THE UNIVERSITY OF MANITOBA

PURIFICATION AND PROPERTIES OF PHOSPHOFRUCTO-KINASE FROM PLANT TISSUES; AND ITS POSSIBLE RELATIONSHIP TO THE PASTEUR EFFECT

A Thesis

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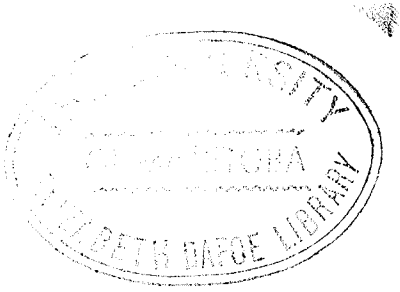

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ABSTRACT

The purification of phosphofructokinase (about 105-fold) from higher plant tissue and some properties of the enzyme are described. Plant tissue phosphofructokinase is activated by sulfhydryl agents, both during maceration and dialysis, and is protected by sodium chloride during fractionation. The enzyme is highly unstable to storage, however, storage in saturated ammonium sulphate solution maintains its activity for 5 days.

Besides adenosine triphosphate, cytosine, guanosine, and uridine triphosphates and adenosine diphosphate can donate phosphate to fructose-6-phosphate. Glucose-6-phosphate and ribulose-5-phosphate are phosphorylated, by the enzyme, but fructose-6-phosphate and magnesium-adenosine triphosphate complex are preferred co-substrates.

The most potent inhibitors of the enzyme from peameal and wheat embryos, are citrate and zinc ($ZnCl_2$); sodium pyrophosphate, imidazole and iron ($FeCl_3$) are activators.

Clear evidence is obtained as to the allosteric nature of the enzyme. Sigmoidal plots of velocity versus fructose-6-phosphate concentrations were obtained. Adenosine triphosphate and substrate for phosphofructokinase at low concentration is a negative effector at high concentrations. Adenosine monophosphate reverses the inhibitory effect of adenosine triphosphate thus acting as a positive effector for the enzyme.

The optimal pH for maximal phosphofructokinase activity is between 8.0 and 8.5. Short duration of heat activates while longer duration leads to rapid loss of enzyme activity.

Benzimidazole (50 mg/litre) appears to maintain the level of phosphofructokinase in wheat leaves.

A part of the studies carried out indicate that the crude extracts from wheat embryos possess a complete glycolytic system, and that the glycolytic activity of the extracts is inhibited by aerobic conditions.

Evidence is also presented that phosphofructokinase is a rate-limiting step in the Embden-Meyerhof-Parnas pathway, and that the control of glycolysis by the inhibition of this enzyme may be responsible for the Pasteur effect in plant tissues.

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ABBREVIATIONS

Abbreviations used throughout the text:

AMP, ADP, ATP - the 5-mono-, di- and triphosphates of adenosine; CoI - coenzyme I; CTP - cytosine triphosphate; DEAE cellulose - diethyl aminoethyl cellulose; NADH-dihydronicotinamide adeninenucleotide; DPN - diphosphopyridine nucleotide; EDTA - ethylene diaminotetracetic acid; F-6-P-fructose-6-phosphate, F-1,6-diP (or FDP)-Fructose-1, 6-diphosphate; Gala-6-P-galactose-6-phosphate; G-1-P, G-6-P-glucose-1- and -6-phosphates; GTP-guanosine triphosphate; HCl - hydrochloric acid; Pi-inorganic phosphate; $MgCl_2$-magnesium chloride; um - micromolar; umole-micromole; NAD - nicotinamide adenine dinucleotide; NADP - nicotinamide adeninine dinucleotide phosphate; PE-pasteur effect; 3-PGA-3-phosphoglyceraldehyde; KCl-potassium chloride; R-5-P-ribose-5-phosphate; Ru-5-P- ribulose-5-phosphate, NaCl - sodium chloride; NaOH - sodium hydroxide; Tris-HCl-Tris (hydroxymethyl) aminomethane hydrochloride; UTP - uridine triphosphate.

Gl(N) - carbon dioxide produced in nitrogen (anaerobic condition)

Gl(O) - carbon dioxide produced in air (aerobic condition).

INTRODUCTION

The purpose of this study was two-fold. Firstly to partially purify the enzyme phosphofructokinase (E.C. No. 2.7.1.11) from higher plant tissues and study the kinetics of the reaction it catalyzes. Secondly to ascertain the role that this enzyme plays in the aerobic inhibition of glycolysis, a phenomenon otherwise referred to as the "Pasteur Effect".

Phosphofructokinase catalyses the phosphorylation of Fructose-6-phosphate according to the following irreversible reaction:

fructose-6-phosphate + adenosine triphosphate ⟶
fructose-1,6-diphosphate + adenosine diphosphate

Amongst all the enzymes in the Embden-Meyerhof glycolytic pathway, the role played by phosphofructokinase may yet prove to be unique.

Investigations of Atkinson *et al* (1964,1965), Mansour and Mansour (1962), Mansour *et al* (1965), Mansour (1965), Bitensky *et al* (1965), Parmeggiani and Bowman (1963) among others have established the kinetics of phosphofructokinase in animal tissues and in microorganisms, while Wu (1964,1965), Passonneau and Lowry (1962) and Tarui *et al* (1965) have elucidated the possible role of this enzyme in the aerobic inhibition of glucose utilisation in animal tissues and

in microorganisms. In plant tissue, however, very little is known about the kinetics and properties of this enzyme.

Phosphorylated compounds essentially form the backbone of most metabolic pathways, and through the mechanism of action of kinases, phosphorylases, phosphatases, pyrophosphorylases and pyrophosphorylases binding of energy, its transference and release are made possible. Since phosphofructokinase plays a primary role in the regulation of the breakdown of carbohydrates, it becomes necessary that its properties be understood. It is therefore with this in mind, that the present studies were undertaken.

The enzyme was extracted from both viable wheat embryos (Triticum aestivum L., var. Selkirk) and pea cotyledons (Pisum sativum L., var. Tall Telephone or Alderman).

Parmeggiani and Bowman (1963), Atkinson et al (1964, 1965),and many others have established that animal tissue as well as phosphofructokinase from microorganisms show allosteric properties. In these studies, the possibility of higher plant tissue phosphofruktokinase being an allosteric enzyme has been investigated.

With the application of the properties of wheat phosphofructokinase as known, its possible relationship to the Pasteur effect has also been investigated. In

plants it has been generally regarded that oxygen affects directly or indirectly one of the enzymes involved in the conversion of fructose-1,6-diphosphate into 3-phosphoglyceraldehyde, Hatch and Turner (1959). However, the results of this study indicate that while 3-phosphoglyceraldehyde dehydrogenase may also be affected, the primary enzyme affected by oxygen in carbohydrate breakdown leading to the establishment of the Pasteur effect is phosphofructokinase.

LITERATURE REVIEW

A. ALLOSTERIC PROTEINS AND METABOLIC REGULATOR SYSTEMS.

Since phosphofructokinase has been considered to have allosteric properties, and it is the main enzyme studied and discussed in this manuscript, the following is a review of literature on the properties of allosteric proteins.

Much progress has been made in the study of regulation and control of cellular metabolism. It is now clearly established that in most organisms, even bacteria, there exists a complex circuit of regulation systems which govern the rate of flow of metabolites as well as the synthesis of protein and other macromolecules.

Umbarger (1956,1958) supplied the initial study on the operation of regulatory controls through specific interactions of synthetic products with an enzyme in its synthesis. He provided the experimental demonstration of such a control by showing that in extracts of disrupted cells, isoleucine strongly and specifically inhibits threonine dehydrase, the first enzyme in its synthetic pathway. He compared such a control to technological feedback control devices. Around the same period Yates and Pardee (1956) unveiled the same type of regulation in the biosynthesis of pyrimidines.

Many of these control systems have been analysed in

detail and it has been shown that the elementary interaction involves a protein with a specific biological activity, and generally a low-molecular weight metabolite. The metabolite either activates or inhibits the system.

These systems although very markedly different both chemically and in metabolic functions, have a number of kinetic features in common. In most cases, the kinetics may vary from 'normal' Michaelis behaviour (formerly considered as first order reaction in substrate interaction with enzyme) with a generally low value of substrate K_m, to a sigmoidal relationship between rate and substrate concentration (formerly considered as higher order reaction) with a much higher substrate concentration being required for half-maximal reaction velocity (Atkinson, 1964).

Monod et al (1963) formulated certain generalisations concerning the functional structures responsible for the regulatory competence of controlling proteins. They proposed a model schematizing the functional structure controlling protein, that,

> ...these proteins are assumed to possess two, or at least two, stereospecifically different, non-overlapping receptor sites. One of these, the active site, binds the substrate and it is responsible for the biological activity of the protein. The other, or allosteric site, is complementary to the structure of another metabolite, the allosteric effector, which it binds specifically and reversibly.

Monod et al further proposed that the enzyme-allosteric effector does not activate a reaction involving the

effector itself, but only brings about a reversible alteration of the molecular structure of the protein. Thus it modifies the properties of the active site, and changes one or more of its kinetic parameters.

Since the absence of any chemical obligatory analogy or reactivity between substrate and allosteric effector appears to be of extreme biological significance, it becomes necessary to establish a distinction between the actions of coenzymes, secondary substrates or substrate analogues before one can establish a clear and proper definition of allosteric effects. In general, it can be concluded that an allosteric effect is obtained if:

(a) A molecule (modifier) unrelated to the substrate is found to bind a particular protein,

(b) This molecule (modifier) after combining with the protein should alter the kinetic properties of the active centre for the substrate, and,

(c) For such a modifier, an enzyme-substrate-modifier complex can be demonstrated.

Monod et al (1963) proposed that the specificity of any allosteric effect and its actual manifestation is exclusively dependent on the specific construction of the protein molecule itself, that it is this construction that enables it to undergo "reversible conformational

alteration", triggered by the binding of an allosteric effector. The complexity of the mechanism of allosteric protein is further intensified by the fact that the action of certain coenzymes or other enzyme-effectors may involve allosteric effects in addition to their classical role as transient reactants or transporters.

Koshland, (1958,1960) proposed the "induced-fit" theory of enzyme action. His theory is based on the following postulate:

(a) a precise orientation of catalytic groups is required for enzyme action;

(b) the substrate may cause an appreciable change in the three-dimensional relationship of amino acids at the active site;

(c) the changes in protein structure caused by a substrate will bring the catalytic groups into proper orientation for reaction, whereas a non-substrate will not.

See also kinetic data (Koshland, 1963,1964).

Monod et al (1963) proposed three models of interaction between a substrate and an inhibitor binding respectively with different groups on enzyme surface, whereby they deduced that allosteric effects are caused by "conformational alterations". To support their conclusion, they reviewed kinetic data on beef liver glutamic

dehydrogenase, acetyl-CoA carboxylase from adipose tissue, muscle phosphorylase b and haemoglobin.

The actual molecular basis for modulation of enzymes' kinetic behaviour by interaction with a small molecule is however, still unknown. This has been a subject of considerable speculation, (Yates and Pardee, 1956; Umbarger, 1958; Koshland, 1958,1960; Gerhart and Pardee, 1962; Monod et al, 1962,1963; Frieden, 1964; Gerhart and Pardee, 1964; Atkinson et al, 1965; Monod et al, 1965).

It is sufficient to mention that the suggestions of most of these investigators, totally or in part advocate stereospecifically specific and functional significant interactions between proteins and small molecules.

Before the direct demonstration of end-product inhibition for isoleucine-valine and pyrimidine pathways by Umbarger (1958) and Yates (1956) respectively, the only type of feedback effects known was that due to repression of enzyme formation. Umbarger (1958) emphasized the unique role of the first and usually only one enzyme of a sequence in feedback inhibition, in contrast, to many or all of the enzymes of a sequence that are often repressed simultaneously - a situation termed "co-ordinate repression", (Umbarger, 1964).

The mechanism of enzyme regulation, through induction and repression of enzyme formation was established in the decade of 1950. Well defined single enzymes were used

to establish this phenomenon, and were shown to occur with many different enzymes in the same pathway. It was discovered, for example, that an enzymic block late in a sequence did not prevent an amino acid from repressing formation of earlier enzyme in the sequence. Furthermore, while intermediates in a sequence could also repress, they could no longer do so when a genetic block prevented them from being converted to the end-product. From these discoveries, it became clear that the end-product plays a special role in repression. Studies on intact cells, of genetics of repression has led to most of the recent developments of the concept of regulatory genes as opposed to genes determining protein structure. It is a product of this regulatory gene that combines with an appropriate small molecule to form the repressor.

End-product repression has been demonstrated (in microorganisms) for most of the well-known biosynthetic pathways. Notable among these pathways are: tryptophan biosynthesis, (Cohen and Jacob, 1959), histidine biosynthesis, (Ames and Hartman, 1962), arginine biosynthesis, (Maas et al, 1963), alkaline phosphate synthesis, (Gallant and Stapleton, 1963), β-galactosidase in E. coli (Loomis and Magasanik, 1963), and lactose utilisation, (Jacob and Monod, 1961). From these studies, a model has emerged, with the following features:

(a) A cytoplasmic repressor, whose formation is genetically determined, regulates the rate at which all the enzymes of a system are produced. In the case of biosynthetic pathways the repressor requires the end-product (for activity).

(b) The genes controlling the production of the enzymes of a pathway are adjacent to each other on the linkage map and their activities are controlled by a part of this genetic segment called the operator (Maas et al, 1963).

This system constitutes a unit under the control of a single cytoplasmic repressor, and a genetic unit; a region of the genetic material controlled by a single operator. This has been called an "Operon", (Jacob et al, 1960).

"Feedback inhibition implies that nature has found it economical to evolve certain enzymes with two kinds of specificity" says Davis (1963), in reference to a group of allosteric enzymes that are capable of binding with their respective substrates to produce biological activity, and at some other site bind with another small molecule (modifier), reversibly to produce or create regulatory metabolic systems. Feedback control of the kinetic behaviour of enzymes augments the repression system briefly discussed above. It has thrown much light on the action of antimetabolites and inhibition of growth by some com-

pounds. It has also provided some interesting problems for protein biochemists.

Of the regulatory effectors thus far studied in detail, adenylates appear to be of very significant importance in the control of cellular metabolism. Recent observations have shown that both the inhibition by ATP and stimulation by AMP play important roles in the regulation of energy metabolism.

Mansour (1963) discovered that both AMP and cyclic AMP stimulate guinea pig phosphofructokinase, which in the present day connotation means that both these mononucleotides act as positive effectors for the enzyme. Similar effects of AMP on phosphofructokinases have been reported by Passonneau and Lowry (1962) and Ramaih et al, (1964). Other systems in which AMP acts as positive or negative effector are cited in Table I. NAD was found to replace AMP as a positive effector for the DPN-specific isocitric dehydrogenase from animal cells.

ATP has also been shown to influence the kinetic properties of citrate synthetase (Hathaway and Atkinson 1965), and AMP aminohydrolase (Cunningham and Lowenstein 1965). The significance of these findings cannot be overestimated, especially in the regulation of phosphofructokinase, DPN-specific isocitrate dehydrogenase and citrate synthetase - all of which participate in the utilisation of carbohydrate which is directly connected with the generation

TABLE I. Regulatory Effectors

Enzyme Affected	Effector	Type of Effector	Reference
Phosphofructo-kinase	AMP ATP	Positive Negative	Mansour, 1963; Passonneau & Lowry 1963; Ramaiah et al 1964
DPN-specific isocitrate dehydrogenase	AMP Citrate	Positive Positive	Hathaway and Atkinson,1963; Chen and Plaut 1963; Bernofsky & Utter, 1964; Sanwal et al,1964; Sanwal & Stachow,1965
Citrate Synthetase	ATP	Negative	Hathaway & Atkinson,1965
Glutamine phosphoribosyl-pyrophosphate amido transferase	AMP ADP ATP GMP,GDP IMP	Negative	Caskey et al 1964
Glutamate dehydrogenase	ADP	Positive	Bitensky et al 1964
Purine Biosynthesis enzymes	Adenine & Guanine Compounds	Negative	May and Kock, 1964
Avian glutamine ribosylpyrophosphate 5-phosphate amido transferase	6-mercapto purine, 8-aza-guanine	Negative	McCollister et al, 1964

continued

TABLE I CONTINUED

Enzyme Affected	Effector	Type of Effector	Reference
L-glutamine-D-F-6-P transaminase	UDP-M-acetyl glucosamine	Negative	Konfeld et al 1964
Glutamate dehydrogenase	L-leucine	?	Bitensky et al 1964
Thymidine kinase	Thymidine ATP	Positive	Ives et al, 1963
Thymidine kinase	d-thymidine triphosphate	Negative	Ives et al, 1963

of energy in form of ATP by oxidative phosphorylation.

Atkinson (1965) has proposed a schematic illustration emphasising the role of adenylates in regulation of energy metabolism.

Other regulatory effectors, the systems affected and references are shown in Table I.

The biological significance of allosteric systems can be appreciated in the light of the fact that this class of interactions plays a special role in the control of living systems. There are other types of mechanisms such as mass action effects which augment cellular control mechanisms. Metabolic feedback regulations appear to work in the same direction as does mass action, although

it is a much more powerful system. Feedback controls and maintains metabolites at relatively constant concentrations, whereas mass action is dependent on thermodynamic equilibrium. The thermodynamic significance of specific control systems (Monod et al, 1963; Atkinson, 1965), is that they successfully circumvent thermodynamic equilibrium. This statement is illustrated by

(a) the synthesis of glycogen from glucose-1-phosphate and,

(b) the interconversion of F6P and FDP.

In case (a) Krebs and Fischer, (1962); Rall and Sutherland (1961); and Leloir (1961) have made it clear that the same pathway is not used by cells in the synthesis and degradation of glycogen, that each of the pathways is governed by specific controls, involving hormones and metabolites, none of which themselves participate directly in the reactions. Atkinson (1965), pointed out that in case (b) if there were no control on the enzymes that catalyse the interconversion, both reaction would occur at the same time and in the direction shown below:

$$ATP + H_2O \longrightarrow ADP + H_3PO_4$$

a condition that would lead to a short circuit in the cell's energy metabolism. "This fatal result is prevented by the control of both enzymes by ATP and AMP". Atkinson called this 'advantage' - "Irreversibility loops".

It would appear that mass action alone cannot satisfy the conditions necessary or required to control these systems, since the synthesis and degradation of glycogen are thermodynamically and physiologically reversible, and the following reactions (interconversion of F6P and FDP):

$$F6P + ATP \longrightarrow FDP + ADP$$

$$FDP + H_2O \longrightarrow F6P + H_3PO_4$$

are catalysed by two different enzymes that are oppositely directed.

The speculation that "genetic repression" may control protein synthesis - when proven - will be of great significance.

Although most of the regulatory systems discussed here have been easiest to study in single-celled organisms, there can be no question that they also exist in the cells of most higher organisms. Control mechanisms have demonstrated that there is a direct parallel between uni- and multicellular organisms. They have also provided that there exists some relevancy between them, differentiation and regeneration phenomena both of which are common to higher organisms. Feedback inhibition and repression involve negative feedback; they keep metabolic levels in a cell constant, while differentiation on the other hand brings about differences among cells that will be maintained in each cell and its progeny. It therefore could

be assumed that differentiation is governed not only by negative feedback but also by positive feedback. On the basis of all these considerations, the most important consequence of feedback control to biology is perhaps the fact that it makes "irreversibility loops" possible.

B. THE PASTEUR EFFECT

With the possibility that phosphofructokinase has allosteric properties, it could be a regulatory enzyme in controlling the Pasteur effect. The following is a review of literature on the Pasteur effect.

The repression of carbohydrate breakdown by living organisms in the presence of oxygen was first observed by Pasteur (1876). Pasteur found that alcoholic fermentation of yeast was severely reduced in the presence of gaseous oxygen. Pasteur also observed that in the presence of increased concentration of oxygen, fermentation was progressively replaced by the synthesis of cellular materials. Several investigators since then have demonstrated and established that carbohydrate utilisation in normally aerobic organisms, e.g. plants, animals and microorganisms, becomes significantly increased under anaerobic conditions. These investigations have equally confirmed that on transfer from anaerobiosis to aerobiosis, the rate of carbohydrate utilisation becomes significantly reduced.

Dixon (1937); Burk (1939); Turner (1951) and Fidler

(1948), observed that carbohydrate loss under anaerobic conditions far exceeded that under aerobic conditions. Fidler (1951) in his experiments with apples observed these same conditions.

Although the rate at which respiratory intermediates are utilized under aerobic conditions is much decreased, aerobic respiration is nevertheless considered a more efficient process. This efficiency is based on energy production by the two processes. There is more sugar breakdown and less energy release under anaerobic conditions while the reverse is true for aerobiosis. The conserving effect of oxygen on carbohydrate and respiratory intermediates leading to the prevention of inefficient drain of foodstuffs that would occur if fermentation were to operate independently and unlimited is called the "Pasteur Effect", after Pasteur who first observed the phenomenon.

