PHOSPHOGLUCOMUTASE, PHOSPHORIBOMUTASE AND PHOSPHOGLUCOSE ISOMERASE IN LINGCOD MUSCLE

by

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A B S T R A C T

In view of the increasing interest in the biochemistry of fish and particularly fish enzymes, a study has been made of some of the glycolytic enzymes of fish muscle. Some important properties of two enzymes of the Embden-Meyerhof pathway, phosphoglucomutase and phosphoglucose isomerase, and one enzyme of the "hexosemonophosphate shunt", phosphoribomutase, are reported.

The procedure for purifying these enzymes by ammonium sulfate precipitation, heating or chromatography on diethylaminoethyl cellulose, is given. However, most of the experiments were carried out with crude extracts, since the partially purified enzymes were not very stable under the usual conditions of storage of enzymes.

The following properties of phosphoglucomutase and of phosphoribomutase are reported: equilibrium of the reaction, optimum pH, requirement for magnesium and cysteine, and the effect of glucose-1,6-diphosphate, ribose-1,5-diphosphate and deoxyribose-1,5-diphosphate on its activity.

A study of the equilibrium and optimum pH of the phosphoglucose isomerase reaction is also reported.

The similarity between the properties of these fish enzymes and the corresponding mammalian enzymes is discussed. The question of identity or non-identity of phosphoglucomutase and phosphoribomutase is also examined.

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I. GENERAL INTRODUCTION

Statement of the problem:

That fish muscle glycogen is broken down very rapidly after death has occurred was well established by MacLeod and Simpson (1927) and by Leim et al (1927). They also demonstrated a rapid post-mortem decrease in muscle glycogen of fish, caught and kept under different conditions. It has been observed (Jones, 1958) that free glucose increases significantly in muscle of dead fish. This phenomenon has not been satisfactorily explained as yet. On the other hand, fish are capable of producing lactic acid during strong physical exercise. This has been demonstrated by various workers among whom is Black (1955, 1957).

Course of formation of lactic acid from glycogen is well established in mammalian muscle. However there has been practically no serious biochemical approach to this problem in fish muscle.

Does the breakdown of glycogen in fish muscle proceed through a series of reactions catalysed by enzymes similar to those occuring in mammalian muscle? This question can only be answered after studies similar to those carried out with mammalian muscle have been made.

Definition of glycolysis:

As a general term, glycolysis means the physiological breakdown of carbohydrates to lactic acid. This breakdown of carbohydrates is achieved through a series of reactions, all of which, except one, are catalysed by enzymes.

Glycolysis differs from the alcoholic fermentation of yeast in two respects. First, fermentation cannot start with glycogen as substrate, but requires glucose or other sugars. Second, and more important, a common intermediate, pyruvic acid, is converted to different substances. In glycolysis, it is converted to lactic acid, while in fermentation, it is first converted to acetaldehyde and then to alcohol. Apart from this, both processes proceed through the same series of reactions, as shown in Fig. 1. This reaction sequence has been termed the Embden-Meyerhof pathway.

Glycolysis (as well as fermentation) is essentially anaerobic, although it can take place in the presence of molecular oxygen. Under either condition, it is an important source of energy, as it yields adenosine triphosphate (ATP), a substance indispensable for muscular activity. Under aerobic conditions, pyruvic acid is oxidized with the formation of additional ATP. Glycolysis is coupled with aerobic metabolism in such a way that lactic acid does not accumulate when there is oxygen available. Thus, when 3-phosphoglyceric aldehyde is oxidized and phosphorylated to give 1, 3-diphosphoglyceric acid, two hydrogen atoms are liberated to give the reduced diphosphopyridine nucleotide (DPN.2H), and this compound in the absence of oxygen, serves to reduce pyruvic acid to lactic acid. In the presence or oxygen, however, DPN.2H is oxidized by the cytochrome system, pyruvic acid is oxidized in the tricarboxylic acid cycle.

