a high pH increases the affinity of the enzyme for fructose-6-phosphate. They suggested that the transient alkaline pH which appeared during muscular contraction due to the hydrolysis of creatine phosphate (228) might promote glycolysis by increasing the affinity of phosphofructokinase for fructose-6-phosphate. The increased affinity for fructose-6-phosphate at a higher pH was confirmed by binding studies (229). Working with rat diaphragm, Ui (230) noticed the stimulatory effect of slightly alkaline medium (pH 7.6) on glycolysis. He suggested that this was due to a decrease of ATP inhibition at slightly alkaline pH.

In summary, an alkaline pH within the physiological range could exert its stimulatory effect on muscle phosphofructokinase in three ways: (1) increasing

the amount of active, soluble enzyme, (2) increasing the affinity of the enzyme for fructose-6-phosphate and (3) decreasing the inhibitory effect of ATP on the enzyme.

The pH stimulatory effects, working in conjunction with the positive regulatory effects of ADP, AMP and Pi, which increase during muscle contraction, might result in an increase in phosphofructokinase activity high enough to support the marked increase in glycolytic flux during muscle contraction (an eight hundred fold increase in glycolytic flux during tetanus has been reported in frog sartorius (72)).

In addition to the low activity of hexokinase in a homogenate and extract and of phosphofructokinase in a slightly acidic extract, the accumulation of triose phosphate and fructose diphosphate in the homogenate and the alkaline muscle extract indicated that glyceraldehyde phosphate dehydrogenase was also rate limiting. Investigations by Garcia-Arocha (56) of glycolysis in rat muscle homogenates showed that the availability of NAD, Pi and the enzyme itself were not responsible for the limiting effect and she postulated that the cause was the marked inhibitory effect of 1,3-diphosphoglyceric acid on glyceraldehyde phosphate dehydrogenase (231). Furfine and Velick (232) in considering the role of glyceraldehyde phosphate dehydrogenase in muscle glycolysis, suggested that any restriction in the activity of the 3-phospho-glycerate kinase, such as might occur during limitation of ADP or magnesium, would lead to a nearly total inhibition of glyceraldehyde phosphate dehydrogenase by its own product.

Further evidence for a limitation by ADP in our system was provided by the observation that in the homogenate, which has a fast rate of lactic acid production, phosphoenolpyruvate was accumulating (Table II) despite the equilibrium constant of muscle pyruvate kinase being highly in favor of pyruvate and ATP formation (K_{app} = 6000) (209). In the presence of ADP all the phosphoenolpyruvate should have been converted to pyruvate. Pyruvate kinase has been reported to be inhibited by ATP (233, 234) but most of the inhibitory effect was found to be due to the chelating action of ATP on magnesium which is required for enzymic activity (235), and when the magnesium level was higher than ATP the inhibitory effect was relieved. In our incubation system, the magnesium/ATP ratio was higher than 1, which means that ATP would not inhibit the pyruvate kinase. The accumulation of phosphoenolpyruvate was therefore probably due to a limitation of the ADP supply. As pointed out by Racker (236) the production and utilization of ADP in the glycolytic pathway is not stoichiometrically balanced. In our incubation system where hexose monophosphates were added as substrate, for each mole of hexose monophosphate converted to lactic acid one mole of ADP was formed in the phosphofructokinase step while 4 moles of ADP were required for the phosphoglycerate kinase and pyruvate kinase reactions. In order to supply the necessary amount of ADP to sustain glycolysis, an "ATPase" reaction which generates ADP from ATP would be necessary.

The optimal activity of the glycolytic enzymes of the rat skeletal muscle

has been assayed in the homogenate (195) and in the cytoplasmic or soluble fraction (237), Table XIV lists these values for comparison. It is clear that with the exception of hexokinase and phosphofructokinase, which exhibit marked soluble-insoluble properties, each enzyme in the glycolytic pathway has a similar activity in the homogenate and in the soluble fraction. In addition, as shown in Table IV, the ATPase activity found in the extract was less than 10% of that in the homogenate. Thus, it is possible to construct glycolytic systems which have approximately equal amounts of each glycolytic enzyme but different amounts of ATPase depending on the proportion of homogenate and extract in the system. Fructose-6-phosphate was added to such systems so that the hexokinase limiting effect was bypassed. Slightly alkaline muscle homogenate and extract were used so that phosphofructokinase was no longer rate limiting.