The existence of the Pasteur Effect has long been established in yeast and in animal tissues. It has also been reported that this phenomenon occurs in the tissues of many higher plants - apple fruit, pea, rice, barley, parsnip roots, potato tubers are a few of the plant tissues in which the Pasteur Effect has been demonstrated.

Blackman (1928) amongst early workers on the elucidation of the Pasteur Effect, prepared a formal catalytical scheme

for apple respiration. "Glycolysis" said Blackman, "signified the conversion of a reactant C to another reactant D" where C is an activated hexose, and D a substrate for the last stages in sugar breakdown. It is this last stage according to Blackman that has alternative fates in air (oxygen) and nitrogen (anaerobiosis). Blackman called his reactant D, trioses.

Turner (1951) expanding on Blackman's views defined glycolysis as "the degradation of hexoses by a series of reactions involving an oxido-reduction with the production of a simpler substrate D, which has alternative fates in air and in nitrogen". Turner conjectured that his substrate D could possibly be pyruvate.

The literature is full of definitions proposed to describe the Pasteur effect, in terms of the rate of carbohydrate utilisation or formation of fermentation end-products. Dixon (1937) proposed a very convincing definition of this effect as "the action of oxygen in diminishing carbohydrate destruction, and in suppressing or decreasing the accumulation of the products of anaerobic respiration". Burk (1939) who did not subscribe to the Dixon school of thought proposed that "Pasteur effect is the suppression of fermentation by oxygen". Burk's arguments against Dixon's definition were:

(a) That there are few experimental determination of carbohydrate loss

(b) That in the aerobic cells, anaerobic processes with carbohydrate as substrates may often go on fast enough to mask the conserving effect of oxygen, and,

(c) That the diminution of carbohydrate destruction does not always accompany suppression of fermentation.

Burk's arguments have become inadequate on the basis of recent studies and reports. The literature now shows that several experiments have been conducted on carbohydrate loss and the significance of this and its relationship to the Pasteur Effect. Also it is now clear that most organisms do not carry out all of the processes of glycolysis at the same time that respiration is going on, so the second argument becomes untenable. Many experimental results have equally shown that there is more formation of fermentation products under anaerobiosis than under aerobiosis, thus liquidating Burk's third argument against Dixon's definition.

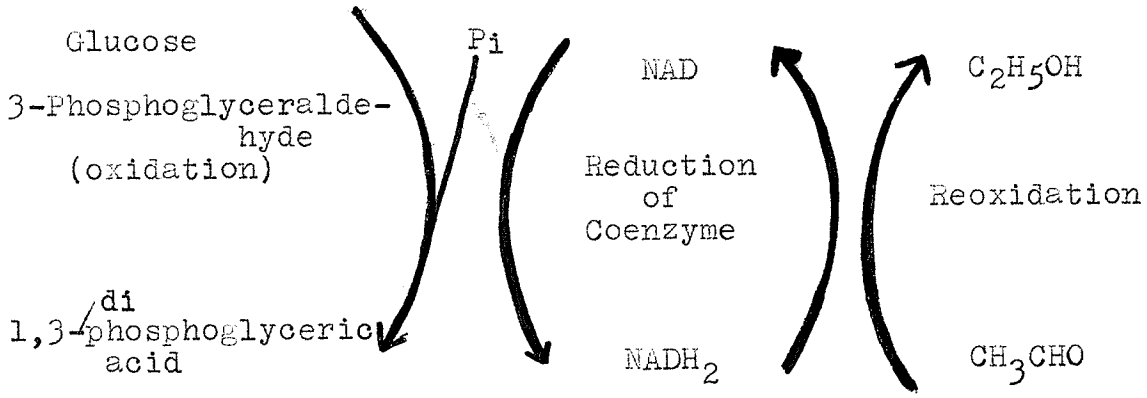
Meyerhof and Fiala (1950) defined the Pasteur effect in a very specific manner as "the strictly reversible and non-progressive inhibition produced in air or oxygen on the steady rate of glycolysis". Another definition proposed by Merry and Goddard (1941) is that "The inhibition of fermentation by oxygen is known as the Pasteur Effect".

Many theories have been put forward for explaining the Pasteur effect, a few of them are mentioned here.

The Pasteur effect is usually ascribed to either increased glycolysis in the absence of oxygen and of its accompanying respiration or to oxidative anabolism accompanying respiration. The above is based on two assumptions:

(1) (a) That Pasteur effect is due to "the oxidative removal of acetaldehyde", Dixon (1937), a substance necessary for the continuation of glycolysis. No substantiative explanation was offered for this assumption until Ball (1939) stated that "Suppression of fermentation is due to the oxidative removal of acetaldehyde, and the subsequent inability of COI (i.e. DPN or NAD) to be returned into the oxidized form after it has been reduced". Gottshalk,(1941), picked the argument up where Ball left it, and postulated that in the presence of oxygen, somehow part of the COI in yeast cell is put out of action because it persists in the reduced state. The application of Gottshalk's theories to higher plant cells yielded very little evidence in support of this theory. Gottshalk's scheme is known to work well if pyruvate were oxidized in air via the Tricarboxylic Acid Cycle. This process produces excess $NADH_2$ which has to be removed oxidatively. Such a mechanism has been shown to exist in barley by James (1946), in pea by Lockhart and Davidson (1939,1950) and in wheat by Waygood (1950). It has now been shown that NAD (DPN) can be

oxidized by the coupled oxidation-reduction system as shown in the following system:



3-Phosphoglyceraldehyde is oxidized to 1,3-diphosphoglyceric acid, and NAD is reduced to $NADH_2$. While $NADH_2$ is reoxidized to NAD, acetaldehyde is oxidized to ethanol. This system works very nicely in Nitrogen (anaerobic conditions). The link between this scheme and the Pasteur effect is that, when acetaldehyde is removed aerobically, reoxidation of $NADH_2$ stops. Under this condition the system is unable to proceed, since $NADH_2$ accumulates and the ratio $NADH_2$/NAD increases so much so that the rate of oxidation of 3-PGA is decreased. The decrease is eventually followed by a decrease in the rate of glycolysis and an increase in fermentation rate. Gottshalk (1941) demonstrated that no oxidative anabolism goes on in yeast cells, and argued that reoxidation of $NADH_2 \longrightarrow$ NAD by acetaldehyde is presumably the cause of the Pasteur Effect in yeast.

(b) Another way in which a change from aerobic to anaerobic conditions might increase the rate of glycolysis thus leading to the establishment of the Pasteur Effect, is ascribed to the phosphate cycle. Gottshalk postulated that the concentration of coenzyme I may limit the rate of glycolysis through the reaction:

3-phosphoglyceraldehyde + P_i + coenzyme I + ADP
$\longrightarrow$ phosphopyruvic acid + coenzyme IH_2 + ATP;

one of the steps in the glycolytic cycle. Johnson (1941) argued that the Pasteur Effect is due to a reduction of glycolysis in air, a condition brought about by the lowering of the concentration of phosphate acceptors, because of the rapid esterification of phosphate in respiration. Direct evidence for this hypothesis has not been reported for plant tissues.

(2) Blackman (1928) proposed the name "oxidative anabolism" to the anaerobic reaction conserving carbon in air. Earlier, Meyerhof (1925) had proposed that the Pasteur Effect was due to the existence of "oxidative resynthesis".

Among other hypotheses proposed to explain the Pasteur Effect are those postulating the inhibition of certain enzymes. Passonneau and Lowry (1961), Lowry and Passonneau (1964), in their experiments with yeast, ascites tumor cells, diaphragm and skeletal muscle proposed that the

enzyme reaction primarily responsible for the Pasteur Effect is phosphofructokinase (PFK). Their study was based on the measurement of substrate levels. They observed that when ATP, the negative effector of PFK, is lowered and P_i, a stimulator of PFK, is raised, the activity of the enzyme is increased 40-fold. The studies of Wu (1965), also based on measurement of substrates confirm that substances that stimulate PFK activity also stimulated glycolysis. Imidazole, high level of inorganic phosphate are two of the agencies reported to stimulate PFK and glycolysis (Wu, 1964). Wu (1965) also reported that inosine and some other compounds that have inhibitory effects on glycolysis do so, by affecting (inhibiting) PFK activity. Mansour and Mansour (1962) suggested the possible relationship between the increase in PFK activity that accompanies low levels of ATP and glycolysis. Salas et at (1965); Parmeggiani and Bowman, (1965), have established that citrate inhibition of PFK has direct relationship to glycolysis, in yeast and rabbit skeletal muscles, respectively.

Other explanations include the role of phosphate. This states that a higher concentration of phosphate is essential for glycolysis than for respiration. High levels of phosphate probably act by stimulating the activity of the kinases, notably hexokinase and PFK.

Barker et al (1964) explained the Pasteur Effect by postulating that mitochondrial ATP which though is readily available to cytoplasmic enzymic systems is not freely accessible to the sugar phosphorylating enzymes. They postulated that there is an initial acceleration of PEP kinase reaction in nitrogen, which results in a greater rate of production of glycolytic G6P and FDP, and this hastens glycolysis. They do not, however, indicate the mechanism by which the PEP kinase reaction is accelerated.

Of the many possible causes of the Pasteur Effect reported in the literature, the following are cited:

(a) Oxidative synthesis of carbohydrates from products of glycolysis (Meyerhof,1925; Blackman, 1951; Turner, 1958).

(b) Disturbance of the phosphate cycle under aerobic conditions (Gottshalk,1941), Johnson,(1941).

(c) Rate of generation of NAD from $NADH_2$ during the oxidation of the triose phosphates (Gottshalk,1941; Ball, 1939; Dixon, 1937; Turner,1951).

(d) High ratio of ATP:ADP in oxygen (Rowan et al, 1956; Rowan and Turner,1957; Wu and Racker, 1959b; Beevers, 1960).

(e) Availability of mitochondrial ATP to the phosphorylating enzymes (Lynen and Hartmann,1959;

Lynen,1963; Barker *et al*,1964).

(f) Inactivation of certain enzymes of the glycolytic cycle - PFK, 3-phosphoglyceraldehyde dehydrogenase and hexokinase - by oxygen or aerobic conditions, and high ATP level (Hatch and Turner, 1959; Passonneau and Lowry,1962; Wu,1964; Salas *et al*, 1965).

The hypotheses (f) regarding the inactivation of Pfk appear to be the most plausible cause of the Pasteur Effect. Recent studies appear to support it.

The existence of the Pasteur Effect has long been established in most living organisms. This is based on several lines of evidence. The Pasteur Effect is considered to be operating in a respiring cell or tissue, if:

(a) On passage of the cell or tissue from that oxygen concentration known as the extinction point (E.P.) or from air to anaerobiosis, carbon loss is relatively increased.

(b) The I/N ratio, where I = CO_2 production in the absence of oxygen, and N = CO_2 production in oxygen or in air, assuming that aerobic CO_2 production is represented by

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6H_2O + 6CO_2$$

and anaerobic CO_2 production by

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2,$$

is greater than 1/3. The ratio 1/3 is expected on the basis of the following assumptions:

(i) that the relationship can be expressed by a simple ratio, that the CO_2 produced in air and in nitrogen do not drift along parallel paths, since, otherwise the ratio will vary with time.

(ii) that the rate in air is the only aerobic rate, since, if aerobic CO_2 production should vary with oxygen concentration, the I/N ratio will also vary and become inconsistent.

(iii) that sugar continues to be consumed at the same rate both in air and in nitrogen.

There have been many arguments against basing the operation of the Pasteur Effect on this ratio, since it was shown that CO_2 production in nitrogen is liable to wide fluctuations. Blackman (1928) extrapolated his data to correct these fluctuations. The assumption that if $I/N > 1/3$ indicates the operation of the Pasteur effect has been seriously challenged. Phillips (1947) pointed out that this ratio can only be validly accepted if it has been demonstrated that the fermentation is purely alcoholic and that all the CO_2 is of the fermentative origin. It is now accepted that if I/N ratio is greater than unity, that one can infer that the Pasteur effect exists.

(c) $X = \left[\left(\frac{12I}{44} + \frac{24Z}{46}\right) - \left(\frac{12N}{44}\right)\right] > 0$

where I = rate of anaerobic CO_2 production, and $\frac{12I}{44}$ represents amount of carbon loss

N = rate of aerobic CO_2 production, and $\frac{12N}{44}$ represents amount of carbon loss, and

Z = rate of alcohol production, and $\frac{24Z}{46}$ represents amount of carbon loss.

These figures are obtained by determining fermentation products.

(d) The Meyerhof Quotient $> 1/3$. The ratio is arrived at by subtracting the amount of CO_2 produced in air from CO_2 produced in nitrogen and dividing by the amount of oxygen produced, i.e.,

$$M\,Q = \frac{CO_2 \text{ in nitrogen} - CO_2 \text{ in air (oxygen)}}{O_2 \text{ uptake}}$$

and

(e) If there is an increase in FDP level in nitrogen over that in air and a corresponding decrease in F6P level in N_2, over that in air; a condition attributed to the activation of PFK by increased contents of AMP, P_i and to a decreased content of ATP, coupled with increased CO_2 production. Different substrates in the glycolytic cycle are measured before and after experiments in air and in nitrogen to arrive at these conclusions.

Each of these lines of evidence or a combination have been used to establish the existence of the Pasteur effect in living organisms.

Inhibitors and Activators of the Pasteur Effect

Certain compounds are capable of abolishing or stimulating the Pasteur effect.

Hatch and Turner (1959) demonstrated that removal of oxygen, increased DPN concentration, addition of cysteine, and reduced glutathione (GSH) reversed the aerobic inhibition of glycolysis by pea extracts. Dinitrophenol, N-ethylmaleimide, and M-ethyldiamino diphenoxyhexane, all of which stimulates glycolysis show inhibitory effects on the P.E. Indoleacetic acid which stimulates glycolysis by lowering the amount of intracellular ATP, is also capable of inhibiting the P.E. (Marre and Bianchetti, 1961). Wu (1965) also demonstrated that high levels of P_i and imidazole will stimulate glycolysis to the extent that the P.E. can be significantly decreased. Sodium fluoride at high concentrations will also act as an inhibitor of the P.E.

Ferricyanide, which asserts its effect by lowering intracellular P_i, uridine, cytosine and 5-fluorouridine, acting by depleting intracellular P_i and lowering the rate of glucose uptake and inosine on the other hand stimulate or activate the Pasteur effect. These compounds are capable of inhibiting aerobic glycolysis while they stimulate or show no effects on anaerobic glycolysis.

C. KINASES

The large majority of phosphorylation utilize ATP as

the phosphate donor. The enzymes involved in the reactions are known as kinases (sub-groups 2.7.1-4; Dixon and Webb). The kinases are of wide distribution in plants, animals and microorganisms. The reactions catalyzed by them which involve the transferring of energy from one system to another in the form of energy-rich phosphate bondsare of very significant importance to the biological systems. In the majority of the cases, the phosphate is transferred to a hydroxyl group in a sugar or an alcohol. The reactions they catalyze may be irreversible as in

ATP + D-Hexose $\longrightarrow$ ADP + D-Hexose-phosphate

and such other reactions catalyzed by galactokinase, phosphofructokinase, ribokinase and fructokinase to mention a few, or reversible as is the case of the reactions catalyzed by acetate kinase, carbamate kinase, aspartate kinase, creatine kinase etc. The extent of reversibility of these reactions is dependent on the location of the high energy bonds. In the first examples given, the high energy bond is only on the substrate side of the reaction while the product has a low energy ester bond, thus making it thermodynamically impossible to reverse the reactions. In the reversible reaction, high energy bonds are located on both sides of the reaction with no thermodynamic barrier preventing reversal.

It has been shown that most of the enzymes that

phosphorylate sugars do so specifically at one or the other terminal carbon. The preference however, as has been shown is for the formation of an ester on the terminal primary hydroxyl group, i.e. position 5 or 6 and position 1 in the ketoses. The enzymes are also capable of forming glycosidic phosphates.

Bublitz and Kennedy (1955) showed that the same end of the glycerol molecule is always phosphorylated by glycerol kinase. Burnett and Kennedy (1955) also demonstrated that protein kinases always and exclusively phosphorylate the serine hydroxyl groups of proteins. These workers demonstrated the specificity of these enzymes.

Another set of kinases that have been recognized are those that catalyze the transfer reactions involving the nucleoside polyphosphates. These groups catalyse a reaction such as

$$ATP + AMP \longrightarrow ADP + ADP$$

To further demonstrate the specificity of these enzymes, there is another group (the adenylate kinases) which catalyses a reaction similar to the one above, however, it is essentially restricted to the adenosine derivatives. Some transfer pyrophosphate groups (e.g. ribosephosphate pyrophosphate kinase) while others form glucosyl-pyrophosphate, (e.g. thiamine pyrophosphokinase), a group that is essential in the synthesis of nucleotides.

Another group of enzymes that have been recently discovered are the pyrophosphorylases. These enzymes catalyze the transfer of a substituted phosphate group from a pyrophosphate to another pyrophosphate group to form a new pyrophosphate bond in the product. NAD pyrophosphorylase which catalyzes the reaction:

ATP + nicotinate ribonucleotide $\longrightarrow$ pyrophosphate + desamino NAD

is typical of this group.

MATERIALS AND METHODS

A. GENERAL

ATP, ADP, AMP, UTP, CTP, ITP, GTP, G-1-P, G-6-P, F-6-P, F,1-6-diphosphate, 3-PGA, R-5-P, galactose-6-P, F-1-P, NAD(DPN), NADH (DPNH), EDTA, cysteine, rabbit muscle aldolase and α-glycerophosphate dehydrogenase + triose isomerase crystals were purchased from the Sigma Chemical Company, St. Louis, Missouri; Cleland's reagent (dithiothreitol) from Calbiochem, Los Angeles, Inosine from Pabst Laboratories, Milwaukee, Winsconsin, U.S.A., benzimidazole and imidazole from Eastman Organic Chemicals, Rochester, New York, U.S.A., DEAE cellulose from Brown Company, Research and Development Department, Berlin, New Hampshire, carbazide reagent and sephadex from British Drug Houses, Toronto, Canada.

The barium salts of the sugar phosphates were converted into their respective sodium salts before use. The auxilliary enzymes were prepared in 0.02 M Tris-HCl buffer, pH 8.0, and dialysed before use.

All other chemical compounds were used without furthere purification.

B. PREPARATION OF DEFATTED PEAMEAL

Approximately 145 g. batches of pea cotyledons (Pisum sativum L. var. Tall Telephone Syn. Alderman) -

obtained from Steele-Briggs Ltd., of Winnipeg were cooled to about 4° and homogenized in a cooled Waring blendor for 20 seconds. The homogenized tissue was sieved through 14 and 28 mesh Tyler sieves. The fractions on and below the 28 mesh were collected while the fraction on the 14 mesh was re-homogenized for 20 secs and sieved. The fractions on and below the 28 mesh were again collected and combined with the first fractions, and were then processed according to the method of Hatch and Turner (1957) as follows.

The sieved peameal was poured into a beaker containing ether (1:3 w/v), stirred for two minutes and allowed to stand for fifteen minutes, after which the ether-fat layer was decanted off. Defatting was repeated three times or until no more chlorophyllous material appeared in the ether-layer. Residual ether was filtered from the defatted peameal, with the aid of suction. The resultant defatted peameal was air-dried in a fume cupboard and stored at 4° until required. The yield was about 72%.

C. PREPARATION OF DEFATTED WHEAT EMBRYOS

Viable wheat embryos (Triticum aestivum L. var. Selkirk) were prepared according to the method described by Johnston and Stern (1948) and stored at 4°. The embryos were defatted by homogenizing for 2-3 minutes with ether (1:3,

w/v) in a mortar. The homogenized tissue was allowed to stand for 15 minutes, following which the ether-fat layer was decanted off. Homogenization was repeated until the ether-layer was clear. Following this, the ether was filtered off by suction, and then the defatted wheat embryos were air dried in a fume cupboard, and stored at 4°.

D. CULTIVATION AND TREATMENT OF WHEAT LEAVES

The primary leaves of Triticum aestivum L. var. Selkirk, were excised when 7-9 days old, except otherwise stated. Selkirk wheat leaves were grown in flats under greenhouse conditions in the summer and fall, and in growth chambers during the winter months when they mature late under greenhouse conditions. Growth chamber conditions included a photoperiod of 16 hours at a light intensity of 1000 ft-c at 27°.

To study the effect of benzimidazole, five gram batches of 7-9 day -old leaves were harvested, washed, dried between paper towels and floated on 500 ml of 50 mg/ litre benzimidazole or deionized water contained in a glass tray. The trays were covered with Saran wrap to maintain a high humidity, and were immediately transferred to a growth chamber, where they were allowed to remain until required for enzyme extraction. This usually was after one, three, and five days.