That formation of lactic acid from carbohydrates is achieved by the series of reactions, as shown on Fig. 1, in most living tissues is very probable. However, it has also been established that microorganisms and

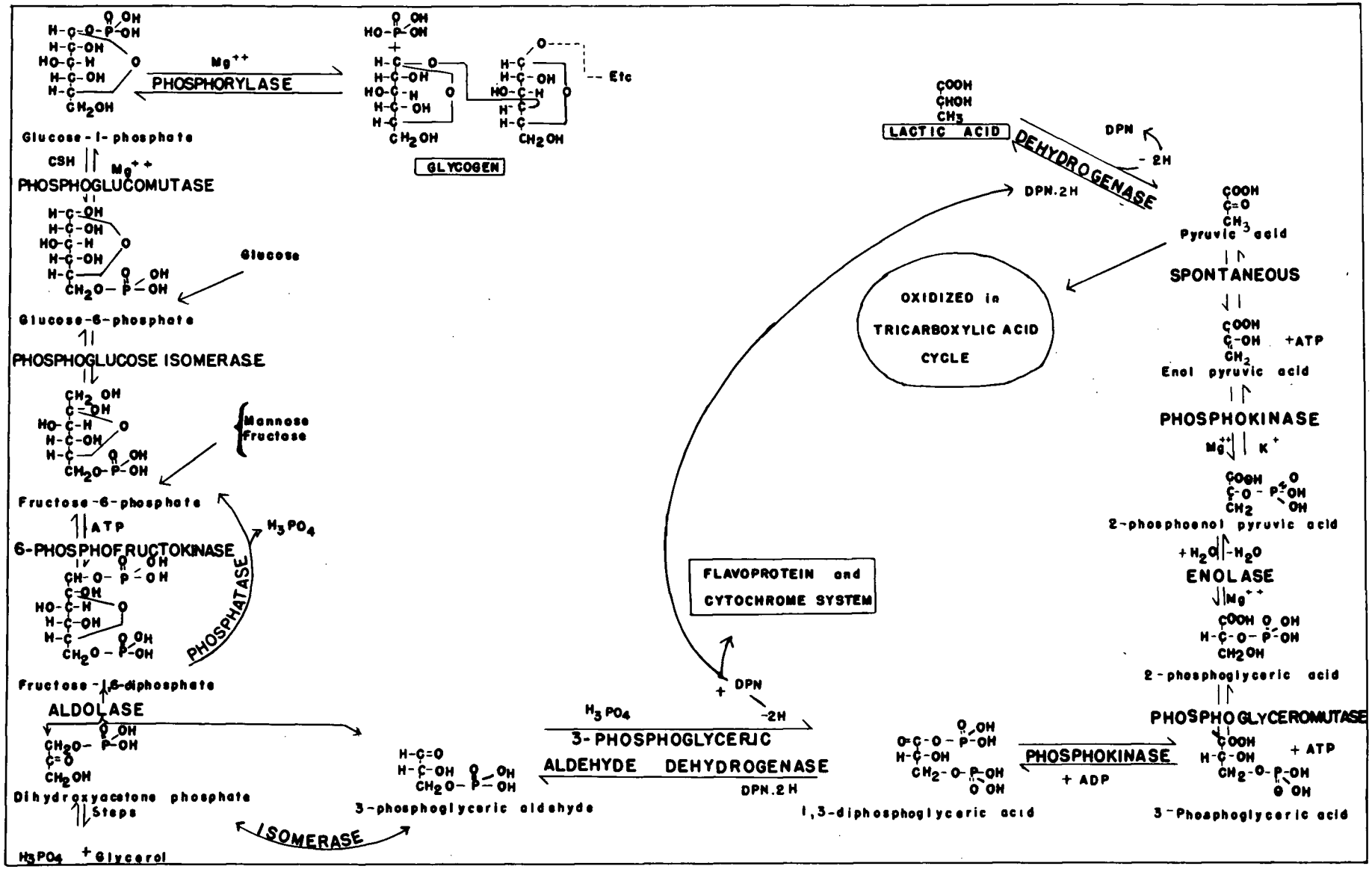


Fig. 1. Anaerobic carbohydrate metabolism.

liver make extensive use of the so called "hexosemonophosphate shunt" or "pentose cycle", as illustrated on Fig. 2. It would therefore be of the greatest interest to investigate the comparative importance of these two series of reactions in the different tissues of fish. Recent experiments (Tarr, 1959) have shown that two of the enzymes of this system, namely phosphoriboisomerase and ribulose 5'-phosphate 3'-epimerase (simply called epimerase in Fig. 2), are present in lingcod muscle.

Formation of pyruvic acid from carbohydrates may bypass these two known pathways. One example is the direct aerobic oxidation, without phosphorylation, of glucose to gluconate and then to 2-keto-6-phosphogluconate and then to pyruvate by some obligate aerobic microorganisms (Gunsalus et al, 1955).

There are also several secondary pathways for breaking down different carbohydrates. These are well summarized by Holzer (1959).