The result of this experiment (Table V, Fig. 12) showed clearly the dependence of the glycolytic rate on ATPase activity. The close correspondence between the concentration of ADP and the lactic acid production indicated the importance of ADP for glycolysis. An increased availability of ADP would provide substrate for the phosphoglycerate kinase and pyruvate kinase reactions and would result in greater lactic acid formation. More important, the increased rate at the phosphoglycerate kinase step would lead to a lower 1,3-diphosphoglyceric acid level, which in turn would relieve the inhibition of glyceraldehyde phosphate dehydrogenase and effectively eliminate the rate limiting step with a resultant increase in glycolytic flux.

Table XIV. Comparison of optimal activities of glycolytic enzymes reported in rat skeletal muscle homogenate* and soluble fraction.**

Enzyme	Enzyme Activity (μmoles/5 min/0.4 g muscle)	
	Homogenate	Soluble fraction
Phosphorylase	-	360 ± 114.4
Phosphoglucose mutase	60 ± 6.6	-
Hexokinase	1.8 ± 0.52	39.8 ± 6.6
Phosphoglucose isomerase	352	62 ± 30.8
Phosphofructokinase	64 ± 10	3.4 ± 1.4
Aldolase	102 ± 11.8	82 ± 9.8
Triosephosphate isomerase	5300 ± 3200	-
Glyceraldehyde phosphate dehydrogenase	588 ± 72	694 ± 254
Phosphoglycerate kinase	338 ± 19.6	244 ± 17.4
Phosphoglycerate mutase	212 ± 36	156.6 ± 6.4
Pyruvate kinase	774 ± 206	432.8 ± 87.8
Lactate dehydrogenase	486 ± 84	768 ± 216

* Reported by Shonk and Boxer (195); muscle homogenates prepared with buffer of the following compositions: 0.15 M KCl, 0.05 M $KHCO_3$, 0.006 M EDTA.

** Reported by Fellenberg et al. (237); cytoplasmic fractions obtained as the 8000 g supernatant of the homogenate prepared from 50 mM Triethanolamine-5 mM EDTA buffer, pH 7.5.

Above an optimal ATPase activity of 75.2 μmoles/5 minutes/0.4 gram muscle, the lactic acid production did not show any further increase with increasing ATPase activity. Since lactic acid production did not increase further, the ATPase was probably inhibited above this level of activity. The exact cause of this inhibition is not known.

Our results are similar to the findings of Racker (238) who reported that in a reconstructed system with a limited supply of both Pi and ADP, the addition of ATPase within a narrow activity range stimulated glycolysis. In his experiment, exogenous ATPase (apyrase) was used; in our system we have used the endogenous ATPase.

At least four types of ATPase are present in muscle: the calcium stimulated myosin ATPase, the magnesium-ATPase present in the mitochondria, the calcium inhibited sarcotubular ATPase (239) and the sodium-potassium activated ATPase associated with the cell membrane and sarcotubular fraction (240). All four types of ATPase can utilize ATP as the sole substrate and could participate in the turnover of ADP for the glycolytic process. The myosin ATPase and the sarcotubular ATPase are conceivably more important in regulating glycolysis during muscular work due to their intimate participation in the contraction-relaxation process.

It is well known that in muscle the pentose phosphate pathway activity is much lower than the activity of the Embden-Meyerhof pathway (172,173). Thus in our investigations of the non-oxidative branch of the pentose phosphate

pathway, most of the intermediates were so low in concentration that their determinations were rather difficult. The concentration of sedoheptulose-7-phosphate, when present in the incubation mixtures, was usually high enough to be assayed quite accurately by the enzymic method. The same was not true for the colorimetric method, especially in the presence of large quantities of hexose monophosphate. Paper chromatographic analysis proved to be an important tool in that it could detect low concentrations of sedoheptulose-7-phosphate and provide an estimate of the relative amount in each sample. Whenever paper chromatography failed to detect the presence of sedoheptulose-7-phosphate, it was also not detected in measurable amount by either the enzymatic or the colorimetric methods. The enzymic method for erythrose-4-phosphate, using transaldolase partially purified from Candida utilis, was tested and found to detect as little as 0.006 μmoles in 2 ml of the assay mixture. The stability of erythrose-4-phosphate towards perchloric acid treatment during deproteinization was also established. Furthermore, paper chromatographic analysis consistently failed to detect the presence of erythrose-4-phosphate in any of the incubation mixtures. For these reasons, it is believed that erythrose-4-phosphate was either absent or present at concentrations below the limits of measurement (0.03 μmoles/6.0 ml deproteinized incubation mixture).