E. ENZYME PREPARATIONS

(1) Peameal

Fifty grams of defatted peameal was suspended in approximately 4 volumes of either 0.02 M Tris-HCl, or one of the following: 0.02 M Tris-HCl, 10^{-3} M EDTA; 0.02 M Tris-HCl, 10^{-3} M cysteine; 0.02 M Tris-HCl, 10^{-3} M EDTA, 10^{-3} M cysteine; 0.02 M Tris-HCl, 0.03 M KF; 0.02 M Tris-HCl, 10^{-3} M Cleland's reagent (dithiothreitol) buffer, pH 7.4; 0.03 M KF, and mechanically stirred at 4^{o} for sixty minutes. The extract was pressed through four layers of cheese cloth, and the homogenate was centrifuged at 20,000 x g for 20 minutes. The supernatant fluid was dialysed against 20 volumes of the extracting buffer at 4^{o} for short periods. The dialysed extract is referred to as the "crude peameal enzyme."

(2) Wheat Embryos

Defatted or non-defatted embryos (6.5 gm) were homogenized in a mortar with 8 volumes of buffer used for the enzyme preparation in (1). The brei was pressed through 8 layers of cheese cloth and the homogenate centrifuged at 20,000 x g at 0^{o} for 30 minutes. The supernatant fluid was dialysed for four hours at 4^{o} against 20 volumes of the extracting buffer. The dialysed extract is referred to as the "crude wheat embryo enzyme". The enzyme

from the defatted embryos generally showed lower activity than the non-defatted embryos, but the former were preferred because defatting offerred some purification.

(3) Wheat Leaves

Five grams of 7-9 days old Selkirk wheat leaves either immediately detached or floated on either water or benzimidazole were ground in a chilled mortar with or without acid-washed sand, and 5 volumes of buffer, preferably 0.02 M Tris-HCl, 10^{-3} M Cleland's reagent, pH 7.4 (see Section E (1), page 36). The brei was pressed through four layers of cheese cloth and the homogenate centrifuged at 20,000 x g at 0^{o} for 15 minutes. The supernatant fluid is referred to as "crude wheat leaf enzyme".

(4) Spinach Leaves

Spinach leaves (obtained from the supermarket) were washed first with tap water and then with distilled water, dried between paper towels and ground in a chilled mortar with buffer as above. The brei was pressed through 4 layers of cheese cloth and the homogenate centrifuged at 20,000 x g at 0^{o} for 15 minutes. The supernatant thereof is referred to as the "crude spinach enzyme".

F. FRACTIONATION WITH AMMONIUM SULPHATE ($(NH_4)_2SO_4$)

Only the "crude peameal enzyme" and crude wheat

embryo enzyme were fractionated with $(NH_4)_2SO_4$. The procedure for fractionation of the peameal enzyme included the addition of solid $(NH_4)_2SO_4$ to a concentration of 33% (v/w) with mechanical stirring at 4^o for 60 minutes. The protein precipitate (if any) was removed by centrifugation at 10,000 x g at 0^o for 10 minutes. The $(NH_4)_2SO_4$ concentration in the supernatant fluid was raised to 65% (v/w) and after a further 60 minutes stirring, the protein precipitate was collected by centrifugation at 10,000 x g at 0^o, for 10 minutes. This was suspended in a small volume of the extracting buffer, and dialysed against 20 volumes of the same buffer for four hours at 4^o. The resulting preparation is referred to as the "33-65% ammonium sulphate fraction". A "20-40% ammonium sulphate fraction" was obtained from the "crude wheat embryo enzyme", by the same procedure described for the enzyme preparation from pea-meal.

G. CHROMATOGRAPHY

(1) Paper Chromatography: This was used for the separation of sugar phosphates, specifically F-6-P, F-1, 6-diP, 3-PGA and G-1-P. Spots or streaks of samples were placed on Whatman No.1 filter paper (unwashed) and developed in phenol:water (72:28) solvent for 10-12 hrs. The chromatograms were sprayed with both aniline hydrogen

phthalate (930 mg aniline and 1.6 gm phthalic acid in 100 ml of water saturated with n-butanol) and acidic naphthoresorcinol (4.75 ml of 2% napthhthoresorcinol + 4.75 ml of 2% aqueous trichloracetic acid + 0.5 ml 60% $HClO_4$), and heated at 85° for 5 minutes.

(2) Column Chromatography

Twenty grams of diethylaminoethyl cellulose was washed with 0.5 N NaOH, 0.5 N HCl, and distilled water successively to remove all yellow colour appearing in the washings. The DEAE cellulose was then suspended in 0.02 M Tris-HCl, pH 8.0, and packed into a column (measuring 35 cm x 2.5 cm) and equilibrated against the same buffer by passing two litres of the buffer slowly through the column. Twenty milliliters of either the "33-65% ammonium sulphate fraction" of the peameal enzyme or "20-40% ammonium sulphate fraction" of the wheat embryo enzyme" was loaded on to the column, and the protein eluted with a linear gradient. Five milliliter-fractions were collected and assayed for aldolase as well as phosphofructokinase activity. Those fractions containing phosphofructokinase activity, the "partially purified enzyme" were pooled and combined. Fifty milliliter-portions of the combined fractions (generally about one hundred milliliters) was loaded on to a G-25 Sephadex column(measuring 55 cm x 2 cm), and

equlibrated against 0.02 M Tris-HCl buffer, pH 8.0 to remove NaCl and excess Tris buffer. The eluate was quantitatively collected in 10 ml aliquots.

H. ASSAYS FOR PHOSPHOFRUCTOKINASE

(1) Colorimetric Method

The method used was based on a modification of that given by Dounce and Beyer (1948) and Sibley and Lehninger (1949) which makes use of colorimetric determination of trioses. Hydrazine is used to fix the trioses formed. At the end of the incubation period (30 minutes), the reaction is stopped by the addition of 10% trichloroacetic acid. The reaction mixture is then centrifuged at 10,000 x g for 2 minutes, and aliquots are removed and made alkaline with 0.75 M NaOH, followed by addition of acid-2, 4-dinitrophenylhydrazine. The mixture is incubated in a water bath at 38° for 10 minutes, and again made alkaline with 0.75 M NaOH. Following this a characteristic color due to a 2,4-dinitrophenylhydrazine derivative of the trioses developed. This derivative exhibits a maximum absorption at 540 mμ, and the intensity of the color is directly proportional to the triose concentration (Sibley and Lehninger, 1949). The color was measured on a Spectronic 20 colorimeter (Baush and Lomb) after 10 min.

The assay system contained:

0.025 M Tris-HCl buffer, pH 8.5	0.30 ml
0.5 M Hydrazine sulfate, pH 8.6	0.15 ml
0.041 M/ml FDP, pH 7.0	0.10 ml
or	
.025 M F6P, pH 7.0	0.10-0.2 ml
0.020 M/ml ATP, pH 7.0	0.05 ml
0.05 M Na_2-KH_2PO_4, pH 7.0	0.1 ml
0.1 M $MgCl_2$	0.05 ml
1 mg protein/ml of .025 M Tris-HCl buffer, pH 8.5 commercial aldolase or Pea aldolase	0.05 ml
Enzyme preparation	0.1-0.4 ml
H_2O to a total volume of	1.50 ml

A unit of PFK is defined as the amount of the enzyme which will cause an OD change of 0.01 at 540 mμ.

(2) Spectrophotometric Method

Phosphofructokinase activity was measured at 25 to 29° by a procedure described by Passonneau and Lowry (1962). This involved coupling the phosphofructokinase reaction to the ∝ - glycerophosphate dehydrogenase and triose isomerase reaction, the favoured end product being glycerol phosphate. The velocity of the reaction was measured in silica cuvettes of 10 mm light path, by following the rate of disappearance of NADH at 340 mμ. The measurement was done on a Gilford optical density converter, model 220, serial 603 attached to a Hilger Spectrophotometer and a recorder with a chart drive. The recorder curves were extrapolated and the slopes taken as initial velocities. The reaction mixture which consisted of:

0.025 M Tris-HCl buffer, pH 8.5	1.0 ml
0.1 M $MgCl_2$	0.1 ml
0.05 M Na_2-KH_2PO_4, pH 7.0	0.1 ml
8 mg/ml NADH in Tris buffer, pH 8	0.06 ml
1 mg protein/ml α-glycerophosphate Dehydrogenase - triose phosphate isomerase	0.05 ml
1 mg protein/ml aldolase (Rabbit muscle)	0.05 ml
0.025 M F6P, pH 7.0	0.20 ml
0.020 M/ml ATP, pH 7.0	0.05 ml
Enzyme preparation and H_2O to a total volume of	0.10-0.4 ml 3.0 ml

was started by adding ATP or F6P. A unit of phosphofructokinase is defined as that amount of it that will cause an optical density change of 0.01 at 340 mμ/minute.

(3) Manometric Methods

Standard Warburg respirometers were used for the estimation of carbon dioxide evolution, aerobically and anaerobically. In the anaerobic experiments, reaction vessels were gassed with 99.995% nitrogen-containing 0.005% oxygen and a trace of hydrogen- for 15 minutes. CO_2 production was measured over 10 minutes and 15 minutes intervals at 25° after equilibration and tipping in, of the substrate and the cofactors. Carbon dioxide was determined by the "direct method", Umbreit et al (1964). The reaction mixture contained:

Enzyme preparation	1.0 ml
0.05 M Na_2-KH_2PO_4, pH 7.0	0.1 ml
0.025 M Tris-HCl, pH 8.5	0.3 ml
0.1 M $MgCl_2$,	0.15 ml

	(0.1 M NAD (DPN) pH 7.0	0.10 ml
*	(0.1 M **GIP, pH 7.0	0.40 ml except otherwise stated.
Sa	(	
	(0.1 M ATP, pH 7.0	0.05 ml
	0.1 M KCl,	0.06 ml
	Water to a total volume of	3.0 ml

Note:
* Sa = (side arm) ** F6P,FDP,3 PGA were also used.

with 0.2 ml of 40% KOH in the central well where required. Ethanol, sugar phosphates etc., were estimated from samples from the reaction mixtures after 1-2 hours.

I. ANALYTICAL METHODS

(1) Protein Determinations

Protein contents of crude enzyme preparations were calculated from determinations of total nitrogen by a Micro-Kjeldahl procedure (Kock and McMeekin, 1924) using peroxide digestion as described by Conway (1957). In the partially purified enzyme preparations, protein was determined according to the method of Warburg and Christian, (1941).

(2) Ethanol Determination

Enzymic digest samples were steam distilled after stopping the reaction with 10% trichloroacetic acid, and the ethanol in the distillate was determined, (a) colorimetrically according to the method of Williams and Reese (1950) and (b) iodometrically according to the method of Peel (1951).

(3) Sugar Phosphate Determination

Sugar phosphates were determined from the same enzymic systems from which CO_2 evolution was estimated. At the end of 30, 60, 90 and 120 minutes of incubation, the reaction was stopped with 10% trichloroacetic acid. Following certrifugation, the aliquots were collected and concentrated. One hundred lambda of the concentrated samples were applied to chromatography papers, developed and sprayed as outlined in chromatography procedure, (Section G (1), page 38). The spots were cut out and eluted with water and estimated using the anthrone reagent test for sugars (Harrow et al, 1960). This procedure consists of placing five milliliters of freshly prepared anthrone reagent (1 gm/100 ml conc. H_2SO_4) in a test tube, and the samples carefully overlaid. This is followed by rapid mixing of the two phases, and boiling at 90^o for 16 minutes. The color developed was measured at 625 mμ. The values in umoles were read off a standard prepared from known concentrations of individual intermediates determined.

EXPERIMENTAL RESULTS

A. PURIFICATION OF PHOSPHOFRUCTOKINASE

Phosphofructokinase from either peameal, wheat embryos or spinach and wheat leaves was extracted as described under Methods. It was found that for maximal initial activity, addition of sulfhydryl compounds to the extracting medium was essential. Of the sulfhydryl compounds tested Cleland's reagent (dithiothreitol) proved most active. Little or no initial activity was obtained in wheat or spinach leaf extracts, if sulfhydryl compounds were not present in the grinding medium.

(1) Purification on DEAE Cellulose Column

Twenty millilitre portions of the 33-65% $(NH_4)_2SO_4$ fraction of the peameal or 20-40% $(NH_4)_2SO_4$ fraction of the wheat embryo enzyme preparations were loaded on to a DEAE cellulose column prepared as described in Methods. The protein was eluted from the column with a gradient of increasing salt concentration. A five-hundred milliliter linear gradient was generated with a gradient devise having two chambers of equal volume and diameters. The mixing chamber initially contained 250 ml of 0.05 M Tris-HCl - 0.2 M NaCl buffer, pH 7.4, and the reservoir chamber, 250 ml of 0.5 M Tris-HCl - 0.2 M NaCl buffer, pH 7.4. Fractions of 5 ml were collected at a rate of one milliliter per minute. The fractions containing enzyme of highest activity

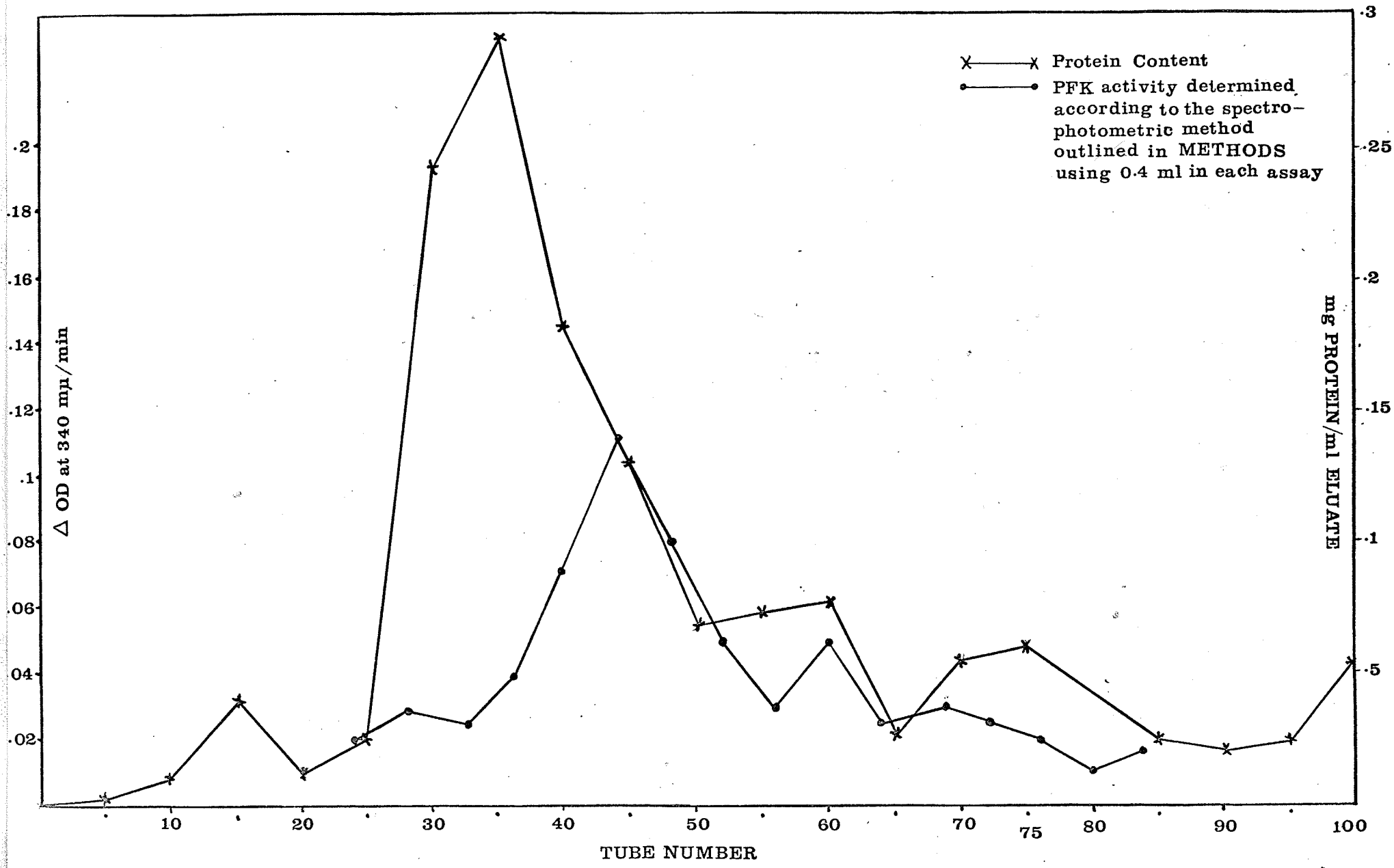


FIGURE 1a. Chromatography of peameal PFK on DEAE cellulose column.

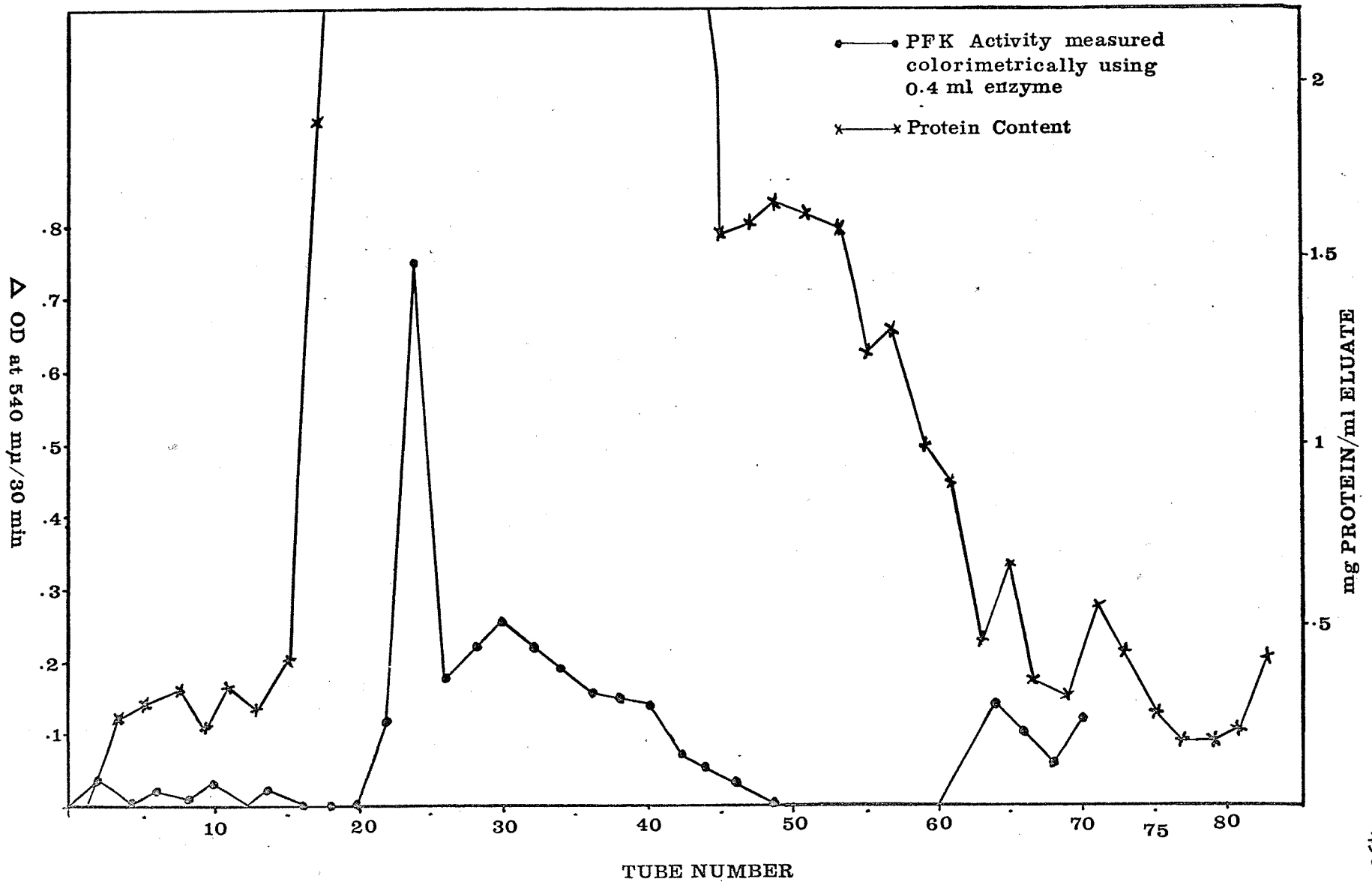


FIGURE 1b. Chromatography of wheat embryo PFK on DEAE Cellulose column.

(fractions 20-40 or 34-54) were pooled (Figs 1a and 1b).

(2) Fractionation on Sephadex G-25 Column

A 50 ml aliquot of the pooled fractions from the DEAE cellulose column was loaded on a Sephadex G-25 column prepared as described in Methods. The protein was eluted with 0.02 M Tris-HCl buffer, pH 7.4. Fractions of 10 ml were collected. This process was included primarily to remove salt and excess Tris-HCl buffer, and resulted in a further purification. However, these fractions were highly unstable. Unsuccessful attempts were made to stabilize these relatively pure fractions with the addition to the eluting buffer of low concentrations of one of the following, F6P, ATP, AMP and Mg-ATP.

A summary of the procedure and the purification for the enzyme from wheat embryos is given in Table II.

B. PROPERTIES

(1) Protection and or Activation by Sulfhydryl Compounds.