Since carbohydrate metabolism is very much influenced by the availability of oxygen in any tissue, it might very well be that fish, living under a limited supply of oxygen, will show some differences in the way their glycogen is metabolized, when compared to mammals which have much more oxygen at their disposal.

General outline of the present work:

After a preliminary discussion of certain general properties of enzymes, with special attention to fish enzymes, a report on a study of two enzymes from lingcod (Ophiodon elongatus) muscle will be given. These two enzymes catalyse two steps of Embden-Meyerhof pathway and are known as phospho-

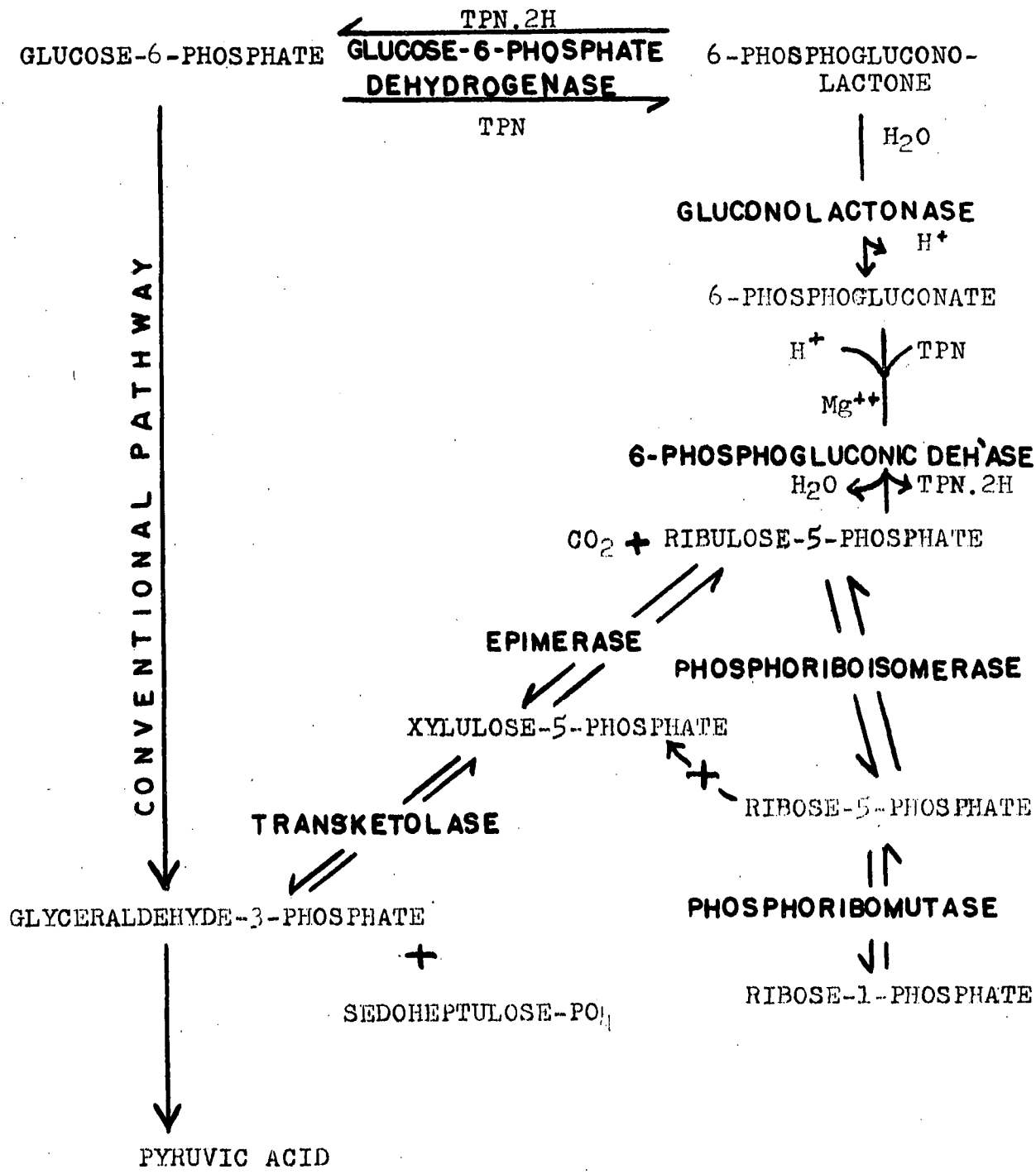


Fig. 2. Hexosemonophosphate shunt.

glucomutase (PGM) and phosphoglucose isomerase (PGI). A third enzyme, phosphoribomutase (PRM) is described in the same chapter as PGM because they were not separated. The possible lack of identity of PGM and PRM will be discussed later.