Using a DEAE-Sephadex A-25 column and NaCl gradient elution, we were able to separate ribose-5-phosphate from the ketopentose phosphates (Figs. 13,14). The elution of ribose-5-phosphate at a lower ionic strength could

be explained by the acid weakening effect of the ring oxygen adjacent to the C-5 phosphate group in ribose-5-phosphate as compared to the acid strengthening effect of the C-4 hydroxyl group on the C-5 phosphate group of xylulose-5-phosphate and ribulose-5-phosphate (241). The identical ionic properties of ribulose-5-phosphate and xylulose-5-phosphate explained why they were not separated in this column. Our result agrees with that of Horecker and coworkers (105) who reported an elution order of sedoheptulose-7-phosphate, ribose-5-phosphate and ribulose-5-phosphate, using a Dowex-1 formate column and formate elution. Complete separation of free ribose, ribulose and xylulose, derived from their respective phosphates by dephosphorylation, has been achieved using a borate column (102). We did not attempt to separate the phosphates using the same technique because of the instability of the ketopentose phosphates at the alkaline pH of the elution medium (191).

The ability of rat muscle extracts to metabolize ribose-5-phosphate was evident from the results of our incubation experiments. The rapid conversion of ribose-5-phosphate to ribulose-5-phosphate and xylulose-5-phosphate was in agreement with results obtained with rabbit muscle extract (102), rat muscle extract (175) and rat and mouse muscle extract (174). However, in these investigations ribulose-5-phosphate and xylulose-5-phosphate were determined together colorimetrically as total ketopentose phosphates.

The phosphoribose isomerase activity, as measured from the initial rate of ribose-5-phosphate disappearance over the first 5 minutes (short interval

incubation, Fig. 19) was 2.75 μmoles/minute/gram muscle at 8 mM ribose-5-phosphate, 37°C and pH 7.5. This rate was comparable with a rate of 0.7 μmoles/minute/gram muscle (2 mM ribose-5-phosphate, 37°C and pH 7.4) measured by Tan and Wood (177), and 1.4 μmoles/minute/gram muscle (2 mM ribose-5-phosphate, 30°C, pH 7.6) obtained by Glock and McLean (174), both in rat muscle extract. By measuring both ribulose-5-phosphate and xylulose-5-phosphate, it was shown that the initial rate of xylulose-5-phosphate formation, as measured from the short interval incubation experiment (Fig. 19), was 1.45 μmoles/minute/gram muscle, indicating an active phosphoketopentose epimerase in the muscle extract. This initial rate, however, does not reflect the optimal epimerase activity because the initial ribulose-5-phosphate concentration was not saturating. An epimerase activity of 0.7 μmoles/minute/gram muscle was obtained by Tan and Wood (177) in rat muscle extract at 2 mM ribulose-5-phosphate, 37°C and pH 7.4.

Tabachnick and coworkers (103) showed that for purified yeast epimerase and isomerase acting on ribose-5-phosphate the equilibrium mixture corresponded to R-5-P/Ru-5-P/Xu-5-P : 3/1/3 and for the yeast isomerase and rabbit muscle epimerase, the equilibrium ratios were also 3/1/3. In all our ribose-5-phosphate incubations, the reaction catalysed by these enzymes had almost ceased after 30 minutes. The ratios of R-5-P/Ru-5-P/Xu-5-P at the end of the incubation are shown in Table XI. Except for the value in the first incubation experiment, the value approached the equilibrium ratios of 3/1/3. It should be noted that true equilibrium could never be reached as long as the ribose-5-phosphate and

xylulose-5-phosphate were reacting via transketolase to form sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate.