Phosphofructokinase is very unstable during maceration and dilution in the absence of sulfhydryl compounds. This is particularly so with the wheat leaf enzyme (Table III). Maceration in the absence of added sulfhydryl compounds yielded very little or no initial activity, whereas on addition of either cysteine, or a combination of EDTA, cysteine and gelatin or β-mercaphoethanol, or Cleland's

TABLE II. Purification of Phosphofructokinase from Wheat Embryos.

Fraction	Protein mg/ml	Total Activity PFK (Units)	Specific Activity Unit/mg.	Purification
I. Crude Extract	702.4	230	0.33	x
II. Ammonium Sulphate (0.20 - 0.40)	390	350	0.90	2.7
III. DEAE Cellulose	0.65	20	30.8	93.3
IV. Sephadex G-25	0.30	10.5	35	106.06

TABLE III. Effect of Some Sulfhydryl Compounds on the Activity of Wheat Leaf Phosphofructokinase.

Extracting Medium	Addition to Extracting Medium	Enzyme Activity in Units
0.02 M Tris-HCl Buffer pH 7.4	-	70
"	10^{-3} M Cysteine	80
"	10^{-3} M Cyst., 10^{-3} M EDTA	60
"	10^{-3} M β-mercaptoethanol	90
"	10^{-3} M EDTA	50
"	10^{-3} M Cyst, 10^{-3} M EDTA, 10^{-4} M $MgCl_2$	120
"	10^{-3} M Cyst, 10^{-3} M EDTA, 1% Gelatin	95
0.02 M K_2HpO_4, pH 8.0	-	25
"	0.05 M Fructose, 10^{-3} M EDTA, 10^{-3} M Cyst.	150
"	10^{-3} M EDTA, 10^{-3} M Cyst., 1% Bovine serum	170
0.02 M Tris-HCl Buffer, pH 7.4	10^{-3} M Cleland's Reagent (Dithiothreitol)	270

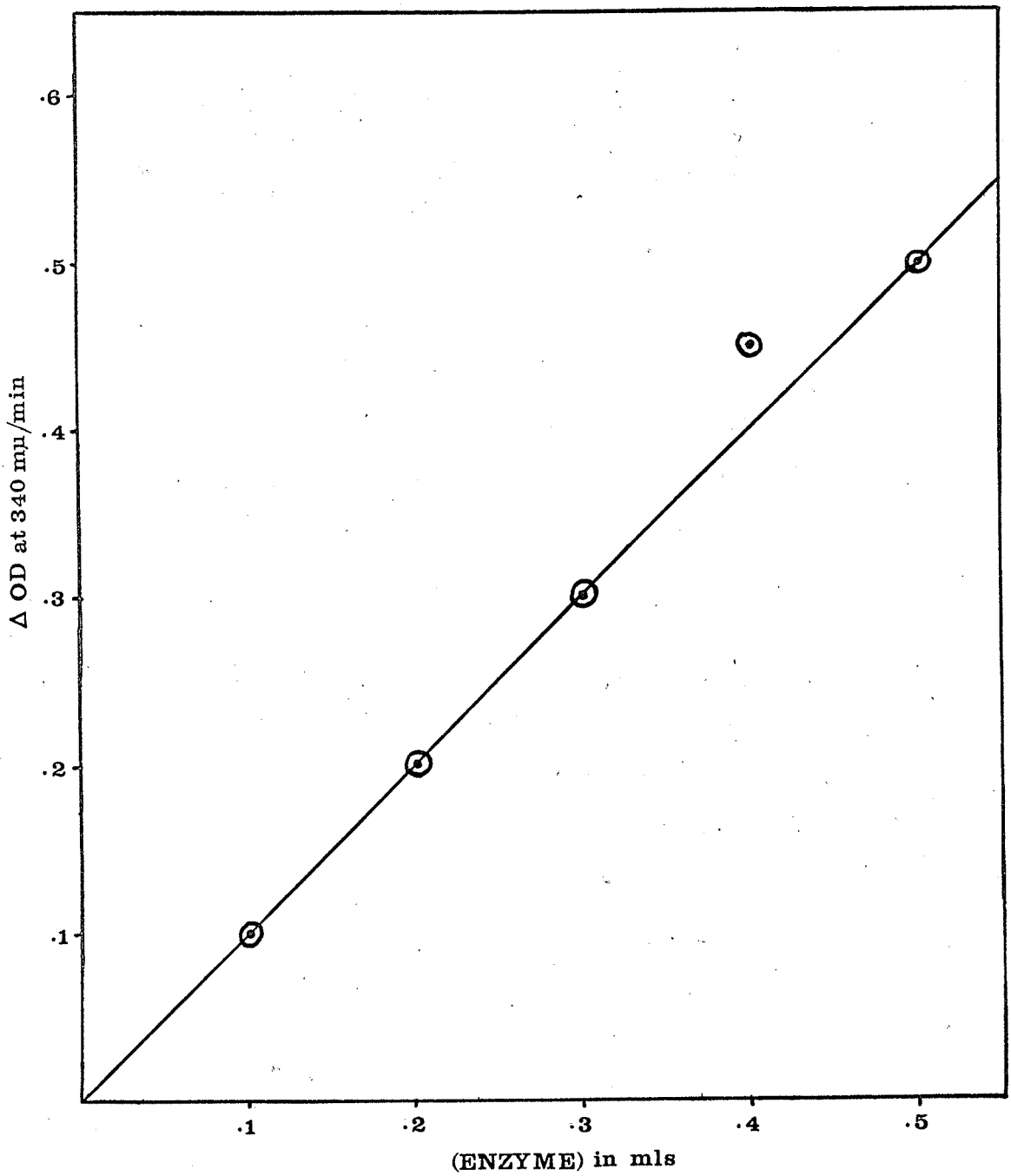


FIGURE 2. Enzyme concentration effect.

Enzyme concentration effect on the partially purified wheat embryo PFK.
Enzyme activity was determined spectrophotometrically.

reagent (dithiothreitol), there is an improved initial activity. Higher initial activities were also obtained for the pea and wheat embryo enzymes following the addition of any of the sulfhydryl compounds shown in Table III. For this reason, a sulfhydryl compound was added to the grinding or extracting media. Table III shows the activation of wheat leaf phosphofructokinase by some of the compounds tested. Dithiothreitol proved to be the most potent activator at a concentration of 1×10^{-3} M. It was also observed that addition of fluoride to the extracting medium or fluoride by itself resulted in increased initial activity.

(2) Protection by Sodium Chloride

Following fractionation on Sephadex G-25, a step devised to remove sodium chloride which was used as the eluting agent with DEAE cellulose, PFK becomes highly unstable.

From many different experiments it appeared that NaCl was stabilizing the enzyme in some manner.

Mansour et al (1965) found that purified sheep heart PFK is in the form of aggregates of several sizes and that these dissociate to smaller polymers when diluted with or in the presence of high concentrations of NaCl. On removal of NaCl by dialysis, however, the smaller polymers revert to the aggregate forms.

It is significant that in the studies carried out here, the partially purified plant PFK becomes highly unstable following removal of NaCl by column chromatography. This suggests that the purified enzyme (in the presence of NaCl) is probably in the form of smaller polymers at which it is fairly stable, and that removal of NaCl reverts the enzyme into the aggregate form which is highly unstable.

(3) Effect of pH

The pH optimum was measured in the range 6.5 to 9.5. It was found that the pea enzyme had a broad plateau of activity from pH 8.0 to 8.5 (Fig 3a) and the wheat embryo enzyme has a broad peak at pH 8.0 (Fig 3b). The activity decreased markedly above and below these values.

(4) Effect of Temperature Treatment

A dialysed ammonium sulphate fraction of the wheat embryo PFK was heated in a water-bath at 59° for 10 minutes, 55° for 10 minutes and at 50° for 60 minutes. Precipitated protein was removed by centrifugation and the supernatant fluid tested for PFK activity at various time intervals. A very short time at 59° results in activation of the enzyme while longer period at 59° results in rapid loss of enzyme activity. Figure 4 shows typical results obtained.

(5) Stability in Storage

Attempts were made to store the enzyme under varying

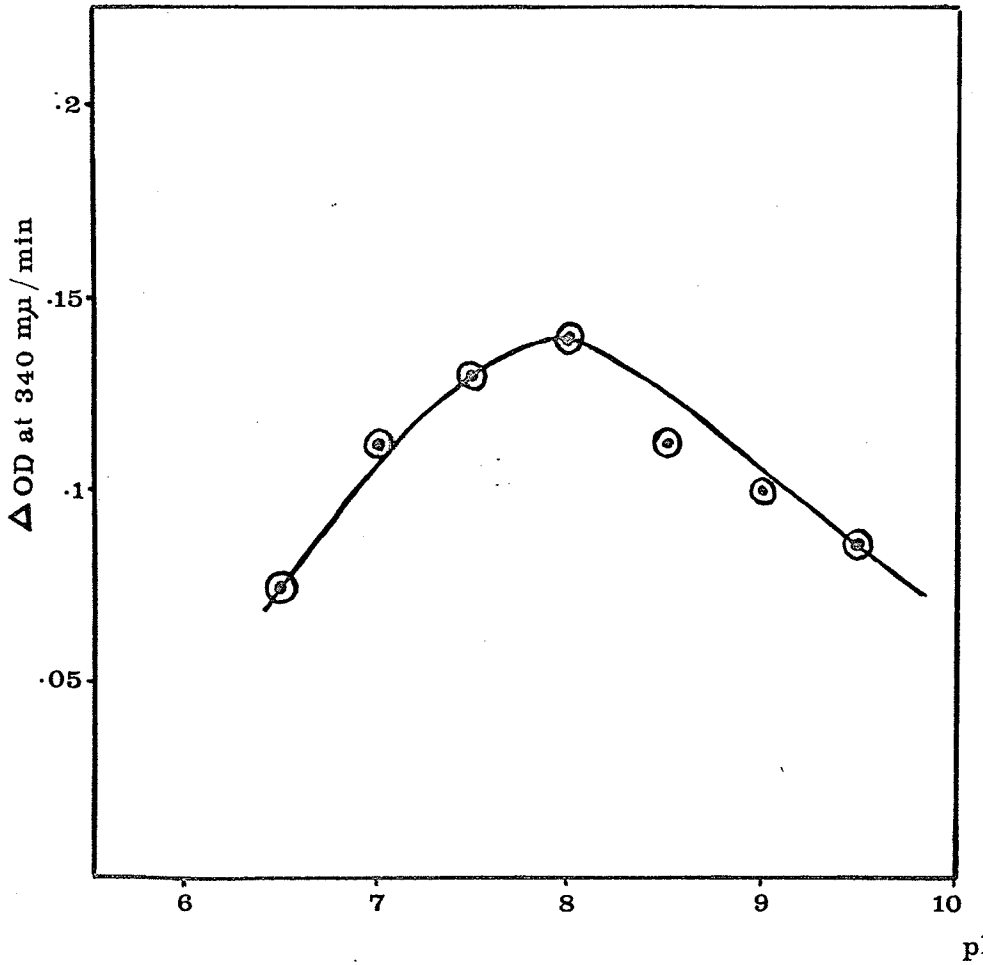


FIGURE 3 (b). Effect of pH on wheat embryo PFK. PFK was assayed via the spectrophotometric method (see text p.41). The system contained 0.40 ml of enzyme.

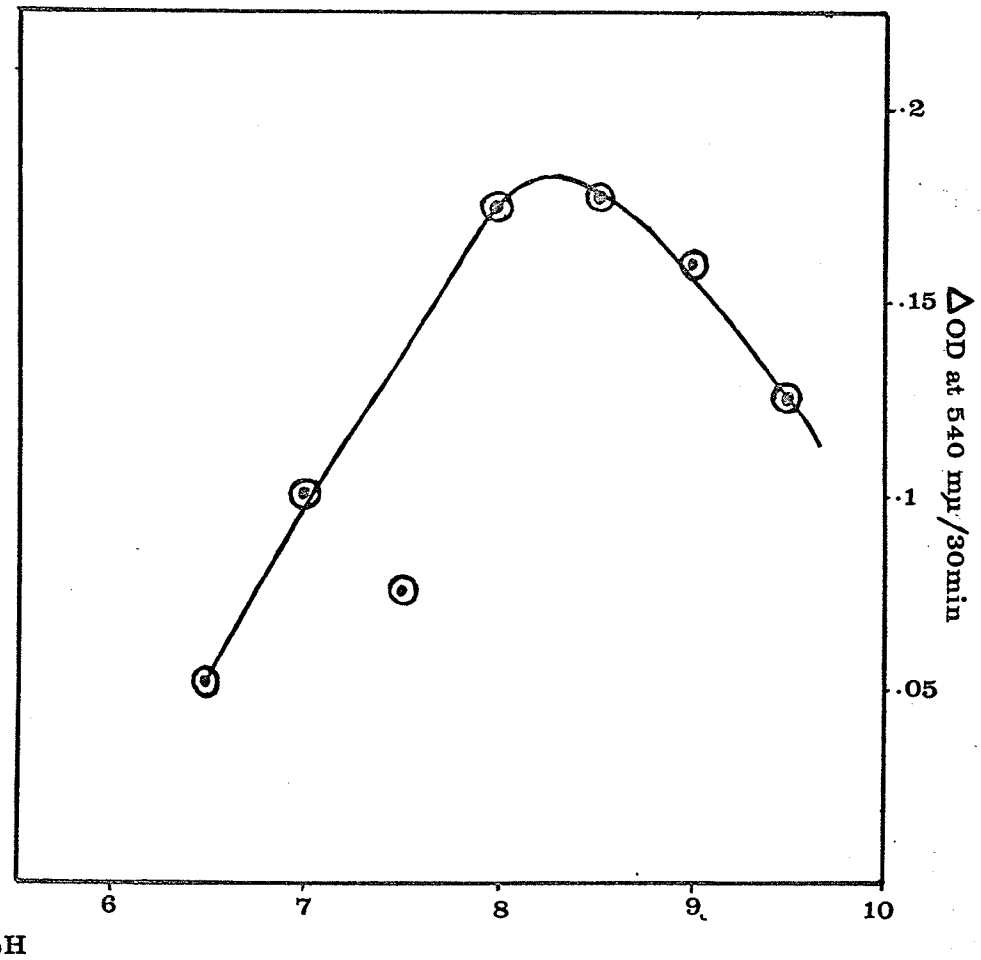


FIGURE 3 (a). Effect of pH on peameal PFK. Activity was measured by the colorimetric method (see text p.40). The system contained 0.40 ml of enzyme.

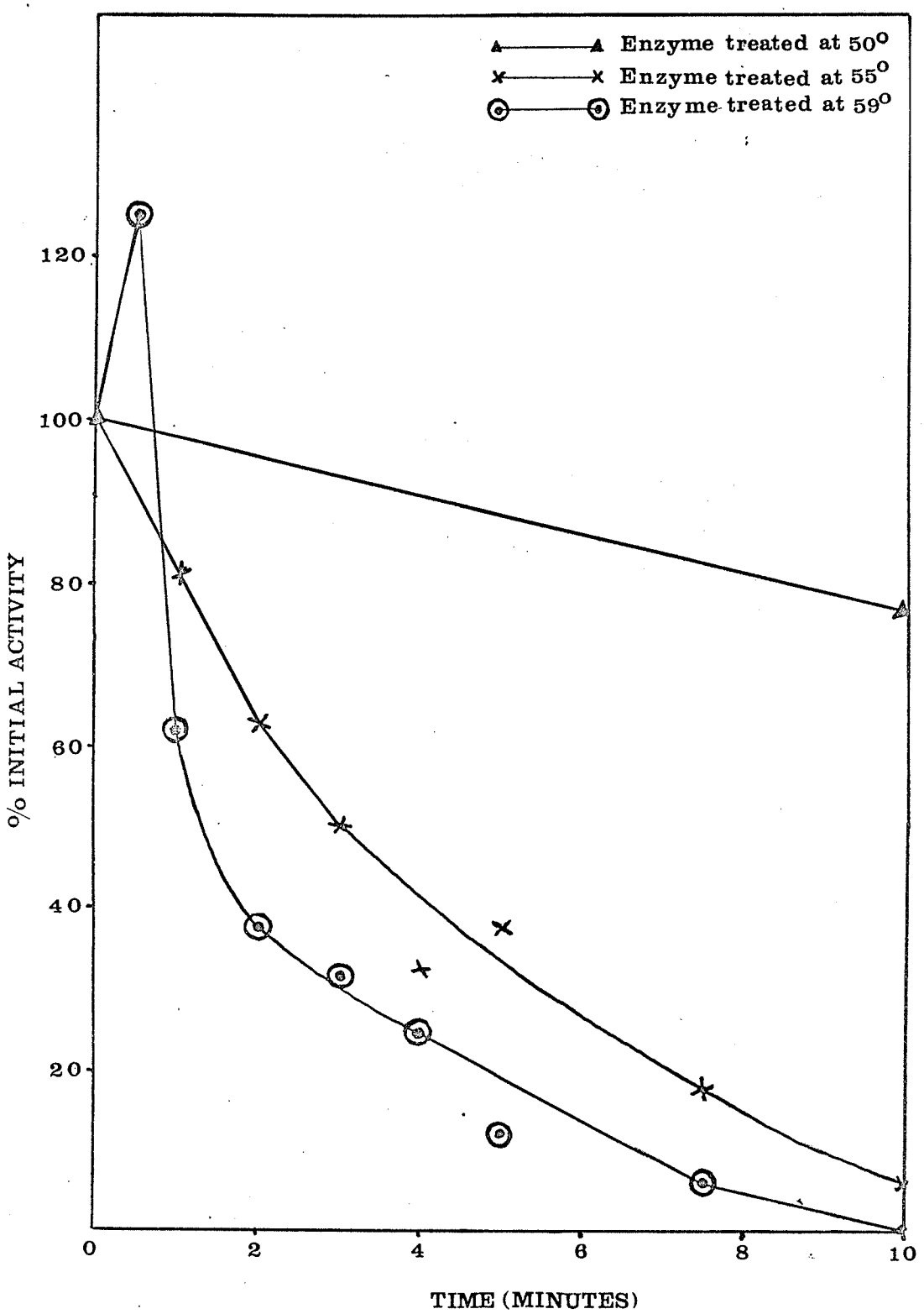


FIGURE 4. Effect of temperature treatment on peameal and wheat embryo PFK.
A 33-65% or 20-40% $(NH_4)_2SO_4$ fraction obtained from either the enzyme preparation from peameal or wheat embryo was used.
0.10 ml of aliquot was used in the colorimetric assay method.

conditions. By far the most successful of these attempts was storage in a saturated solution of ammonium sulphate. A dialysed ammonium sulphate fraction of the enzyme was stored in 65% $(NH_4)_2SO_4$ at 4^o for a period of 14 days and assays were made on the dialysed enzyme at various time intervals. At 5 days, the enzyme preparations had retained about 80% of its initial activity. After 6 days storage, the enzyme rapidly lost its activity, and at the end of 8 days less than 20% of the original activity was retained (Table IV).

(6) Reactivation of Inactive Phosphofructokinase

Enzyme preparations that had undergone complete inactivation were incubated with ATP, ATP-Mg, ADP, AMP, P_i and F6P at various temperatures for varied periods. Very weak reactivation was obtained with AMP, AMP and P_i together, while no measurable activity was recorded after incubation with ATP, ADP, F6P, or P_i alone.

C. SUBSTRATE STUDIES

(1) Effect of Fructose-6-Phosphate Concentrations.

It was found from many experiments that similar to the phosphofructokinase from the yeast and animal tissues, the enzyme from plant tissues falls into the category of those enzymes the kinetics of which are expressed by sigmoidal curves of reaction rate against substrate concentration. A typical sigmoid curve in the plots of velocity

TABLE IV. Effect of Storage on the Activity of Wheat Embryo Phosphofructokinase.

Days Stored	Activity* in Enzyme Units	% Activity
0 (freshly prepared enzyme)	550	100
1	500	90.9
2	505	92
3	500	90.9
4	480	87.3
5	450	81.8
6	300	55.5
7	100	19.2
8	50	9.6
9	30	5.5
10	-	

*Results represent the mean of three different experiments, each of which followed the same trend. 20-40% $(NH_4)_2SO_4$ fractions of the enzyme preparation from wheat embryo were stored in 65% saturated solution of $(NH_4)_2SO_4$ at 4°. Two millilitre aliquots were withdrawn and dialysed before assay by the colorimetric method. The amount of enzyme used in each assay is 0.1 ml.