Some of the most important properties of these enzymes will be described, but no mention will be made of the effect of temperature on their activity, because it is considered that the optimum temperature is not a real characteristic of an enzyme. This is clearly discussed by Dixon and Webb (1958, p. 150) who state:

> "The optimum temperature is determined by the balance between the effect of temperature on the rate of the enzyme reaction and its effect on the rate of destruction of the enzyme; therefore the actual values of the optimum temperature have no special significance".

Much attention was given to the purification of these enzymes by ion-exchange chromatography. Unfortunately it was not possible to use purified preparations for all our experiments since they were not always stable at $0^{\circ}C$ or in the frozen state, and consequently could not be stored for use.

A general discussion will follow the report of the experimental results.

II. SOME GENERAL CHARACTERISTICS OF ENZYMES

All known enzymes are proteins and their study in any tissue is a study of a particular protein of this tissue. On the other hand, proteins are generally accepted as the basis of specificity of most living tissues. It can then be speculated that most enzymes, representing a certain portion of the total protein content of tissues, will share this specificity with

the structural proteins which are not believed to have any enzymatic function.

The properties of enzymes are still often studied using crude or partially purified tissue extracts. This procedure is sufficient for determining particular aspects of the activity of an enzyme, requirements for co-factors, optimum pH, etc. This procedure must still be employed in many cases since the purification of enzymes is usually achieved by long and difficult techniques and in many instances highly purified preparations are very unstable. That the enzymes which have been purified so far are all perfectly pure is very doubtful. It appears very clear that the whole question of species-specificity of enzymes, for which there is an unlimited field of investigation, will eventually have to be studied on perfectly pure preparations.

That the sequence of amino acids may vary with enzymes from different sources is rather probable but has never been proven experimentally. There are, however, some enzymes for which the amino acid composition (not the sequence) is known. These are listed by Dixon and Webb (1958, p. 483). Only a few peptides, having a much smaller molecular weight than the enzymes, have so far given the secret of their structure. Among these is insulin for which the species differences were established by Harris et al (1956).

It is believed that most enzymes are globular in shape. It is then easily understood that the peptide, which constitutes the molecule, must be folded in one way or another so that the pattern might differ from one species to another even if the amino acid sequence was almost identical.

There are some physical properties of enzymes which may or may not be criteria of differentiation. The form of the crystals, for instance, may often be related to the experimental procedure. In fact, the same enzyme, coming from the same tissue and even from the same preparation has often given different crystalline forms (Dixon and Webb, 1958, p. XIX to XXXIII).

Differences in enzymes coming from different sources may often be detected by some physical properties, such as solubility, electrophoretic mobility, sedimentation constant and immunological properties, provided these properties are studied under identical conditions and preferably by the same experimenter.

There are not many known cases of enzymes requiring different co-factors when coming from different tissues. Clark and MacLeod (1954) found some evidence that glycolysis in Lactobacillus arabinosus needed manganese, instead of magnesium as in other organisms, to form lactic acid from carbohydrates. This is an indirect proof that a given enzyme may require different metallic ions, since glycolysis is achieved by enzymes.

What do all these small differences mean in terms of enzymic activity? It looks very much as if a particular chemical reaction cannot be catalysed by two completely different enzymes. As a general rule, as Dixon and Webb (1958, p. 653) say, "the catalytic properties, specificity, activity, affinities, etc., of a given enzyme vary little with the source".

Is there, then, any reason to believe that fish enzymes differ in specificity and other properties from mammalian enzymes?

The metabolism of fish as a whole is rather similar to the metabolism

of mammals. Many of the reactions, common to both groups, are likely to proceed at different rates due to such factors as supply of oxygen, ambient temperature, differences in sexual behaviour, feeding habits, etc. Only by investigation can possible differences of enzymes coming from different sources be established.

There is some good evidence to claim that the amino acid composition of fish muscle differs from that of mammalian muscle but the importance of these differences is very difficult to establish. Block and Bolling (1951) have given a complete summary of the amino acid composition of different food materials: although the figures given were extracted from several different sources, and are not always easily comparable, they show that there is some difference between mammal and fish muscular proteins.