Although the conversion of ribose-5-phosphate to ribulose-5-phosphate and xylulose-5-phosphate was rapid, the subsequent conversion beyond pentose phosphate through the transketolase and transaldolase reactions was very slow, judging from the very slow rate of disappearance of total pentose phosphate over 60 minutes of incubation. In the second incubation (Table VIII, Figs. 16a,b), where the disappearance of total pentose phosphate was accompanied by a small but measurable increase in sedoheptulose-7-phosphate, the amount of total pentose phosphate lost over 60 minutes was 2.4 μmoles or 9% of the total pentose phosphate. This is in agreement with the 10% loss of pentose phosphate reported by Dickens and Williamson (102) in rabbit muscle extract over 120 minutes incubation. They also estimated the formation of hexose monophosphate, heptulose phosphate and triose phosphate in the same extract to be 2%, 5% and less than 2%, respectively, of the pentose phosphate. In our incubation with rat muscle extract, the triose phosphate and hexose monophosphate were no more than 0.02 to 0.08 μmoles while the highest sedoheptulose-7-phosphate production, recorded in the dialysed extract of Experiment-3 (Table IX) was 0.16 μmoles or about 1.3 % of the total pentose phosphate. Sable (175) has also reported a very slow conversion of ribose-5-phosphate beyond the ketopentose phosphate. On the other hand, Glock and McLean (174) have reported active resynthesis of hexose monophosphate from

ribose-5-phosphate in rat and mouse muscle extract. Dickens and Williamson (102) suggested that these differences were due to the different strains or species of animal used.

The optimal activity of transketolase has been reported to be 0.27 μmoles/minute/gram muscle (177). Since the ribose-5-phosphate concentration was saturating and the xylulose-5-phosphate concentration was also saturating during most of the incubation, the maximum amount of sedoheptulose-7-phosphate that could be formed in the incubation mixtures, which contained an equivalent of 0.4 gram muscle, would be 6.48 μmoles in 60 minutes. The fact that the sedoheptulose-7-phosphate level in all the incubation mixtures were never higher than 0.16 μmoles, together with the extremely low level of other non-pentose phosphate intermediates suggested that the transketolase in the muscle extract was inhibited. A regulatory role for transketolase has been suggested in rat liver (168), rat adipose tissue (169) and ascites tumor cell (170). However, the regulatory mechanism itself is far from understood. Transketolase has been reported to be inhibited by inorganic phosphate (187,188). However the dialysed extract (Table IX, Fig. 18b) did not show any significant increase in the level or the rate of formation of sedoheptulose-7-phosphate over the nondialysed extract (Fig. 17b), indicating that inorganic phosphate or any other dialysable materials present in the extract were not responsible for the inhibition.

Dische and coworkers (242) in their studies of ribose-5-phosphate incubation with human red blood cell hemolysates, suggested that the slow rate of formation

of hexose monophosphate was due to inhibition of transaldolase by high concentrations of pentose phosphate. Our data on rat muscle extract does not permit interpretation of the transaldolase activity. However, it seems that the primary reason for the absence of hexose monophosphate and erythrose-4-phosphate was the low concentration of sedoheptulose-7-phosphate and the almost total absence of triose phosphate which made further reaction through the transaldolase step extremely slow. The absence of triose phosphate was unexpected since the transketolase reaction produced sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate in equimolar amounts. One possible explanation is that the triose phosphates have combined through the muscle aldolase reaction to form fructose-1,6-diphosphate. The equilibrium constant of the aldolase reaction favours the direction of synthesis ($K_{eq} = 10^{-4}$). Assays for fructose-1,6-diphosphate should therefore be done in future experiments.

Since glyceraldehyde-3-phosphate is an intermediate of both the glycolytic and the pentose phosphate pathways, this compound could play an important role. Regulation of one pathway over the other could involve competition for this intermediate. With active glycolysis, the triose phosphate is oxidized through the glyceraldehyde phosphate dehydrogenase step and the nonoxidative reactions of the pentose phosphate pathway would be limited due to the low levels of triose phosphate. When glyceraldehyde phosphate dehydrogenase was partially inhibited, which was the case in our homogenate and extract glycolytic system, triose phosphate would accumulate. Provided