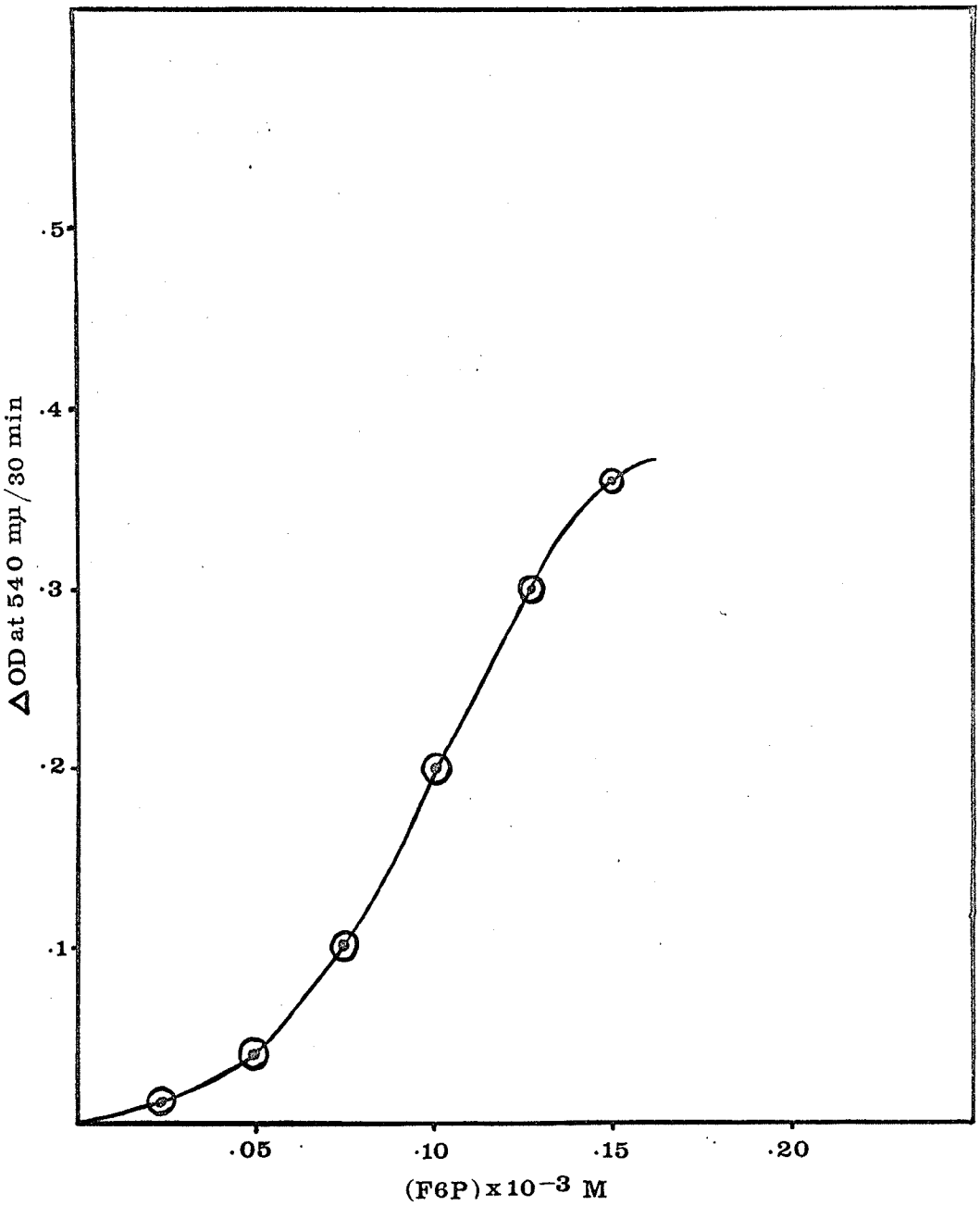


FIGURE 5. Effect of increasing F-6-P concentration on wheat embryo PFK. A total of 0.40 ml of the partially purified enzyme was used in the colorimetric assay method.

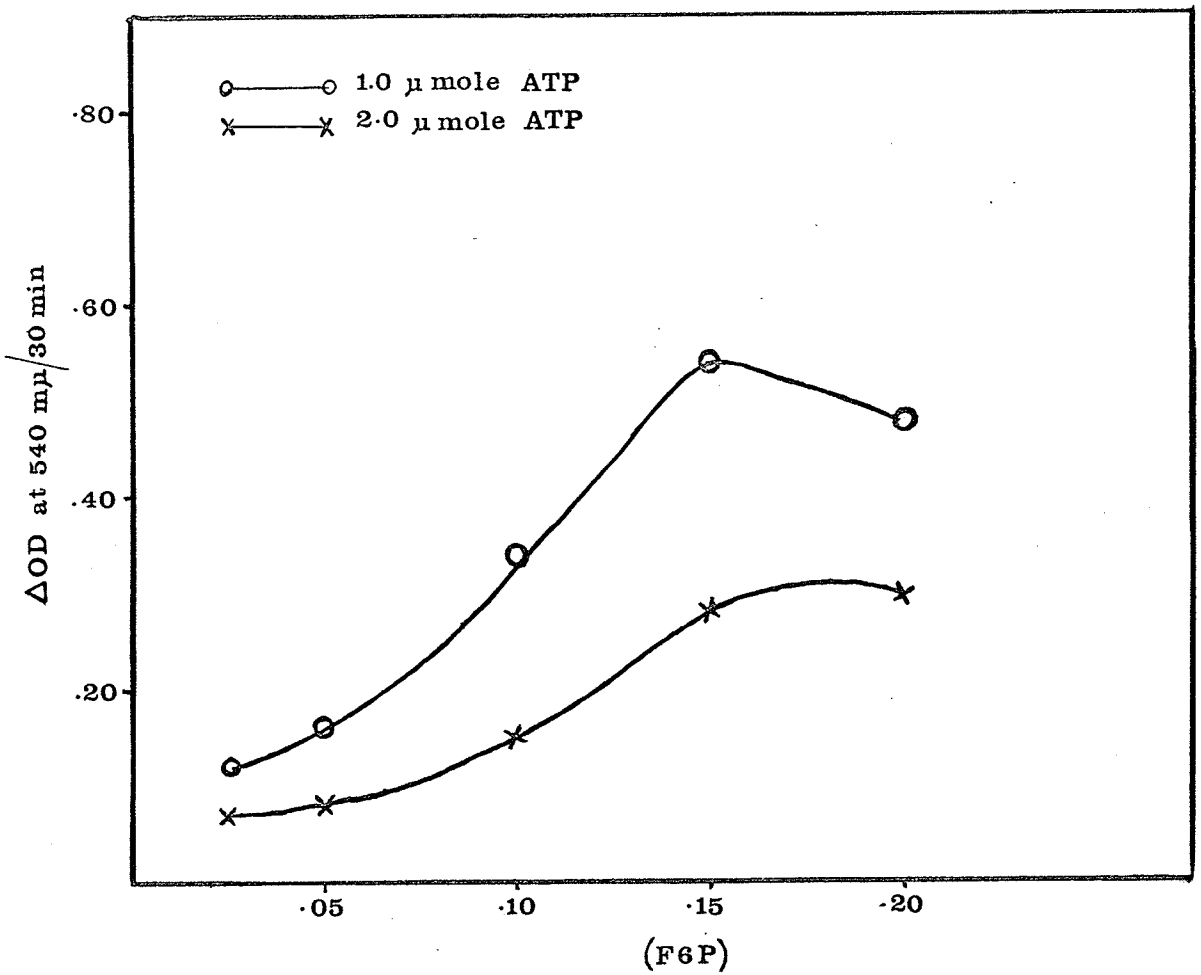


FIGURE 6. Initial velocity pattern for wheat embryo PFK, with F6P as varied substrate. 0.40 ml of partially purified wheat embryo PFK was used in the colorimetric assay method.

against F-6-P concentration is shown in Figure 5. The double reciprocal plots (same data as the ones plotted in Fig 5) gave a parabolic curve instead of the linear relationship found with classical enzymes.

(2) Effect of Adenosine Triphosphate Concentration

Adenosine triphosphate together with F-6-P and Mg^{++} form the substrate complex for the reaction:

$$\text{F-6-P} + \text{ATP} \longrightarrow \text{F-1,6-DiP} + \text{ADP}$$

catalysed by phosphofructokinase. It is relevant to note that this nucleotide which serves as a substrate for PFK at low concentrations has been shown to have inhibitory effects on the enzyme at high concentrations. The inhibitory effect of ATP on the enzyme from liver fluke (Mansour and Mansour, 1962); rabbit muscle (Passonneau and Lowry, 1962), Escherichia coli (Atkinson and Walton,1964) and yeast (Vinuela et al, 1963; Atkinson et al, 1965) has been investigated and it has been concluded that ATP may have a regulatory effect on PFK.

In the present studies, it was observed that phosphofructokinase from higher plant tissues - particularly from pea cotyledons and wheat embryos, is also inhibited by high concentration of ATP. Beyond a concentration of 1.0 µmole, with 2.50 µmoles of F-6-P, ATP begins to show inhibitory effects (Figs 7 and 8). The K_m of 2.7×10^{-5} M calculated from Fig 7 compares very well with 3.0×10^{-5} M

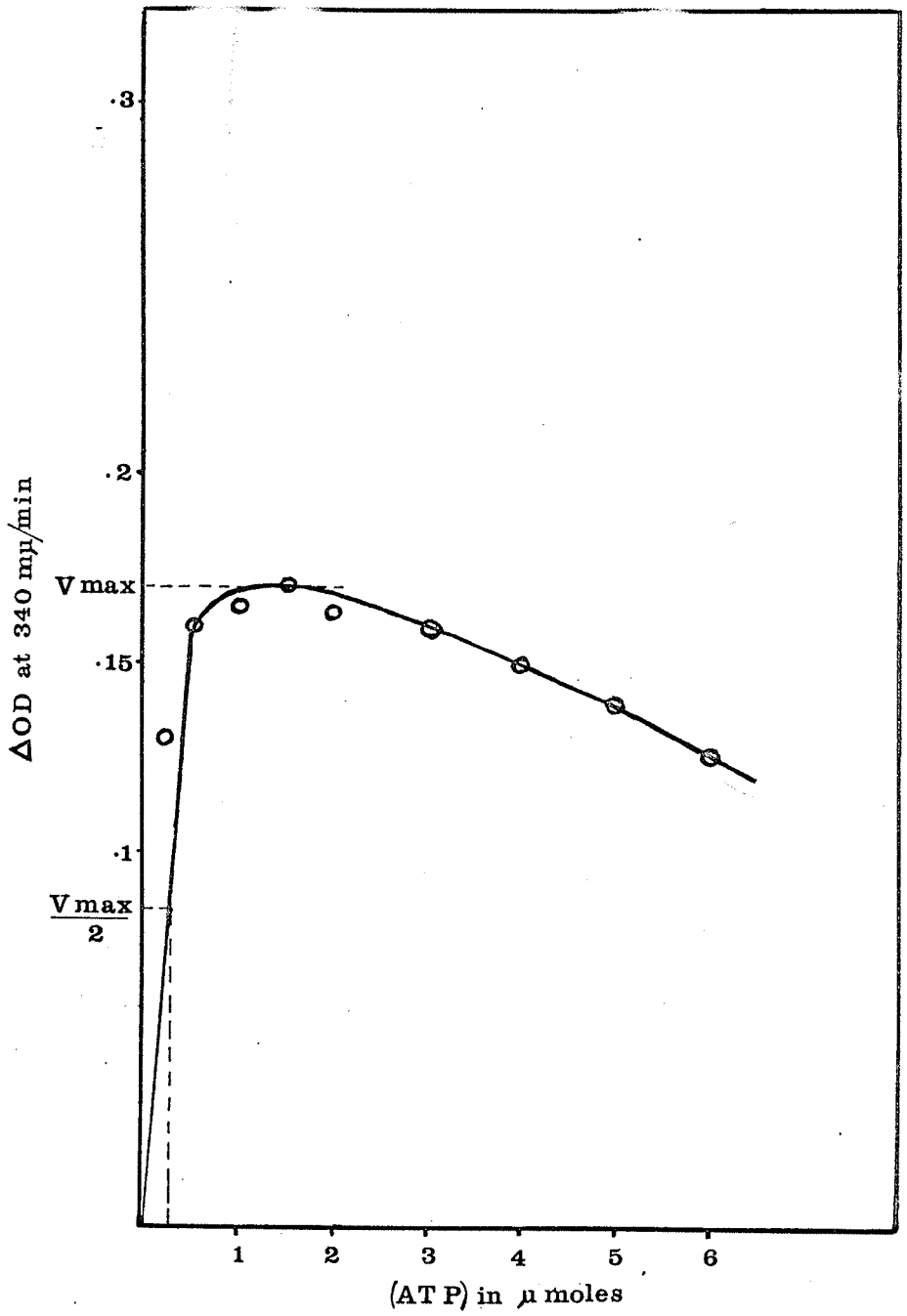


FIGURE 7. Effect of increasing ATP concentration on wheat embryo PFK. Enzyme was assyed via the spectrophotometric method (see text p.41). The system contained 0.40 ml of the enzyme. $K_m = 2.7 \times 10^{-5}$ M.

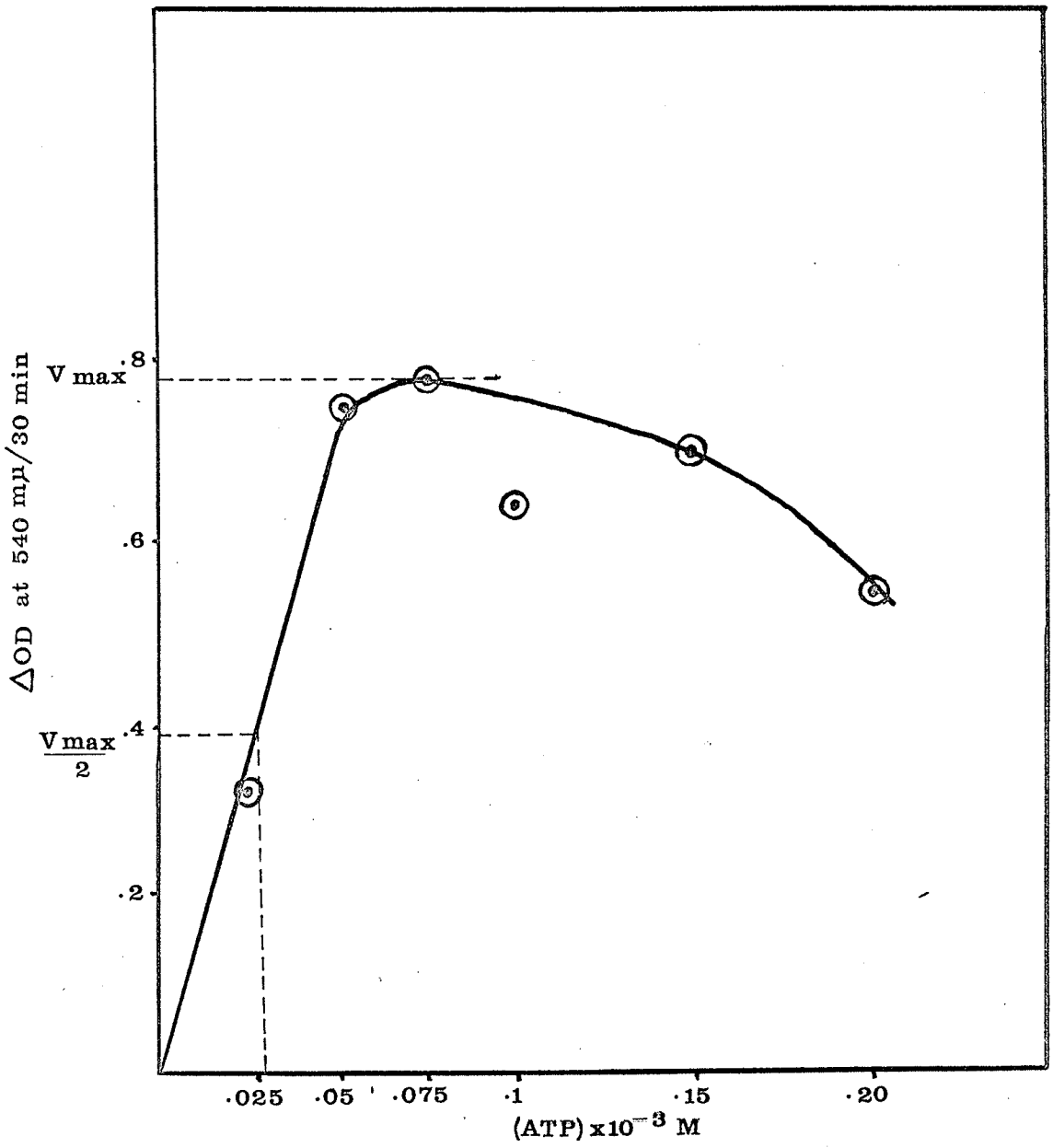


FIGURE 8. Effect of increasing ATP concentration on peameal PFK. Enzyme was assayed via the colorimetric method (see text p.40). The system contained 0.40 ml of enzyme. $K_m = 2.75 \times 10^{-5}$ M.

reported for the rabbit muscle enzyme by Ling et al (1955).

Several investigations have been carried out on the mechanism of action of ATP as a negative effector for PFK. It has been suggested that the enzyme is probably a flexible protein (Atkinson and Walton,1965), the conformation of which is changed by binding of ATP or AMP, at one or more effector sites. Vinuela et al (1963) suggested that ATP may be considered as the end product of the pathway whose first irreversible step is the PFK reaction, that the end-product inhibition by ATP possibly acts as a feedback control. In this investigation, no attempt has been made to demonstrate how ATP may regulate PFK activity. It is however shown that other nucleotides tested do not have such a pronounced inhibitory effect on the enzyme, also that AMP, Pi and Mg^{++} are capable of reversing or overcoming ATP inhibition.

D. SUBSTRATE SPECIFICITY

The purified wheat embryo PFK requires F6P, ATP, Mg^{++} and aldolase (Colorimetric Assay), F6P, ATP, Mg^{++}, aldolase, α-glycerophosphate dehydrogenase and triose isomerase (Spectrophotometric Assay), for activity. Table V shows the specificity of wheat embryo PFK. From the data it would appear that the enzyme can only phosphorylate the No.1 carbon atom. Glucose-6-phosphate and not G-1-P was phosphorylated up to about 75% of the control (F6P).

TABLE V. Substrate Specificity of Phosphofructokinase.

Substrate and Concentration	Co-Substrate and Substitutes	Activity in Enzyme Units	Activity As % of F6P+ATP (Control)
*2.5 µmoles F6P	*1 µmole ATP	59.0	100
"	" ADP	26.0	44
"	" AMP	nil	nil
"	" CTP	20.0	34
"	" GTP	47.0	80
"	" UTP	30.0	50.8
2.5 µmoles GIP	1 µmole ATP	nil	nil
" G6P	" "	44.0	74.5
" FDP	" "	190.0	322.3
" 3PGA	" "	nil	nil
" Ribulose-5-P	" "	4.0	6.8
" Galactose-6-P	" "	nil	nil

*The concentrations given were in a total volume of 1.5 ml.

The system for the assay is the same as described for colorimetric methods under Materials and Methods. Purified wheat embryo enzyme was used.

The phosphorylation of G-6-P probably may be due to the presence of a glucose phosphate isomerase (E.C.No.5.3.1.9). Ribulose-5-phosphate was very weakly phosphorylated while Galactose-6-phosphate showed no activity. The results obtained with F-1,6-diP serving as substrate agrees with that of Atkinson and Walton (1965), who found that in their control experiments in which F-1,6-diP was added to assay mixtures lacking F6P, the change in absorbance at 340 mu corresponded to F-1,6-diP levels between 97 and 100% of the known amounts added.

Fructose-1,6-diphosphate is probably an activator of the wheat embryo PFK. In the assay systems in which F-1,6-diP served as substrate instead of F6P (Table V), it is noteworthy that a higher activity than that obtained for F6P, was recorded. Although the possibility of this high activity being due to aldolase, which has to be added to the assay system is not over-ruled, but in other control experiments, the assay of which did not require aldolase, a higher activity with F-1,6-diP as substrate over F6P was also recorded.

It is also interesting that ADP which actually inhibits the activity of the enzyme in the presence of ATP, can by itself serve as a phosphate donor. Also of interest is the fact that some of the sugars that were not phosphorylated (Table V) do not appear to have any

particular effect on the enzyme. Adenosine monophosphate which is unable to donate phosphate to F6P, reversed the inhibitory effect of high ATP concentration.

E. EFFECT OF NUCLEOTIDES AND COFACTORS

(1) Effect of Nucleotides

Table V shows the effect of the nucleotides tested. Adenosine diphosphate, cytosinetriphosphate, guanosine triphosphate and uridinetriphosphate can all donate phosphate to F6P. These results do not agree completely with those of Atkinson and Walton (1965) who reported that CTP and GTP have only slight activity as phosphate donors, with the enzyme from Escherichia coli. Although CTP, GTP, and UTP are less effective than ATP at low concentrations, these nucleotides are not inhibitory at concentrations much greater than that optimal for ATP. Beyond a concentration of one µmole, ATP begins to inhibit the activity of wheat embryo and peameal PFK, whereas no inhibition by GTP for example, is observed until well beyond 1.5 µmoles.

(2) Effect of Cofactors

Of the divalent cations tested magnesium showed the highest activity in the reaction. Other cations tested with the exception of Fe^{+++} showed inhibitory effects (Table VI), even in the presence of Mg^{++}. The results obtained are consistent with the hypothesis that an Mg-ATP complex is the actual substrate for the kinase reactions.