Certain specific fish proteins, like collagen for instance, have been found to be different from mammalian collagen in some respects (Eastoe, 1956). The review of the subject by Tarr (1958) suggests that several other groups of fish proteins might show significant differences when compared to mammalian proteins.

The importance of studies of fish enzymes is probably not well appreciated by those whose lot it is to study fish, but there is no doubt that more emphasis should be given to it. It is possible that studies of fish enzymes will eventually serve as an indispensable tool for taxonomists as well as physiologists and biochemists. Food technologists are already aware of the importance of enzymes in food preservation. Enzymes have obviously an important function in this fast expanding area of research.

It is beyond the scope of this work to present a list of all enzymes that have been studied in fish muscle and to compare them with mammalian enzymes. There have been but a few dozens of fish enzymes studied so far and of these, few were prepared from muscle. Tarr (1958) has given an interesting summary of this subject.

Little is known regarding the glycolytic enzymes in fish muscle. The presence of PGM and PRM activities was demonstrated in a crude enzyme preparation from lingcod muscle (Tarr, 1958a), and the latter was used to prepare deoxyribose-1, 5-diphophate for the first time (Tarr, 1957). Several glycolytic enzymes were found to be present in different tissues of fish by MacLeod (1959). Ono (1957) studied the properties of phosphorylase in carp muscle, while Shibata (1958) studied a partially purified aldolase from the same source. These two enzymes did not show any marked difference from their corresponding mammalian enzymes.

In addition to this, several acid-soluble phosphorous compounds concerned in the breakdown of glycogen have been found in fish skeletal muscle by Tarr (1950).

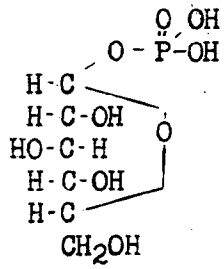
These very few observations suggest that the breakdown of glycogen to lactic acid in fish muscle follows a pathway similar to that found in mammalian muscle, but they are insufficient to draw any final conclusions.

III. PHOSPHOGLUCOMUTASE and PHOSPHORIBOMUTASE

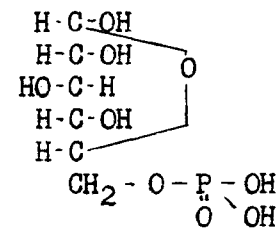
INTRODUCTION

The conversion of glucose-1-phosphate (G1P) to glucose-6-phosphate (G6P) was observed for the first time by Cori and Cori (1936). This is

a reversible reaction that can be written as follows:



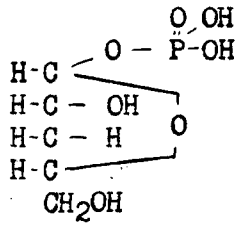
G1P



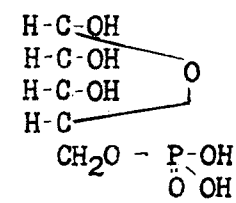
G6P

The presence of PGM that catalyses this reaction was demonstrated in different mammalian tissues and yeast by Cori et al (1938a, 1938b). Since that time, PGM had been found in many other tissues. It was observed in smooth muscle of hen stomach (Mejbaum, 1955) where it was found to be as active as in striated muscle. Its presence was also established in mammary tissues from lactating cows (Gander et al, 1956), in hibernating glands of animals (Grigor'eva. 1949), in brown adipose tissues (Mirshi, 1942), in the liver of fetal guinea pig (Nemeth et al, 1954) and in calcifying bones of young rabbits, rats and cats (Leynse, 1952). At least one protozoon was found by Ryley (1955) to contain it. Achromobacter Fischeri (Friedman, 1954) and probably most bacteria contain it. As noted previously, PGM was found in lingcod muscle by Tarr (1958a).

PRM catalyses a reaction similar to that catalysed by PGM. It is also reversible and can be written as follows:



Ribose-1-phosphate (R1P)



Ribose-5-phosphate (R5P)

Its presence in living tissues was first demonstrated by Schlenk and Waldvogel (1946). It was also found in muscle (Sable, 1952) and in liver (Abrams and Klenow, 1951). So far it has been generally accepted as identical with PGM.

MATERIALS

G1P. Dipotassium salt with 2 molecules of water, was obtained from the Sigma Chemical Co., Saint Louis, Mo., U.S.A., and also from the Nutritional Biochemicals Co., Cleveland, Ohio, U.S.A. Both gave the same amount of labile phosphorus. In the study of the effect of sugar diphosphates on PGM activity, only the NBCo product was used as substrate.