that fructose-6-phosphate was also present, the enzymes of the nonoxidative pentose phosphate pathway would be expected to convert these phosphates into erythrose-4-phosphate, sedoheptulose-7-phosphate and the pentose phosphates. The fructose-6-phosphate and fructose-1,6-diphosphate incubation experiment (Table XII, Fig. 20a,b) simulated such a condition. Interpretation of the results of this experiment was complicated by the unexpected finding that the triose phosphate level changed markedly with time of incubation. This temporal change in triose phosphate level was not related to the formation of sedoheptulose-7-phosphate both in the time course and in the magnitude of the changes. Hexose monophosphate, instead of decreasing, was actually found to be increasing. It can only be postulated that this change in hexose monophosphate and triose phosphate was not related to the nonoxidative pentose phosphate pathway reactions but was in some way involved in the aldolase and fructose diphosphatase (224, 243) reactions. Although pentose phosphates and erythrose-4-phosphate were not detected in the incubation mixtures, the time course dependent formation of sedoheptulose-7-phosphate nevertheless indicated the presence of a weak nonoxidative pentose phosphate pathway activity operating from the direction of fructose-6-phosphate and triose phosphate.

We have investigated an enzymatic method which specifically assays sedoheptulose-1,7-diphosphate, and using this assay, we were able to show that in the presence of ATP, rabbit muscle phosphofructokinase catalysed the

formation of sedoheptulose-1,7-diphosphate from sedoheptulose-7-phosphate. Thus confirming the finding of Ling et al. (211). The formation of sedoheptulose-1,7-diphosphate in human red cells has been reported by Bucolo and Bartlett (244). Most investigators have emphasized the synthesis of sedoheptulose-1,7-diphosphate from erythrose-4-phosphate and dihydroxyacetone phosphate (119,120,121,124) and its conversion to sedoheptulose-7-phosphate by the action of sedoheptulose diphosphatase (119,122,123,124). The possibility of sedoheptulose-1,7-diphosphate being formed through phosphorylation by ATP should not be discounted, considering that in our in vitro incubation, at a physiological concentration of ATP (3.8 mM), 80% of the sedoheptulose-7-phosphate was phosphorylated in 15 minutes (Table XIII, Fig. 22).

If phosphofructokinase mediated phosphorylation of sedoheptulose-7-phosphate does occur in vivo, this would mean the participation of yet another glycolytic enzyme in the pentose phosphate pathway. More important would be the role of ATP which, until now, has not been implicated in this pathway. Further experiments on the pentose phosphate pathway, with the inclusion of ATP in the incubation mixtures, might provide a different picture from that observed in the absence of ATP.

The method for the assay of sedoheptulose-1,7-diphosphate has been further developed into a method for the assay of sedoheptulose-7-phosphate (Fig. 23). This assay is quicker and less complicated than the assay with transaldolase (Fig. 5) and has the advantage of using only commercially

available enzymes. It should be particularly useful for assaying sedoheptulose-7-phosphate in mixtures low in hexose phosphates.

SUMMARY

1. In both rat skeletal muscle homogenates and extracts, hexokinase was shown to be the first rate-limiting step in the glycolytic process.

2. In slightly acidic muscle extracts, the phosphofructokinase step was rate-limiting. This was related to the amount of soluble and insoluble forms of the enzyme. The relative amounts of each form depended on the pH. This rate-limiting effect was eliminated when the musle extracts were prepared with a slightly alkaline medium.

3. In addition to the steps at hexokinase and phosphofructokinase, a further rate-limiting process was found to be the ATPase activity of the system and the sypply of ADP.

4. Some metabolic intermediates (ribulose-5-phosphate, xylulose-5-phosphate, sedoheptulose-7-phosphate) of the pentose phosphate pathway was prepared. Transaldolase was partially purified from Candida utilis.

5. Various enzymatic and colorimetric methods for the assay of intermediates of the pentose phosphate pathway were studied.

6. The formation of intermediates of the pentose phosphate pathway when rat muscle extracts were incubated with ribose-5-phosphate or fructose-6-phosphate and fructose-1,6-diphosphate was studied. The step catalysed by transketolase appeared to be rate-limiting. Phosphoribose isomerase and phosphoketopentose epimerase in the extracts were quite active.

7. The ability of rabbit muscle phosphofructokinase to phosphorylate sedoheptulose-7-phosphate in the presence of ATP, with the formation of sedoheptulose-1,7-diphosphate, was confirmed.

8. An enzymatic method for the assay of sedoheptulose-7-phosphate was developed.

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