TABLE VI. Effect of Activators and Inhibitors on Higher Plant Tissue Phosphofructokinase.

Ion	Enzyme Source	Ion Concentration	% Activity
Sodium Acetate	a	10^{-4} M	116 (-)*
Ferric Chloride	a,b	"	114.7 (103)
Pyrophosphate (Na)	a,b	"	110 (106)
Imidazole	b	"	- (107)
Ammonium Chloride	a	"	105 (-)
Formate (Na)	a,b	"	104.4 (103)
Fluoride (Na)	a,b	"	104.1 (102)
Mercuric Chloride	a	"	97 (-)
EDTA	a,b	"	95.5 (98)
Cyanide (K)	a,b	"	94.1 (70.8)
Arsenate (Na)	a,b	"	92.6 (94.3)
Sodium Azide	a,b	"	85.3 (82)
Ammonium Molybdenate	a	"	83.8 (-)
Cupric Chloride	a,b	"	82.3 (75.4)
Inosine	b	"	- (80)
Na-K-Tartarate	a	"	76.4 (-)
Zinc Chloride	a,b	"	54.4 (47.2)
Citrate (Na)	a,b	"	45 (50)

'a' refers to Peameal enzyme
'b' refers to Wheat embryo enzyme.

* Figures in bracket are those for wheat embryo enzyme.

Magnesium significantly reversed ATP inhibition of wheat embryo (and peameal) PFK, while P_i did so very weakly. Figure 9 shows the effects of Mg^{++} and P_i on wheat embryo PFK in the presence of an inhibitory level of ATP.

F. EFFECTOR FOR THE WHEAT EMBRYO PFK

Direct evidence for the inhibition of PFK by excess ATP and its counteraction by some metabolites has been reported for Fasciola hepatica, muscle, yeast and Escherichia coli (Mansour and Mansour, 1962; Passonneau and Lowry, 1962; Ramaiah et al, 1964; Atkinson and Walton, 1965; and Atkinson et al, 1965).

Following the observation that high ATP concentration inhibits the activity of both peameal and wheat embryo PFK, some experiments were carried out to test the effect of AMP, ADP, P_i and Mg^{++} on the enzyme at an inhibitory level of ATP.

As shown in Table VII, the effector for peameal and wheat embryo PFK is similar to that of yeast and Escherichia coli enzymes. Adenosine monophosphate serves as a positive effector while ATP at high concentrations serves as a negative effector. It is also observed that beyond a certain concentration of AMP, at which perhaps the enzyme is saturated, further addition of AMP to the system tends to decrease the rate. These results (Table VII) are similar with those of Atkinson and Walton (1965).

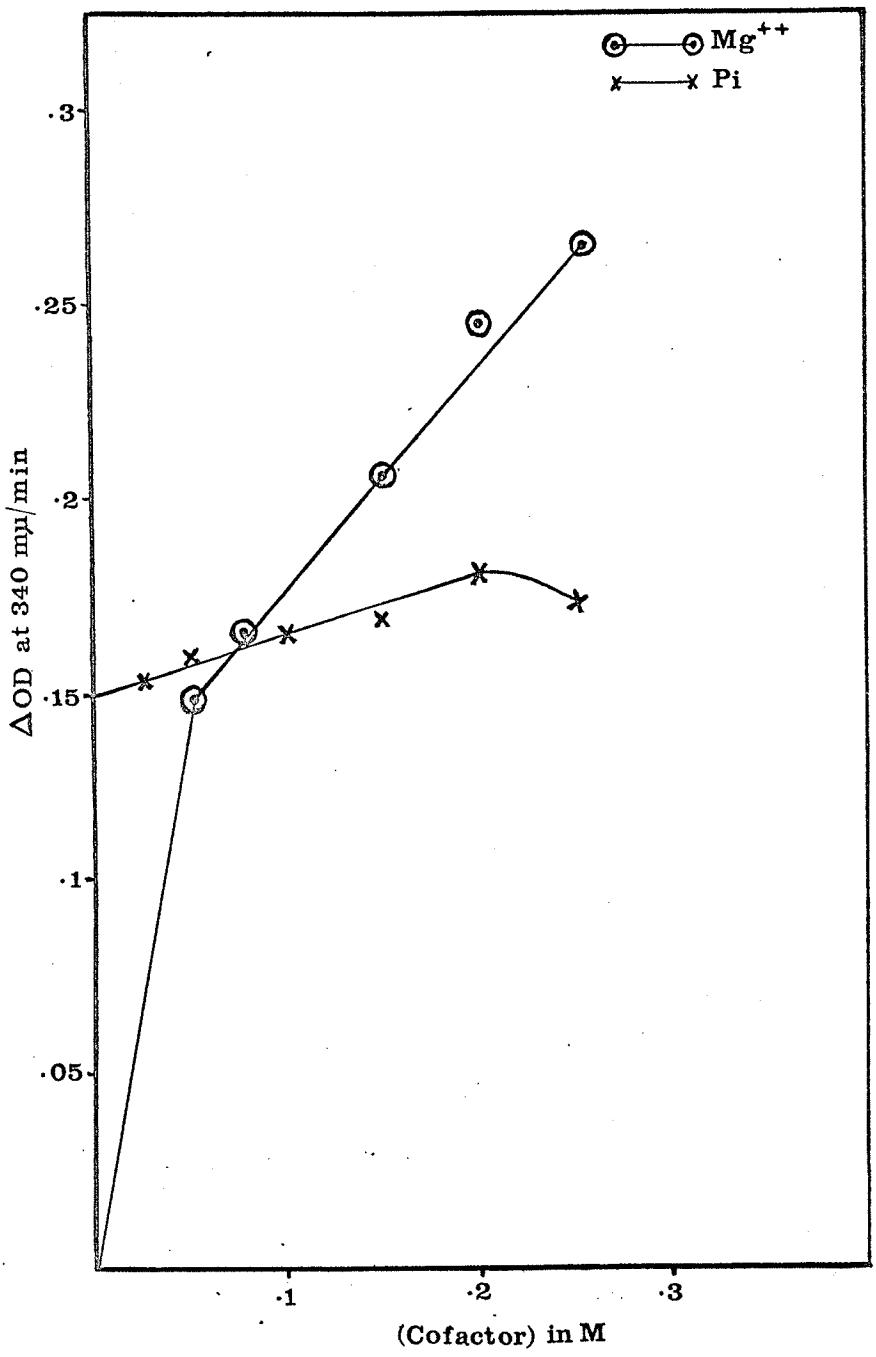


FIGURE 9. Effect of increasing Mg^{++} and P_i concentrations (at an inhibitory level of ATP) on wheat embryo PFK. Enzyme was assayed via the spectrophotometric method (see text p.41). The system contained an inhibitory concentration of ATP and 0.40 ml of the enzyme.

TABLE VII. Adenosine-5'-monophosphate as a Specific Positive Effector for Peameal and Wheat Embryo Phosphofructokinase.

Experiment No.	Concentration of ATP	Addition to System and Concentration	PFK Activity in Enzyme Units
I	1.0 µmoles	-	14.5
	2.0 µmoles	-	10
	"	0.5 µmoles ADP	11
	"	1.0 µmole ADP	8
	"	2.0 " "	6
	"	0.5 " AMP	18
	"	1.0 " "	15
	"	2.0 " "	12
II	1.0 µmole	-	14.0
	2.0 "	-	6
	"	0.5 µmoles ADP	5
	"	1.0 " "	4
	"	2.0 " "	2
	"	0.5 " AMP	17
	"	1.0 " "	14
	"	2.0 " "	8
III	0.50 µmole	-	55
	2.0 "	-	26
	"	0.5 µmoles ADP	21
	"	1.0 " "	21
	"	0.2 " "	18
	"	0.5 " AMP	50
	"	1.0 " "	41
	"	2.0 " "	31
IV	1.0 µmole	-	18.5
	2.0 "	-	16.5
	"	0.5 µmoles ADP	16.5
	"	1.0 " "	14.5
	"	2.0 " "	15
	"	0.5 " AMP	17.5
	"	1.0 " "	18
	"	2.0 " "	16

Partially purified peameal PFK was used in Experiments I and II; an $(NH_4)_2SO_4$ fraction of the peameal enzyme preparation in Expt.III, and partially purified wheat embryo enzyme in IV; and the activity in IV was measured with spectrophotometer.

Atkinson and Walton, suggested that the decrease in rate is due to the fact that AMP, beyond these concentrations at which it reverts ATP inhibition of Escherichia coli, depresses the maximal velocity. It is quite possible that AMP also depresses maximal velocity in its interactions with F6P, ATP and the peameal and wheat embryo PFK. It has been mentioned previously (Table V) that AMP does not react with F6P in the absence of ATP. It also shows no discernible effect at low ATP concentrations. This probably suggests that the effector site is unoccupied in these cases.

Adenosine diphosphate which was also tested (Table VII) shows no activity as a positive effector for either the peameal or the wheat embryo enzyme. Any conclusions drawn on the role of ADP in these studies are probably not plausible, since no specific attempt was made to ascertain that the partially purified enzyme preparations were absolutely free of adenylate kinase.

G. EFFECT OF INHIBITORS AND ACTIVATORS

A number of substances were tested for possible effects on the activity of PFK from peameal and wheat. It is significant to observe that citrate at 1×10^{-4} M was approximately 50% inhibitory. This is consistent with the findings of Salas et al (1965) who showed that citrate inhibited the activity of PFK, and thereby triggers the

operation of the Pasteur effect. Table VI shows that Zn^{++}, sodium azide and inosine inhibited the activity of the enzyme from wheat embryo and peameal. The inhibition by inosine and azide, while imidazole, NaPP, KF, Fe^{+++} activate these enzymes is significant. Wu (1965) pointed out that inosine inhibits aerobic glycolysis, perhaps it does so indirectly by its effect on PFK activity. Wu (1965) also reported that imidazole stimulates aerobic glycolysis. On the basis of the results in this study, it is quite possible that imidazole stimulates PFK activity in such a way as to overcome aerobic inhibition of glycolysis.

H. EFFECT OF DIALYSIS

Several dialysing media were tested. Table VIII shows that the effect of dialysis on plant tissue PFK is dependent on both time and the dialysing medium. With the addition of sulfhydryl compounds more of wheat leaf PFK activity is preserved.

Attempts made to preserve the total initial activity after dialysis failed. Although dialysis does not have such a severe effect on wheat embryo and peameal PFK activities, nevertheless, it was observed that additions of sulfhydryl compounds to the dialysing medium, coupled with short period of dialysis maintained and often increased the enzyme activity.

TABLE VIII. Effect of Dialysis on the Activity of Wheat Leaf Phosphofructokinase.

Expt. and Age of Leaves	Dialysing Medium	Addition to the Dialysing Medium	Duration of Dialysis	Activity in Enzyme Units	Activity as % of Control
I 9 day old leaves	Control (no dialysis)	-	-	100	100
	0.02 M Tris-HCl Buffer, pH 8.0	-	16 hrs.	0	0
	0.02 M Tris-HCl Buffer, pH 8.0	-	8 "	27.5	27.5
	0.02 M Tris-HCl Buffer, pH 8.0	-	4 "	30	30
II	Control (no dialysis)	-	-	115	
	0.02 M Tris-HCl Buffer, pH 8.0	-	4 "	35	30.4
	"	10^{-3} M Cleland's Reagent	4 "	37.5	32.6
	"	10^{-3} M EDTA	4 "	15	13.04
	Deionized Water	-	4 "	7.5	6.5

I. INHIBITION AND ACTIVATING EFFECT OF WHEAT LEAF EXTRACT

Because of the low activity of the enzyme from wheat leaves, it was tested for its inhibitory effect on the enzymes in pea and wheat embryos. It was observed that crude wheat leaf extracts prepared as described in Methods significantly inhibited the activities of the enzyme from pea and wheat embryo. The neutralized supernatant of the extract after perchloric acid precipitation showed no inhibitory effect, but in some experiments it activated the system, to some extent. However, no detailed studies were made on the active and inhibitive principle of the crude wheat leaf extracts.

J. EFFECT OF BENZIMIDAZOLE ON THE LEVEL OF PFK IN WHEAT LEAVES.

Cultivation and light treatment of wheat leaves were carried out as described in Methods. Leaves were floated for 1, 3 and 5 days respectively. At the end of each floatation period, enzymes were extracted from the leaves according to the procedure described under Methods. In leaves floated on water, the level of PFK activity remains steady after 24 hours and decreased rapidly thereafter. On the other hand, in leaves floated on benzimidazole (50 mg/litre) the level of PFK activity was found to be comparable to those in the immediately detached leaves (Table IX) even after 5 days.

TABLE IX. Effect of Benzimidazole on Phosphofructokinase Level in Detached Selkirk Wheat Leaves.

Expt. No.	Days after detachment	Enzyme units in immediately detached leaves	Enzyme units leaves floated on Water	Enzyme units leaves floated on Benzimidazole
I[1]	0	110	-	-
	1	-	87.5	100.0
	3	-	75.0	102.5
	5	-	62.0	107.25
II[1]	0	100	-	-
	1	-	92.5	110.0
	3	-	82.50	107.25
	5	-	60.0	106.25
III[2]	0	50	-	-
	1	-	45.0	55.0
	3	-	43.75	52.0
	5	-	46.25	-
IV[2]	0	35	-	-
	1	-	25.0	37.50
	3	-	20.0	36.25
	5	-	25.0	32.50
V[2]	0	45	-	-
	1	-	15.0	42.50
	3	-	15.0	36.25
	5	-	5.0	37.50
VI[3]	0	72.5	-	-
	1	-	50.0	73.75
	3	-	45.0	72.5
	5	-	25.0	72.5

Superscripts 1, 2 and 3 refer to leaves grown in the Green House in the Fall and in the Winter, and Growth Chamber at constant temperature and light intensity (in winter) respectively.

K. WHEAT EMBRYO PHOSPHOFRUCTOKINASE AND THE PASTEUR EFFECT

Inhibition of fermentation or of glucose uptake in the presence of oxygen has been observed in many organisms and tissues and it is generally referred to as the "Pasteur Effect". The relation of the phosphofructokinase reaction to the Pasteur effect has been suggested for many years. Recent evidence clearly suggests that the regulation of phosphofructokinase activity in animals and in micro-organisms, is fundamental in the control of glycolysis.

It has been suggested that the control of the Pasteur effect could be achieved via a series of feed-back mechanisms (Sols et al, 1963; Passonneau and Lowry, 1962, 1963, 1964), or through the endproduct inhibition of phosphofructokinase activity by ATP in certain tissues (Vinuela et al, 1963; Wu, 1965), and or by an additional feed-back involving the effect of citrate on phosphofructokinase activity in yeast cells (Salas et al,1965).

In the light of this evidence, some experiments were conducted in order to ascertain whether the properties of the phosphofructokinase from wheat embryos (studied in the previous sections)could be related to the control of glycolysis in extracts of wheat embryos.

(1) Glycolysis by the Crude Wheat Embryo Extracts

Preliminary investigations showed that the crude extracts from wheat embryo possessed complete glycolytic activity. Glucose-1-phosphate or glucose-1-phosphate and

fructose were utilized and carbon dioxide was produced (Fig 10). The glycolytic activity of the crude extract measured by CO_2 output was lower, if either G-1-P or ATP was omitted from the system. Maximum activity in the absence of these compounds was generally about 30% of the control (Fig 10). The absence of ATP always increased the lag period.

The partially purified extract (20-40% $(NH_4)_2SO_4$ fraction with high PFK activity possessed little or no glycolytic activity (Fig 10). Accordingly, crude wheat embryo extracts were used in all subsequent studies.

(2) Aerobic Inhibition of Glycolysis

It was found that in the presence of oxygen (air) glycolysis by the wheat embryo extract was considerably inhibited. There was a significant decrease in both carbohydrate loss and CO_2 output in systems incubated under aerobic conditions (Fig 11 and Tables X and XI). The amount of carbon dioxide produced in air is only about 35% of that produced under anaerobic condition.

Clear evidence that the Pasteur effect operates in the wheat embryo extract was obtained. In all experiments conducted and reported here

$$\frac{Gl(N)}{Gl(O)}$$

where $Gl(N)$ = CO_2 production in anaerobic glycolysis and,

$Gl(O)$ = CO_2 production in aerobic glycolysis

clearly exceeds unity.

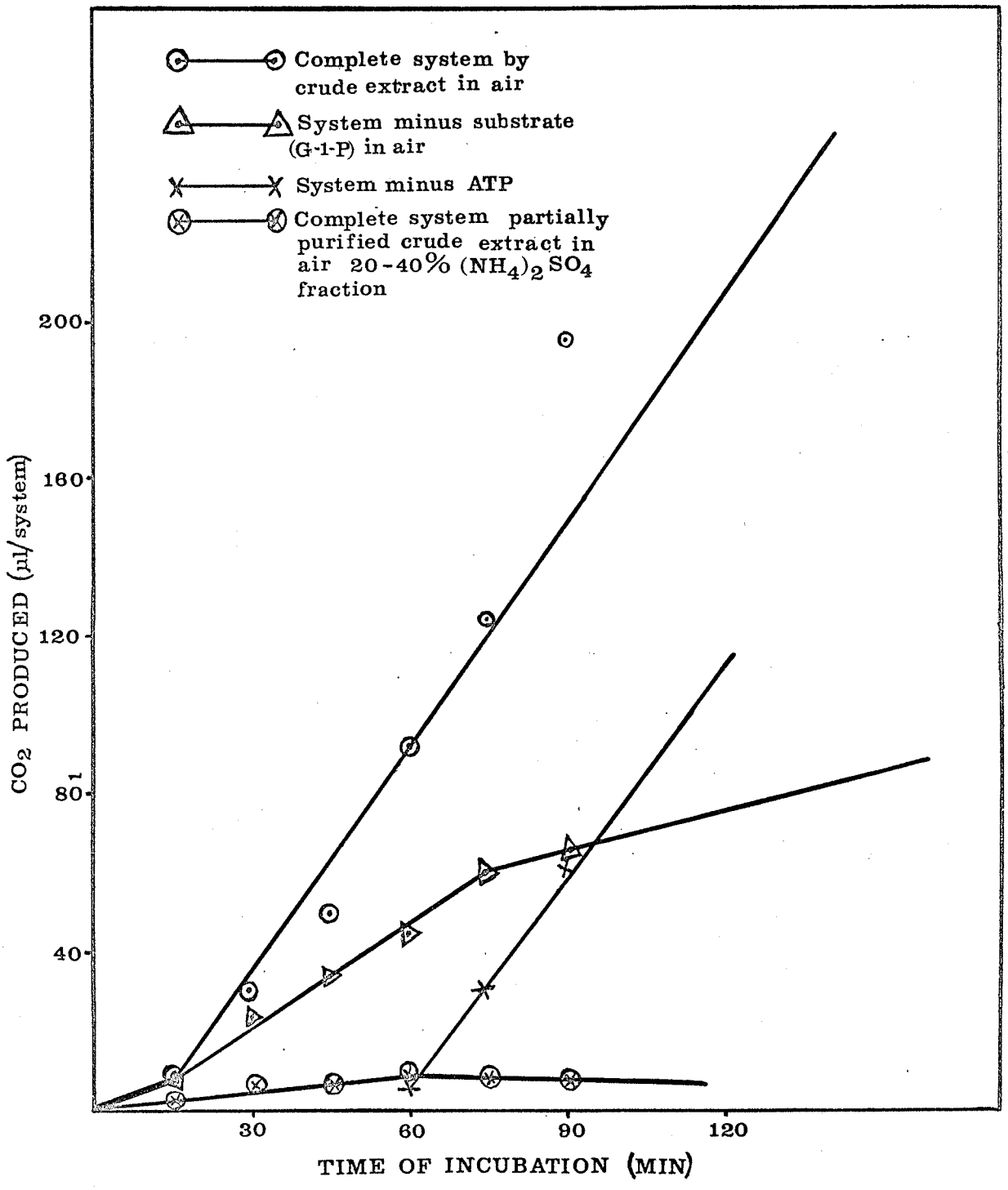


FIGURE 10. Glycolysis by crude wheat embryo extract. Reaction mixture contained 1.0 ml of the enzyme preparation and the reagents indicated in Methods, manometric assays (see text p.42).