G6P. Dipotassium salt with $1\frac{1}{2}$ molecule of water, was purchased from the Sigma Chemical Co.

R1P. The dicyclohexylammonium salt prepared enzymatically by Tarr (1958) was used. It was at least 95% pure.

Ribose-1, 5-diphosphate ($R1,5P_2$). The tetracyclohexylammonium salt was used. This was an analytically pure, chemically synthesised product (Tener and Khorana, 1958).

Deoxyribose-1,5-diphosphate ($DR1,5P_2$). The tetracyclohexylammonium salt was used. This was prepared enzymatically by Tarr (1957).

Glucose-1,6-diphosphate ($G1,6P_2$). This was prepared enzymatically as the dibarium salt ($C_6H_{10}O_{12}P_2Ba_2$) from brewer's yeast (Carling Breweries) by Dr. H.L.A. Tarr, following the method of Cardini et al (1949).

L-cycteine.HCl was obtained from the California Foundation for Biochemical Research, Los Angeles, Calif.

Magnesium sulfate ($MgSO_4.7H_2O$), sodium molybdate ($Na_2MoO_4.2H_2O$), sodium bisulfite ($NaHSO_3$), sodium carbonate (Na_2CO_3), copper sulfate ($CuSO_4.5H_2O$) were obtained from the J.T. Baker Chemical Co., Philippsburg, N.J., U.S.A.

Magnesium chloride ($MgCl_2.6H_2O$) was obtained from Merck and Co., Ltd., Montreal, P.Q.

Tris (hydroxymethyl) aminomethane was purchased from the Sigma Chemical Co.

Diethylaminoethyl cellulose (DEAE-cellulose) Type 20 was obtained from the Brown Co., Berlin, New Hampshire.

Dowex 50 x 8 (200-400 mesh, analytical grade) was obtained from the California Foundation for Biochemical Research.

8-hydroxyquinoline was purchased from Eastman Organic Chemicals, Rochester 3, N.Y. Division of Eastman Kodak Company.

Sodium tartrate ($Na_2C_4H_4O_6.2H_2O$) was obtained from the Fischer Scientific Co., Fair Lawn, N.J.

Bovine albumin (crystalline) was obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

Triphosphopyridine nucleotide (TPN), as monosodium salt, was purchased from the Sigma Chemical Co.

Source of enzyme: For most experiments, lingcod muscle, kept at -20°C (up to three months) was used.

METHODS

A Beckman DU spectrophotometer (with 1 cm. cells) was used for all measurements of optical density.

A Beckman pH meter model G was used for all pH measurements.

Enzyme assay for PGM: Tris (hydroxymethyl) aminomethane-HCl was used as buffer with a final concentration of 0.02M at approximately 7.5. In some preliminary assays, however, no buffer was used, all solutions being adjusted to proper pH before adding the enzyme. The final concentration of cysteine and Mg^{++} (as $MgCl_2$ or $MgSO_4$) was 0.01M and 0.001M respectively unless otherwise stated. The dipotassium salt of G1P was used as such for substrate. The extract containing the enzyme was diluted in cold water immediately before being used. When a greater dilution was needed (e.g. 100 or 200 fold), it was found that it was much preferable to dilute it in albumin (0.1%) since a dilution in water in such a case caused rapid destruction of enzyme and brought erroneous results. The temperature used was 30° C in all cases. A water bath was used, the temperature being controlled by an ultra-thermostat (Colora, no. K-0992).

Usually all ingredients were incubated for 5 minutes before adding the enzyme. In some cases, however, the enzyme was added to a cold substrate (0°C). In this latter case, only a few seconds were necessary to reach 30°C in the thermostat. Since a very small volume was used, no

detectable difference could be found between the two methods; this was checked in several different experiments.

The reaction was stopped with an amount of 2N H_2SO_4 equal to the final volume of the reaction mixture in order to attain a final concentration of 1N H_2SO_4. The products of the reaction could then be analysed by the following methods:

1) Qualitative detection of G6P: Paper chromatography was used to check the appearance of G6P when G1P was incubated with the extract. The reaction mixture, which was deproteinized by 2N H_2SO_4, was neutralized with 2N NaOH after removal of protein by centrifugation. An aliquot of this mixture was then spotted on Whatman No. 1 filter paper. Chromatographic separation of the products of the reaction was then carried out using 16-hour descending development with n-propanol: 28% NH_4OH:H_2O (6:3:1) as solvent, at room temperature (Hanes and Isherwood, 1949). The distance travelled by the solvent was approximately 40 cm. The paper was dried in air and then sprayed with the following reagent prepared according to Hanes and Isherwood (1949) as follows:

60% w/w perchloric acid.....	5 ml
1N hydrochloric acid.....	10 "
4% w/v ammonium molybdate.....	25 "
H_2O.....up to	100 "

The paper was then dried in an oven at 100° C for 3 minutes. G1P appeared very readily when paper was allowed to reabsorb moisture over a steam bath. It was sometimes necessary to expose the paper to ultraviolet light in order to make G6P appear. G1P gave a yellow spot with an R_f value of 0.16 while G6P gave a blue spot with an R_f value of 0.13. In order to identify these compounds, pure G1P and G6P were spotted and developed

under the same conditions on the same paper. It was found that, with both these compounds, 2 to 4 µg of phosphorus was adequate to produce good spots.

2) Quantitative measurement of G6P: G6P can be measured by its capacity to reduce TPN. This reaction is catalysed by glucose-6-phosphate dehydrogenase (Horecker and Wood, 1957).

A few attempts were made to use this method for measurement of PGM activity, but for several reasons it was abandoned. One lot of highly purified enzyme (from the Sigma Co.) that was first used had lost too much activity after having been kept at -20°C for about 6 months. It was also found to contain some PGI, an enzyme which obviously interferes with this specific dehydrogenase. A different cruder preparation (from Nutritional Biochemicals Corp.) was very active. At this time, because the partially purified PGM was rather unstable, the crude enzyme preparation was used to study PGM activity. In this case the use of glucose-6-phosphate dehydrogenase could not easily be employed. Nevertheless the procedure for enzymic determination of PGM is recorded below.

A standard curve (Fig. 3) was prepared using different amounts of a standard solution of G6P. The following ingredients were mixed in a 1-cm quartz cell with the following final concentrations:

TPN (as monosodium salt)	0.001M
Tris-HCl buffer (pH 7.5)	0.0375M
Mg^{++} (as $MgCl_2$)	0.01M

Glucose-6-phosphate dehydrogenase: 0.02 ml of the original suspension bought from The Nutritional Biochem. Corp. and diluted twentyfold was used.