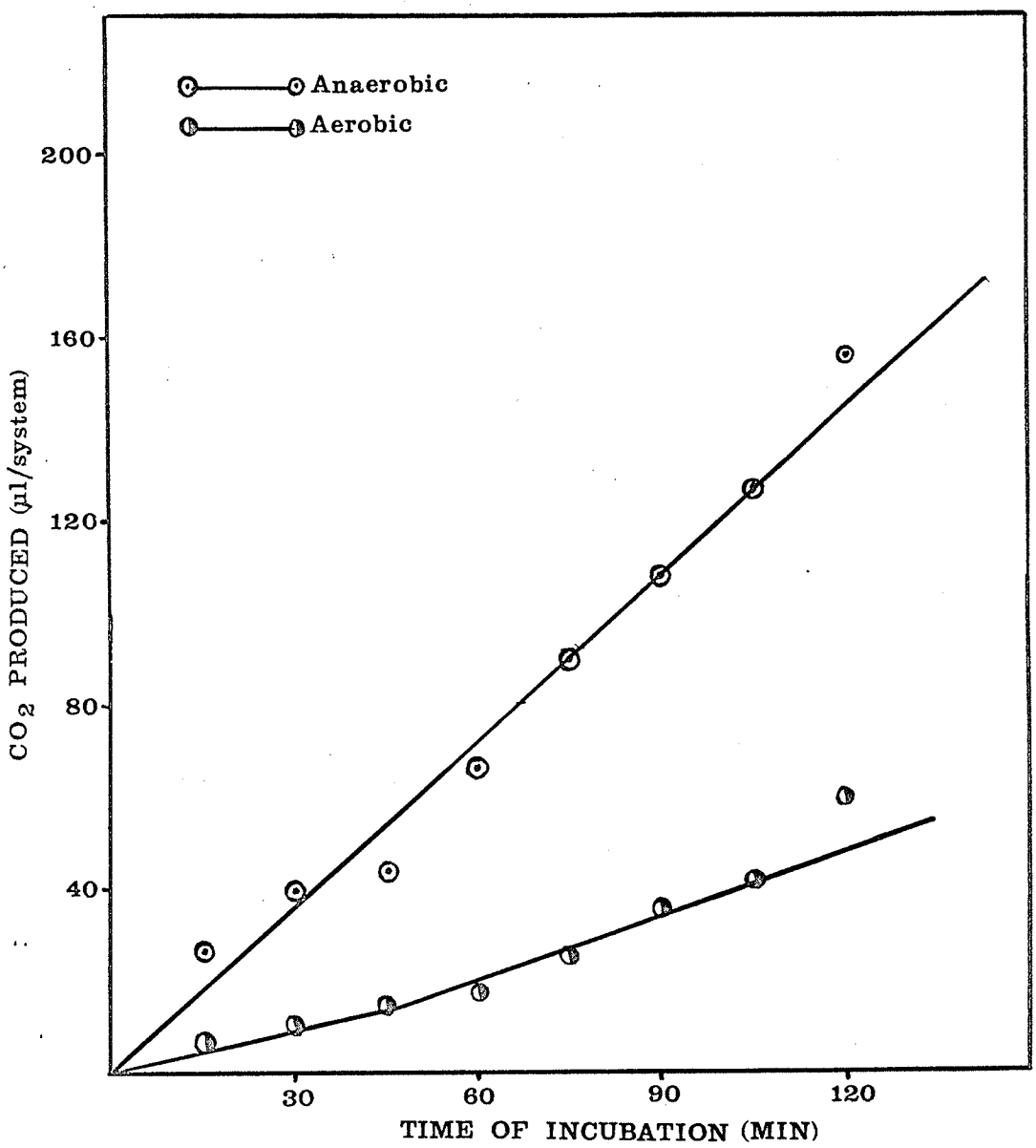


FIGURE 11. Aerobic inhibition of glycolysis of crude wheat embryo extract.
Assay system: The same as described in Methods (see text p.42).

TABLE X. Aerobic Inhibition of Glycolysis

Expt. No.	Substrate	Glycolytic Activity GL(N)	Glycolytic Activity GL(O)	GL(N)/GL(O)
I	*Glucose-1-phosphate	51	48	1.1
	"	113	91	1.2
II	"	190	174	1.1
	"	107	97	1.1
III	"	63	42	1.5
IV	*Glucose-1-phosphate and **Fructose	160	139	1.2
		79	51	1.5
V	"	51	31	1.6
	"	99	56	1.7
VI	"	44	22	2.0

* GlP concentration = 40 µmoles

** Fructose concentration = 10 µmoles

System contained 1.0 ml of wheat embryo extract in a total volume of 3.0 ml.

GL(N) and GL(O) are expressed in µl CO_2/hour/3.0 ml.

TABLE XI. Carbohydrate Loss and CO_2 Production by Crude Extracts from Wheat Embryos Under Aerobic and Anaerobic Conditions.

Expt. No.	Carbohydrate	Initial Conc. of Carbohyd.	Final Conc. of Carbohyd.		CO_2 Produced		GL(N)/GL(O)	Time of Incubat. in Min.
			Anaerobic	Aerobic	GL(N)	(GL(O)		
I	GlP	40.60	1.40 (-39.20)	5.20 (-35.40)	40	28.2	1.4	30
	F6P	1.80	5.20 (+ 4.0)	9.20 (+ 7.40)				
	F-1,6-diP	2.70	9.80 (+ 7.10)	9.00 (+ 6.30)				
	3-PGA	2.10	0.80 (- 1.30)	2.60 (+ 0.50)				
II	GlP	42.10	2.0 (-40.10)	5.40 (-36.70)	115.6	56.80	2.03	60
	F6P	5.55	6.60(+ 1.05)	7.40 (+ 1.85)				
	F-1,6-diP	6.0	13.80(+ 7.80)	11.00 (+ 5.0)				
	3-PGA	1.65	0.80(- 0.85)	2.60 (+ 0.95)				
III	GlP	40.66	0.20(-40.46)	0.48 (-40.18)	51.43	31.02	1.7	60
	F6P	1.65	1.0 (- 0.65)	4.0 (+ 2.35)				
	F-1,6-diP	4.05	4.20(+ 0.15)	1.60 (- 2.45)				
	3-PGA	1.80	1.20(- 0.60)	4.16 (± 2.36)				
IV	GlP	40.66	0.60(-40.06)	4.40 (-36.26)	29.20	8.46	3.4	30
	F6P	2.10	3.20(+ 0.11)	7.20 (+ 5.10)				
	F-1,6-diP	3.30	7.0 (+ 3.70)	6.20 (+ 2.90)				
	3-PGA	1.95	2.20(+ 0.25)	4.90 (+ 2.85)				

In these experiments (Table XI) 40.0 µmoles of GlP were added as substrates and endogenous levels of the substrate and indicated intermediates were determined in the extracts. The values are given in µmoles/3.0 ml containing 1.0 ml of enzyme preparation. GL(N) and GL(O) are as previously stated.

Studies based on measurement of the level of substrate and intermediates of glycolysis clearly indicate that there is less carbohydrate breakdown in air than in nitrogen, (Table XI). The ratio of breakdown of F6P in nitrogen as compared to air indicates a greater loss of this intermediate under anaerobic conditions. In the case of F-1,6-diP the ratio indicates a greater breakdown of this compound under aerobic conditions, or that F-1,6-diP accumulates to a greater extent under anaerobic conditions. This would be expected if PFK is involved in the Pasteur effect. A carbon balance sheet has not been calculated owing to the large losses of GIP which is probably due to phosphatase activity in the crude extracts. Further evidence in support of this conclusion is reported later in the manuscript. Although the results obtained (Tables XI and XII) confirmed the findings of Hatch and Turner (1959) that glyceraldehyde-3-phosphate dehydrogenase is affected by oxygen, however in these studies, it is clearly shown that more than one enzyme is affected by oxygen, in the aerobic inhibition of glycolysis. Hatch and Turner (1959) indicated that the inhibition of glycolysis in pea-seed extract was due to "oxidative inactivation of glyceraldehyde-3-phosphate dehydrogenase", that oxygen exerts its effect "on a step beyond F-1,6-diP in the glycolytic system". The results thus far given which are similar to

TABLE XII. Aerobic Inhibition of Glycolysis of Various Substrates. Glycolytic activity is Expressed as an Average of the First 2 Hours of Steady-rate Activity.

Expt. No.	*Substrate	Glycolytic Activity GL(N)	Glycolytic Activity GL(O)	GL(N)/GL(O)
I	G1P	127	116	1.09
	F6P	51	16.5	3.09
	F-1,6-diP	80	72	1.11
	3-PGA	93.2	74	1.25
II	G1P	94.5	81.5	1.15
	F6P	71	50	1.42
	F-1,6-diP	73.2	61.2	1.19
	3-PGA	110.6	84	1.31
III	G1P	92.5	85.6	1.08
	F6P	48	40.5	1.19
	F-1,6-diP	56	54	1.03
	3-PGA	54.6	45	1.20
IV	G1P	112	94.6	1.18
	F6P	62	60	1.03
	F-1,6-diP	59.5	59	1.0
	3-PGA	72.6	57.2	1.26

* 40.0 μmoles of each substrate were used in the assay systems; and GL(N) and GL(O) are as previously stated.

those reported for animal tissues and micro-organisms (Passonneau and Lowry, 1962, 1963, 1964; Parmeggiani and Bowman, 1963; and Wu,,1965) strongly support that an enzyme (PFK) operating prior to glyceraldehyde-3-phosphate dehydrogenase in the E.M.P. pathway is also affected by oxygen and that it is probably primarily responsible for the Pasteur effect.

(3) Effect of Glucose-1-Phosphate Concentration on the Glycolysis in the Extracts from Wheat Embryos.

The data plotted in Figure 12 reveal that the availability of G-1-P is also capable of controlling aerobic glycolysis. Increasing the concentration of G-1-P led to a greater stimulation of both aerobic and anaerobic glycolysis, but the Pasteur effect was almost abolished. An increase from 20 µmoles to 80 µmoles of GIP decreased the Pasteur effect by about 30%. The Gl(N)/Gl(0) ratio at low G-1-P concentration (20 µmoles) is equal to 1.2, whereas with G-1-P at 80 µmoles, the ratio is reduced to 0.89 (Fig 12). These results closely parallel those reported by Wu (1965) for Ehrlich Ascites tumor cells. He showed that when the concentration of glucose was increased 10-fold, the rate of aerobic glycolysis was doubled, and an increase in the PE from 60% at high glucose levels to 80% at low glucose levels was obtained.

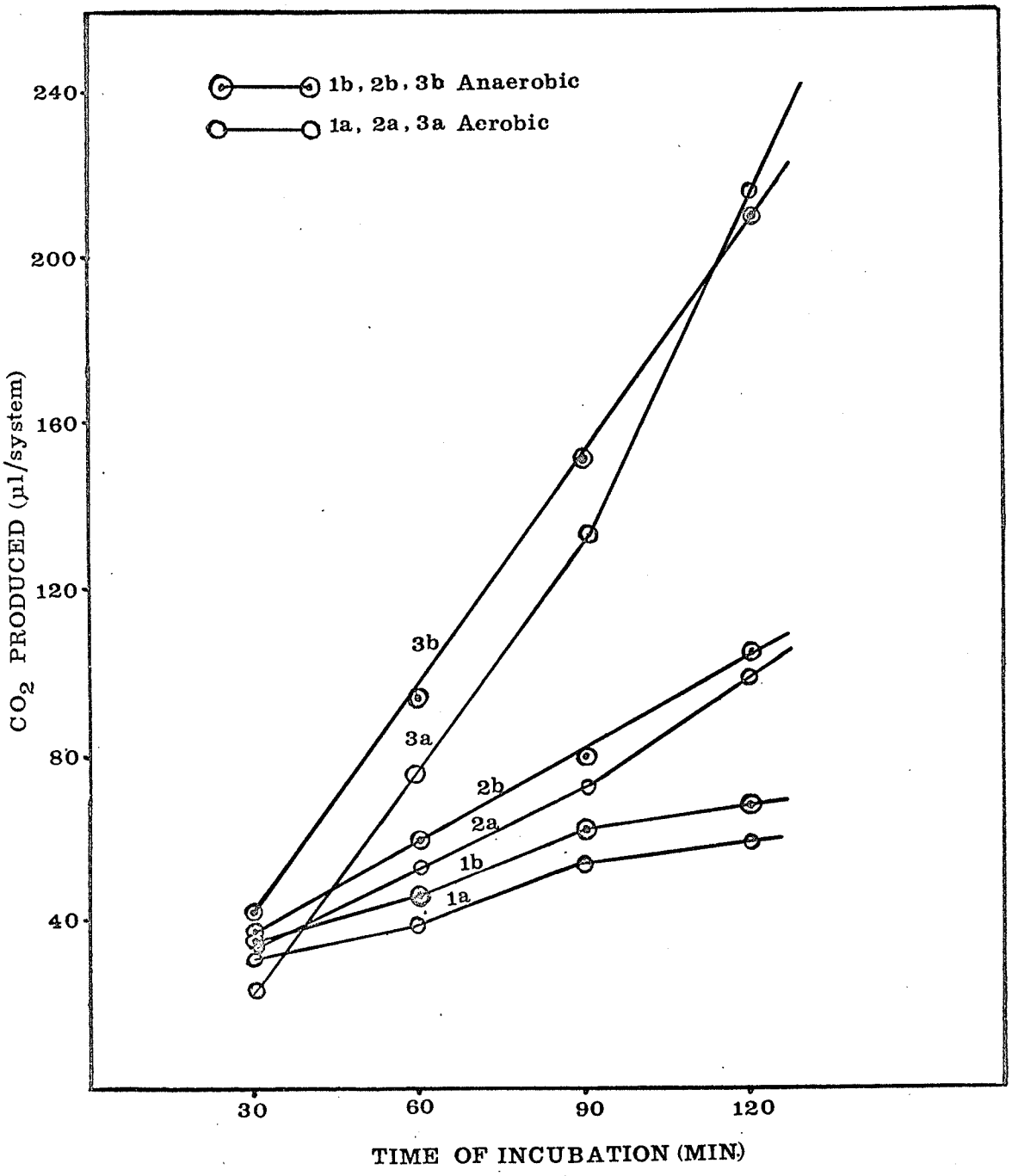


FIGURE 12. Effect of increasing glucose-1-phosphate on glycolysis by crude wheat embryo extract. Reaction mixture contained 1.0 ml of the enzyme preparation.
Systems 1a, 1b contained 20 µmoles G-1-P; 2a,2b contained 40 µmoles G-1-P; and systems 3a,3b contained 80 µmoles G-1-P in addition to the other reagents indicated in Methods (see text p.42).

(4) Effect of Aerobic Conditions on the Utilisation of Glycolytic Intermediates Serving as Substrates

In order to further determine the point (in the EMP pathway) at which oxygen asserts its effect, some experiments were carried out in which the crude wheat embryo extract was incubated with G-1-P, F-6-P, F-1,6-diP and 3-PGA respectively.

The breakdown of F-6-P was generally inhibited in air more than any of the other compounds (Table XII). This appears to indicate that the activity of the enzyme converting F6P $\longrightarrow$ F-1,6-diP, i.e. phosphofructokinase is being inhibited by oxygen, rather than any of the enzymes converting F-1,6-diP to the Trioses. There is however some evidence that one, perhaps more enzymes are affected. Table XI also shows that under anaerobic condition the ratio of F6P accumulating generally exceeds that of 3-PGA accumulating - this also is a clear indication that phosphofructokinase is perhaps more severely effected than glyceraldehyde-3-phosphate dehydrogenase. These results supplement those of Hatch and Turner (1959) in that the aerobic utilisation of F-6-P and 3-PGA are both inhibited.

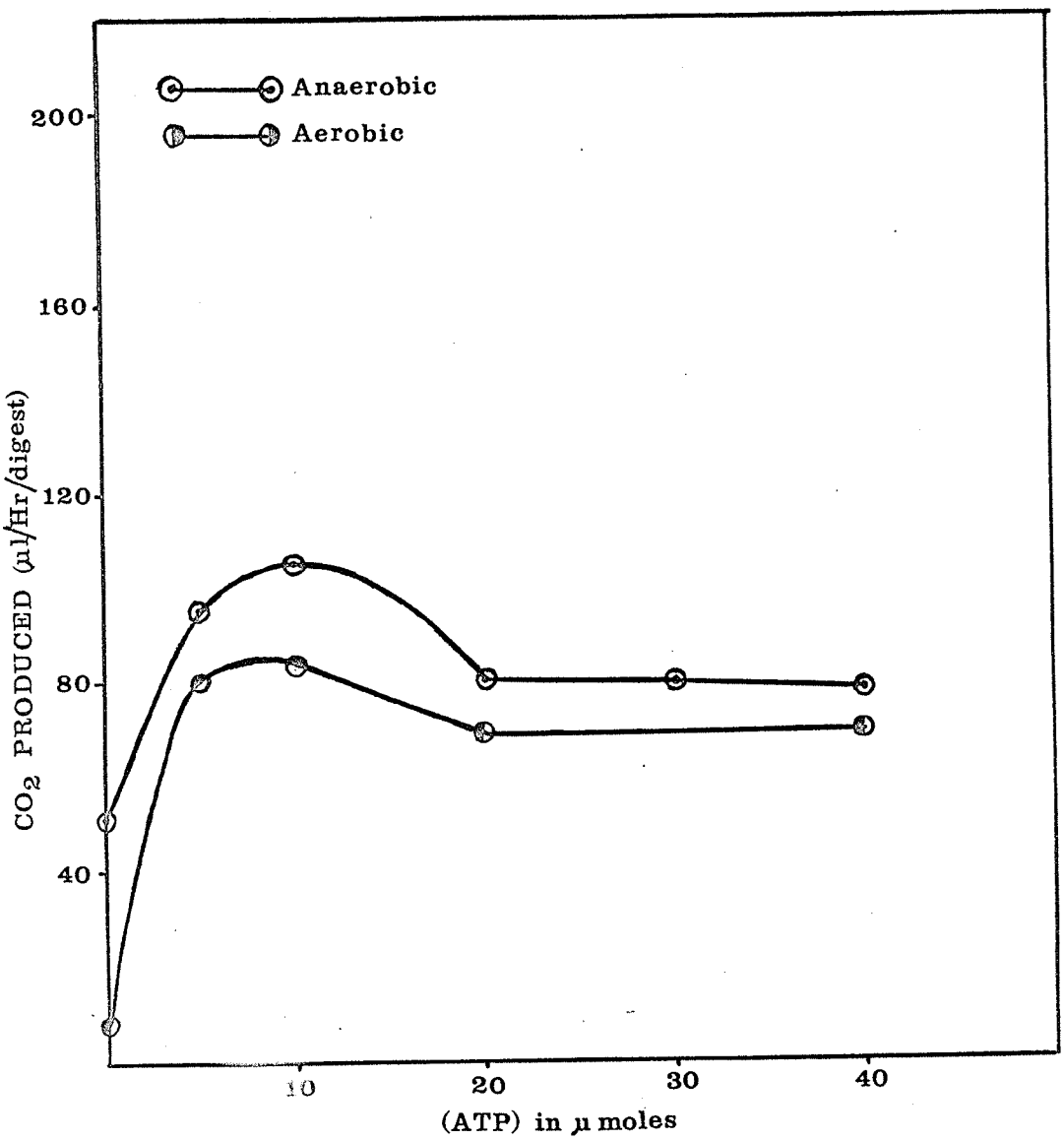
(5) Effect of Adenosine Triphosphate Concentration on the Aerobic Inhibition of Glycolysis

It has been reported in this manuscript (see page 53 that ATP at low concentrations serve as substrate for PFK, while at higher concentrations it serves as a negative

effector, an observation that is consistent with those of others for the enzyme from other sources (cf.) Atkinson and Walton (1965); Atkinson et al (1964); Salas et al (1963); Passonneau and Lowry (1962,1963), and Mansour and Mansour (1965). It has been speculated that the regulatory effect of ATP on PFK probably confers on it the unique property of controlling glycolysis (Lardy and Park,1955; Mansour and Mansour, 1962; and Wu,1964). It is interesting that the results (Fig 13) show that ATP at low concentrations operates as a substrate, while at higher concentrations both aerobic and anaerobic glycolysis of wheat embryos extract are inhibited. As shown in Table XIII, both anaerobic and aerobic CO_2 production are decreased at a high ATP concentration. From these results it can be concluded that this effect of ATP on glycolysis is brought about by its control on PFK, a speculation that will be consistent with the view that the inhibition of PFK by high level of ATP may be responsible for the Pasteur effect (Lynen et al, 1959; Mansour and Mansour, 1962 etc).

(6) Effect of AMP

Passonneau and Lowry (1962); Atkinson and Walton (1965), amongst others reported that the inhibition of PFK activity by ATP was relieved by AMP (see also results obtained on AMP as a positive effector for wheat embryo PFK, page 59). When the crude wheat embryo extract was incubated with AMP at an inhibitory level of ATP (Fig 14)



FIGU 3. Effect of increasing ATP concentration on the glycolysis of crude wheat embryo extract. System contained the reagents indicated in Metho (see text p.42).

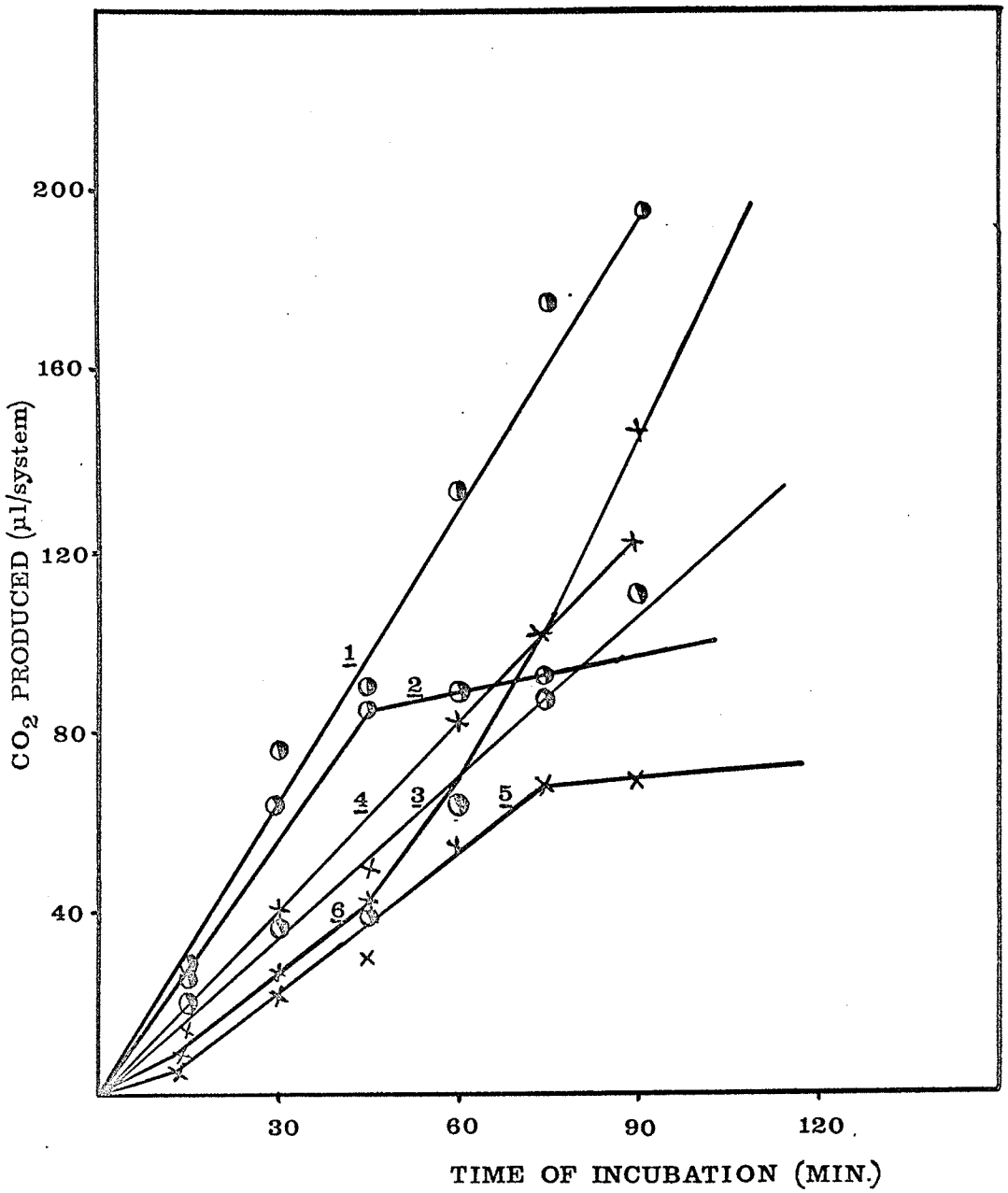


FIGURE 14. Effect of AMP (at an inhibitory ATP level) on glycolysis by crude wheat embryo extract.
(1) Anaerobic control system contained 10.0 µmoles ATP.
(2) Anaerobic system contained 20 µmoles ATP (inhibitory conc.)
(3) Anaerobic system contained 20 µmoles ATP + 10 µmoles AMP
(4) Aerobic control system contained 10.0 µmoles ATP.
(5) Aerobic system contained 20 µmoles ATP (inhibitory conc.)
(6) Aerobic system contained 20 µmoles ATP + 10 µmoles AMP.

In addition to the above, system contained the reagent listed in text, p.42.

TABLE XIII. Effect of Some Compounds on Aerobic and Anaerobic Glycolysis in Crude Wheat Embryo Extracts.

*Substrate	Addition	Concentration.	Glycolytic Activity GL(N)	Glycolytic Activity GL(O)	GL(N)/GL(O)
G-1-P	Control (no addition)	-	82	62	1.32
	P_i	10	90	72	1.25
	"	20	87	76	1.14
"	No addition	-	130	88	1.47
	ATP (inhibitory)	20	90	69	1.30
	Inh.ATP + AMP	4	82	83	0.98
	"	10	86	102	0.84
"	no addition	-	140	123	1.13
	Inosine	10	139	118	1.17
"	-	-	43	37	1.16
	Imidazole	10	69	45	1.53
"	-	-	84.6	83	1.01
	Benzimidazole	10	63	-45	-
"	-	-	133	93.8	1.41
	Benzimidazole	10	71	-85	-

*Systems contained 40.0 µmoles of G1P as substrate. These sets of results were repeated 3 to 4 times with similar results, and those on inosine, imidazole and benzimidazole are averages of three such results.

it was found that it reverses the aerobic inhibition of glycolysis markedly, while it appeared to act as a negative effector on anaerobic glycolysis. In some of the experiments conducted, after 60 - 90 minutes of incubation, it was observed that aerobic CO_2 production in the presence of AMP far exceeded anaerobic CO_2 production. The stimulation of aerobic glycolysis found in these experiments is significant, in that it again makes it possible to agree with the postulate that, PFK most certainly controls glycolysis. Wu (1964) showed that in aerobic experiments with kidney cortex silices, there was always a high level of ATP and a low level of AMP. The reversal of the inhibition of aerobic glycolysis by AMP is therefore significant. The level of intracellular AMP must have been raised by its addition, thus enabling it to play its role as a positive effector for PFK.

(7) Effect of Imidazole, Inosine etc., on Aerobic and Anaerobic Glycolysis of Wheat Embryo Extracts

Wu (1965) has shown that imidazole activates and that inosine inhibits the glycolysis of Ascites tumor cells. Accordingly these compounds and others were tested for their effect on glycolysis in wheat embryo extracts. Typical experiments are shown in Table XIII. The crude wheat embryo extracts were incubated in the usual assay system. Inorganic phosphate and imidazole activated both

aerobic and anaerobic glycolysis. On the other hand inosine inhibited aerobic glycolysis, and showed no discernible effect on anaerobic glycolysis. Adenosine-5'-monophosphate stimulated and reversed the inhibitory effect of ATP on aerobic glycolysis, and intensified the inhibition of anaerobic glycolysis. The results obtained here are consistent with those of Wu and Racker (1959,1963); and Wu (1964,1965).

DISCUSSION

Experiments have shown that the properties of the phosphofructokinase from pea (Pisum sativum L. var. Tall Telephone syn. Alderman) and wheat (Triticum aestivum L. var. Selkirk) show some unique features, which are consistent with the view that this enzyme falls into a class of enzymes that serve a regulatory function in vivo. The term 'allosteric protein' was coined for this group of enzymes (Monod et al, 1963).

Phosphofructokinase was isolated from various plant material and partially purified. During the course of isolation and purification, it was observed that maceration leads to inactivation of the enzyme particularly the enzyme from wheat and spinach leaves. It is however relevant to add that addition of a sulfhydryl compound e.g. Cleland's reagent (dithiothreitol) partially stops this inactivation. The protection offered by Cleland's reagent for example suggests that in all likelihood, PFK possesses an -SH group in its active centre or centres, that this group or groups are probably exposed and being exposed they are easily destroyed or interferred with during maceration. The addition of sulfhydryl compound probably supplies the lost -SH group.

It is also suggested that PFK is a flexible protein, the flexibility of which confers on it, the ease of undergoing conformational changes at maceration. It is also

significant that in some experiments in which 0.05 M fructose was added to the extracting medium a 6-fold increase in initial activity was obtained; addition of 10^{-4} M $MgCl_2$ also led to a 3-fold increase (Table III). This suggests that these compounds probably bind to the enzyme surface, in such a way that the active centre or centers are no longer exposed to inactivation by maceration.

Experiments also show that plant tissue PFK is not only sensitive to maceration, but also to dialysis. After dialysis, it was observed that wheat leaf PFK retained little or no activity. The behavior of this enzyme is similar to most others particularly glucose-6-phosphate dehydrogenase which is inactivated by dialysis and reactivated by warming or addition of NADP (Kirkman and Hendrickson,1962). The fact that dialysis leads to reduction or complete loss of activity suggests that the activity of the enzyme is due to a small molecule or molecules. Chromatography on Sephadex G-25 column (a step used to remove NaCl) leads to instability of the enzyme. It is probable that loss of activity during dialysis is due to excess loss of NaCl, and this will be comparable with the result obtained following removal of salt from the partially purified enzyme. That this might be the case, is supported by the fact that after the enzyme has been dialysed for 16 hours, no activity was detected, whereas after dialysis

for 8 hours or 4 hours, 25-30% of the initial activity was retained (See Table VIII, in the text). Although the addition of Cleland's reagent to the dialysing medium restored the activity to about 35% of the undialysed extract, the mechanism of action of this sulfhydryl compound, in this respect, is not understood.

Other treatments that inactivate this enzyme include, storage for over five days, heat under any conditions beyond a time of 30 seconds and high acid and alkaline pH.

Adenosine-5'-monophosphate, in the presence of P_i weakly reactivated the inactive enzyme. It is relevant again to note that this is consistent with the view that AMP is a positive effector for the phosphofructokinase from wheat embryo and peameal.

It has been shown in these studies that certain divalent metals and some compounds are potent inhibitors or activators of plant tissue PFK. Notable amongst the inhibitors are citrate, inosine, Cu^{++}, Zn^{++} and cyanide, while acetate, imidazole, pyrophosphate and Fe^{+++} are activators. The inhibition by citrate, inosine and Zn^{++}, and the activation by imidazole, P_i and Mg^{++} are of particular interest, since all these compounds have been shown to be tied up with the controlling effect of PFK on glycolysis (Salas et al, 1965, Parmeggiani and Bowman, 1963; and Wu, 1964, 1965).

The generally low activity obtained from the wheat leaf

extract led to experiments in which the effect of these extracts were tested on phosphofructokinase partially purified from both peameal and wheat embryos. The finding that the extracts inactivated peameal and wheat embryo PFK also led to the speculation that some inhibitory principle might have been involved. Neutralised supernatants after perchloric acid precipitation however showed no inhibitory effect. Since no conclusive result was obtained, it is suggested that a different approach for the isolation of the inhibitory principle be made to obtain a more successful result.

In these studies experiments show that benzimidazole maintained the level of PFK in Selkirk wheat leaves even 5 days after detachment. Results in this laboratory have indicated that benzimidazole treated Khapli wheat leaves generally show high soluble sugar levels (Mishra,1963). There is probably a link between these two observations. The increase in soluble sugar content in benzimidazole treated leaves may be due to more active phosphorylation due in part to ample or sufficient supply of phosphofructokinase.

The enzyme is probably specific for F-6-P, although G-6-P and R-5-P were phosphorylated (Table V). The doubt as to the specificity of the enzyme for F-6-P is based on the fact that the partially purified enzyme might not be

absolutely free of glucose isomerase. On the other hand, it is quite clear that the enzyme is non-specific for ATP, the second substrate. Cytosine, guanosine and uridine-triphosphates and even ADP can substitute for ATP. In fact it is significant that if ATP is substituted by GTP, there is no inhibition by excess nucleotide within the same range. This finding does not completely support the view of Atkinson and Walton (1965) who indicated that nucleotides other than ATP only have slight activity as phosphate donors.

The effects of changes in concentrations of substrates and or other components in a reaction, such as cofactors, on the binding of other reaction components by the enzyme is a standard method employed in the study of enzyme kinetics. For a reaction which follows the true Michaelis prediction, optimal concentrations for each reaction component can be calculated. The V_{max} and the K_m in such circumstances vividly define the rate-substrate curve. In the case of an enzyme (allosteric) reaction which deviates markedly from the normal Michaelis pattern, calculation of K_m or V_{max} and inhibition constants mean very little, if at all, in elucidating the true and actual properties of the enzyme. These conventional methods for the study of allosteric enzymes are inadequate, but they would have to be employed, if only for the fact that they aid in determining what enzyme reaction follows the normal Michaelis predictions, and which ones do not.

Results in these studies show that the plot of rate as a function of F-6-P concentration is markedly sigmoid, when ATP serves as a phosphate donor. The curve appears much less so when GTP serves as a phosphate donor. Also when reaction rate is plotted as a function of ATP concentrations, with F-6-P at 2.5 or 5.0 µmoles (Figs 7 and 8) the velocity attains a maximum value at about 1.0 µmole ATP, and decreases at higher concentrations. The affinity of the enzyme for F-6-P appears to be decreased by high ATP concentrations. The inhibition caused by ATP is most probably due to its effect on enzyme-substrate binding sites.

Adenosine-5'-monophosphate which increases the affinity of the enzyme for F-6-P, increases the reaction rate at high ATP concentrations, probably by replacing ATP at the effector site. As the concentration of AMP is increased, however, a point is reached at which further addition of AMP tends to decrease rate. This is probably due to the fact that F-6-P becomes the limiting factor. Since the binding of ATP to the effector site on the enzyme appears to favour the inactive form of the enzyme, and the binding of AMP favours the active form, it could be concluded that ATP is a negative effector, while AMP is the positive effector for both peameal and wheat embryo phosphofructokinase.

Although no specific model is proposed, it would appear

that the conformation of both the peameal and wheat embryo PFK are similar to the Escherichia coli enzyme (Atkinson and Walton,1965) which is easily changed or modified by the binding of AMP or ATP at some specific site.

Passonneau and Lowry (1962,1964); Sols et al (1963); Vinuela et al (1963); and Wu (1965) have supplied evidence that the regulation of phosphofructokinase activity from various tissues of living organisms may control glycolysis in these organisms.

Experiments reported here support this view. Firstly, the breakdown of hexose and hexose phosphates to carbon dioxide and ethanol by the crude extracts from viable wheat embryos suggest the operation of a complete glycolytic system. Additional evidence was obtained by the conversion of F-6-P, F-1,6-diP and 3-PGA into carbon dioxide and ethanol by the extracts.

There is a clear evidence that no alternative pathway was operating to any significant level. The ratio of CO_2: ethanol was generally less than 60%, and this is far short of a greater than unity ratio, expected, if the breakdown of hexose and hexose phosphates was by the pentose phosphate pathway.

Secondly experiments conducted under aerobic and anaerobic conditions showed that there is more CO_2 production in anaerobiosis than in aerobiosis - a condition suggestive of

the operation of the Pasteur effect. Generally, in all experiments, the ratio

$$\frac{Gl(N)}{Gl(O)} \left(\frac{CO_2 \text{ production in nitrogen(anaerobic)}}{CO_2 \text{ production in air (aerobic)}}\right)$$

far exceeded unity. Further evidence for the operation of the Pasteur effect was provided by experiments based on measurement of the levels of certain intermediates of the glycolytic system . The breakdown of sugar phosphates was found to be much higher under anaerobic conditions than under aerobic conditions. Critical evaluation of the data presented, clearly indicate that the control of glycolysis and consequently the operation of the Pasteur effect is due to inactivation of PFK, partly by ATP and possibly by other compounds. Salas et al (1965) and Parmeggiani and Bowman (1963), suggested, the inhibition of PFK by ATP alone cannot explain the control of the Pasteur effect. They showed that citrate also participates in the inactivation of the enzyme, and in the control of glycolysis. Although the effect of citrate on glycolysis of the extracts from wheat embryo was not directly investigated, the fact that citrate is one of the potent inhibits of the enzyme appears to support this view.

Raising the concentration of ATP is paralleled by a decrease in CO_2 output, both under aerobic and anaerobic conditions. This again offers a clear evidence that

inhibition of PFK certainly controls glycolysis. It will be recalled that in the kinetic studies high ATP concentration markedly inhibited PFK from wheat embryos.

Perhaps the most interesting and significant finding is that AMP should again relieve the inhibition of glycolysis by a high concentration of ATP. It would be recalled that

(a) AMP increases the affinity of PFK for F-6-P

(b) It has been suggested that AMP possibly replaces ATP at the effector site, on PFK molecule, in order to achieve situation (a),

with this in mind, the conclusion that the regulation of PFK activity controls glycolysis of wheat embryo extracts can again be made.

Analysis of data obtained by measuring the level of intermediates, showed that F-1,6-diP accumulates more under anaerobic conditions than under aerobic conditions, fructose-6-phosphate on the other hand accumulates more under aerobic conditions than under anaerobic conditions. This suggests that the enzyme converting F-6-P$\longrightarrow$F-1,6-diP is being inactivated in air, or that it is more active in nitrogen than in air. The extent of inhibition of PFK by ATP has been attributed to the level of intracellular P_i (Wu, 1965). The lower the P_i level, the greater the inhibition. Although the P_i level has not been measured in these studies, evidence

was obtained in support of this view. By raising the concentration of P_i in the assay system, both anaerobic and aerobic glycolysis, increased, with a more pronounced increase in the aerobic system. Imidazole showed the same effect as P_i, while inosine and benzimidazole showed inhibitory effects on aerobic glycolysis. Benzimidazole also inhibited anaerobic glycolysis, while inosine shows no discernible effect. Wu (1965) suggested that inosine inhibited aerobic glycolysis in ascites tumor cells by lowering the intracellular P_i level. No evidence is presented on this view, but it would appear reasonable to assume that the effect of inosine on aerobic glycolysis of wheat embryo extracts probably follows a similar pattern, remembering that the lower the P_i level, the higher the inhibition of PFK by ATP. If inosine lowers the P_i level as Wu (1965) suggested, then ATP will show more inhibitory effect on PFK and consequently on glycolysis.

Other than the activation and inactivation of PFK, there appears to be some other subsidiary rate-limiting factors. Hatch and Turner (1959) reported that the aerobic inhibition of glycolysis in pea extracts was due to oxidative inactivation of 3-phosphoglyceraldehyde dehydrogenase. There was more CO_2 production under anaerobic condition than under aerobic condition when 3-PGA served as substrate. There was more accumulation of 3-PGA in air

than in nitrogen. All these would appear to support that there is in fact inactivation of 3-PGA dehydrogenase. The ratio Gl(N)/Gl(0) was however generally less in experiments where 3-PGA served as substrate than that obtained when F-6-P served as substrate. The ratio of F-6-P lost in air to that lost in nitrogen is generally higher than the ratio of 3PGA lost under the same conditions.

Another factor that contributes to the control of glycolysis observed in these studies is the availability of glucose-1-phosphate. Raising the concentration of G-1-P is closely paralleled by an increase in the rate of aerobic CO_2 output, whereas the rate of CO_2 output anaerobically is not dependent on the supply of G-1-P.

The ratio Gl(N)/Gl(0) is higher when F-6-P served as substrate than the ratio obtained when any other intermediate served as substrate. High concentration of ATP (the negative effector for PFK) inhibits aerobic glycolysis and this inhibition is relieved by AMP (positive effector for PFK). The level of F-1,6-diP is lower under aerobic than under anaerobic conditions. These suggest that the phosphorylation of F-6-P is rate limiting in the glycolysis of the extracts from wheat embryos.

These findings with the extracts from wheat embryos confirm the results reported for Fasciola hepatica, muscle,

yeast and ascites tumor cell PFK (Mansour and Mansour, 1962; Passonneau and Lowry, 1962; Wu, 1964,1965; and Salas et al, 1965). These reports state that aerobic glycolysis is controlled by the regulation of PFK activity and that the inhibition of the enzyme by some compounds, notably high concentration of ATP, may be responsible for the Pasteur effect.

SUMMARY

A method for the preparation of a 105-fold purified phosphofructokinase from pea (Pisum sativum L. var. Tall Telephone syn. Alderman) and wheat (Triticum aestivum L. var. Selkirk) has been described.

The crude extract is highly sensitive to maceration and dialysis. Addition of sulfhydryl groups to grinding medium protects the inactivation of the enzyme during maceration and dialysis.

The partially purified enzyme is stable in the presence of sodium chloride.

The kinetic behaviour of phosphofructokinase from both peameal and wheat embryos is generally similar to that of the enzyme from Escherichia coli, yeast and animal tissues.

Activity is maximal at pH 8.0 to 8.5. Besides fructose-6-phosphate G-6-P was found to be readily phosphorylated by the enzyme.

Nucleotides other than adenosine triphosphate, especially guanosine and uridine triphosphates and adenosine diphosphate, can serve as phosphate donors.

The affinity of the enzyme for fructose-6-phosphate is decreased by high concentrations of adenosine triphosphate, while the affinity is increased by adenosine monophosphate, magnesium and inorganic phosphate.

Sigmoid rate curves, characteristic of regulatory enzymes, were observed for the plots of rate versus fructose-6-phosphate concentrations.

Observations recorded are consistent with the model

proposed for the Escherichia coli enzyme, that the binding of adenosine-monophosphate or adenosine triphosphate at some effector site leads to conformational changes that modify the affinity of the enzyme for fructose-6-phosphate.

Crude extracts from wheat embryos supplemented with necessary cofactors possessed complete glycolytic activity.

Glycolysis by the crude enzyme preparation was inhibited by aerobic conditions, a finding that is consistent with the definitions of the Pasteur effect.

The degree of aerobic inhibition of glycolysis leading to the establishment of the Pasteur effect appear to depend largely on the regulation of phosphofructokinase activity.

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