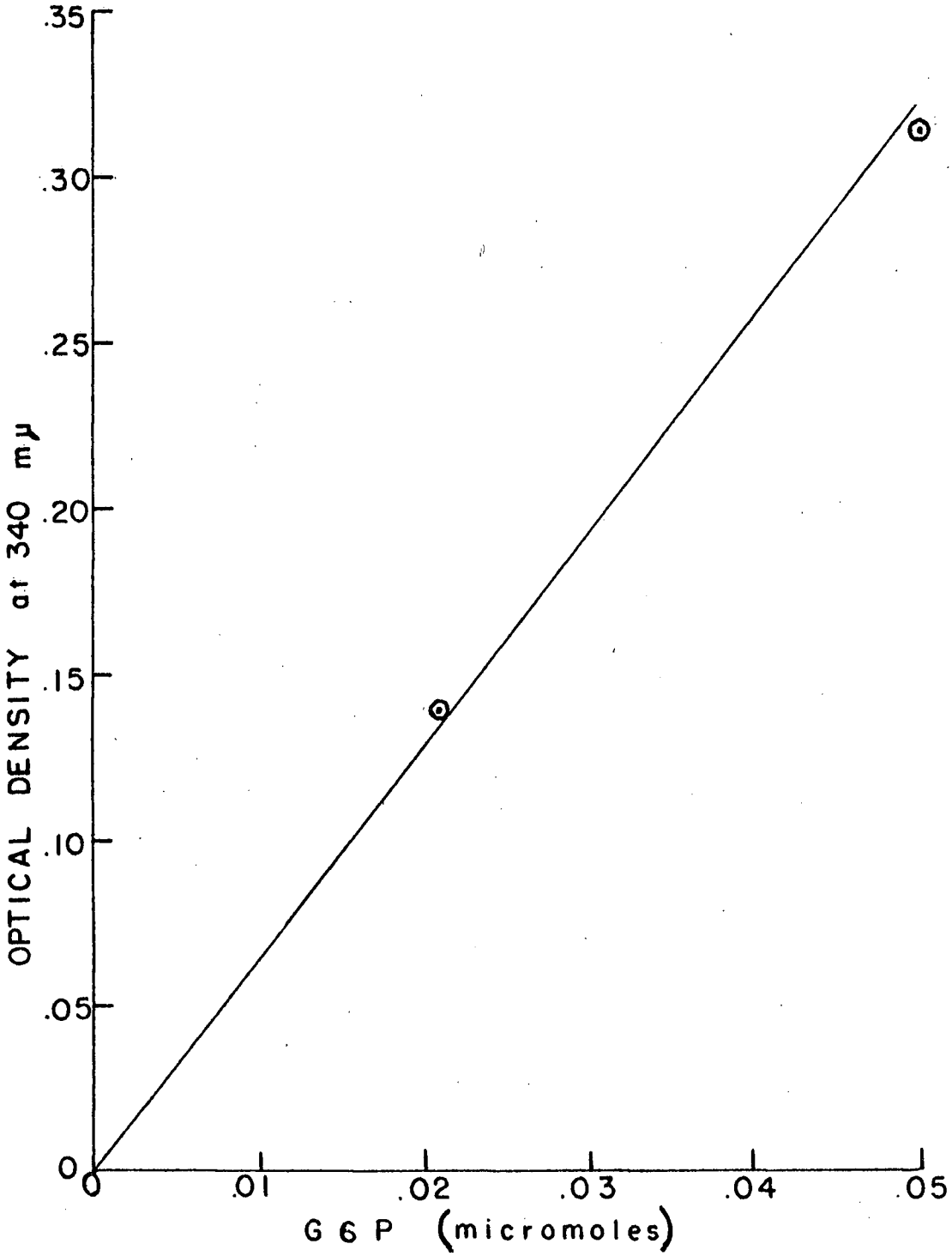


Fig. 3. G6P determination by reduction of TPN in the presence of glucose-6-phosphate dehydrogenase.

G6P	0 to 0.05 micromole
Total volume	1 ml

The optical density, at 340 mμ, was adjusted to zero just before the G6P was added. The substrate was added with a long-tip pipette to permit an even distribution. The increase in optical density was then followed until a constant value was obtained. The value was recorded and used in preparation of the standard curve.

The amount of G6P which is formed from G1P by the action of PGM was estimated by two methods.

The first method consisted of adding an excess of TPN and glucose-6-phosphate dehydrogenase to the PGM reaction mixture at the start and then following the change in optical density. Thus the rate of change in optical density was a measure of the PGM activity.

The amount of G6P was also measured after the PGM reaction had been stopped by immersing the tube containing the reaction mixture in boiling water. The amount of G6P formed was then measured using an appropriate aliquot, the method followed being the same as that used for preparing the standard curve.

3) Quantitative measurement of G1P: The reaction catalysed by PGM is reversible, and since the equilibrium is heavily in favor of G6P, G1P was employed as substrate. The activity of the enzyme was measured by the disappearance of G1P. The phosphorus on the C-1 position of G1P is very acid labile while that on the C-6 position is very stable under the same conditions. Therefore, the amount of acid labile phosphorus may be

used as a measure of the G1P concentration.

The hydrolysis of G1P was carried out using the H_2SO_4 deproteinized enzyme mixture by heating at 100°C for 7 minutes. The amount of labile phosphorus was then determined using the method of Gomori (1942), as recorded below.

An aliquot (1.075 ml) containing from 0 to 10 µg of phosphorus was pipetted into a small test tube (8 mm x 75 mm) followed by 0.125 ml of acid molybdate reagent and 0.05 ml of Elon reagent.

Reagents:

Acid molybdate: 2.5 g. of $Na_2Mo_4.2H_2O$ are dissolved in 50 ml of distilled water followed by 25 ml of 10 N H_2SO_4 and 25 ml of distilled water.

Elon: 0.5 g. of Elon (methyl-p-aminophenyl sulfate) is dissolved in 50 ml of 3% $NaHSO_3$.

The tube was then well shaken and the optical density observed at 750 mµ after 90 minutes or more. Fig. 4 illustrates different standard curves prepared with the same series of tubes but read after different periods of time. The standard curve as drawn when optical density was read after 90 minutes was used throughout the present experiments. In fact, it is observed from the curve that a longer time is needed for color development when higher concentrations are used.

A blank was prepared for each enzyme assay. In this case, no substrate was added and its optical density was taken as zero.

Enzyme assay for phosphoribomutase. Unless otherwise stated, the reaction mixture contained 4×10^{-4}M RIP and 0.01M Tris-HCl buffer, pH 7.5, in a total volume of 0.375 ml.

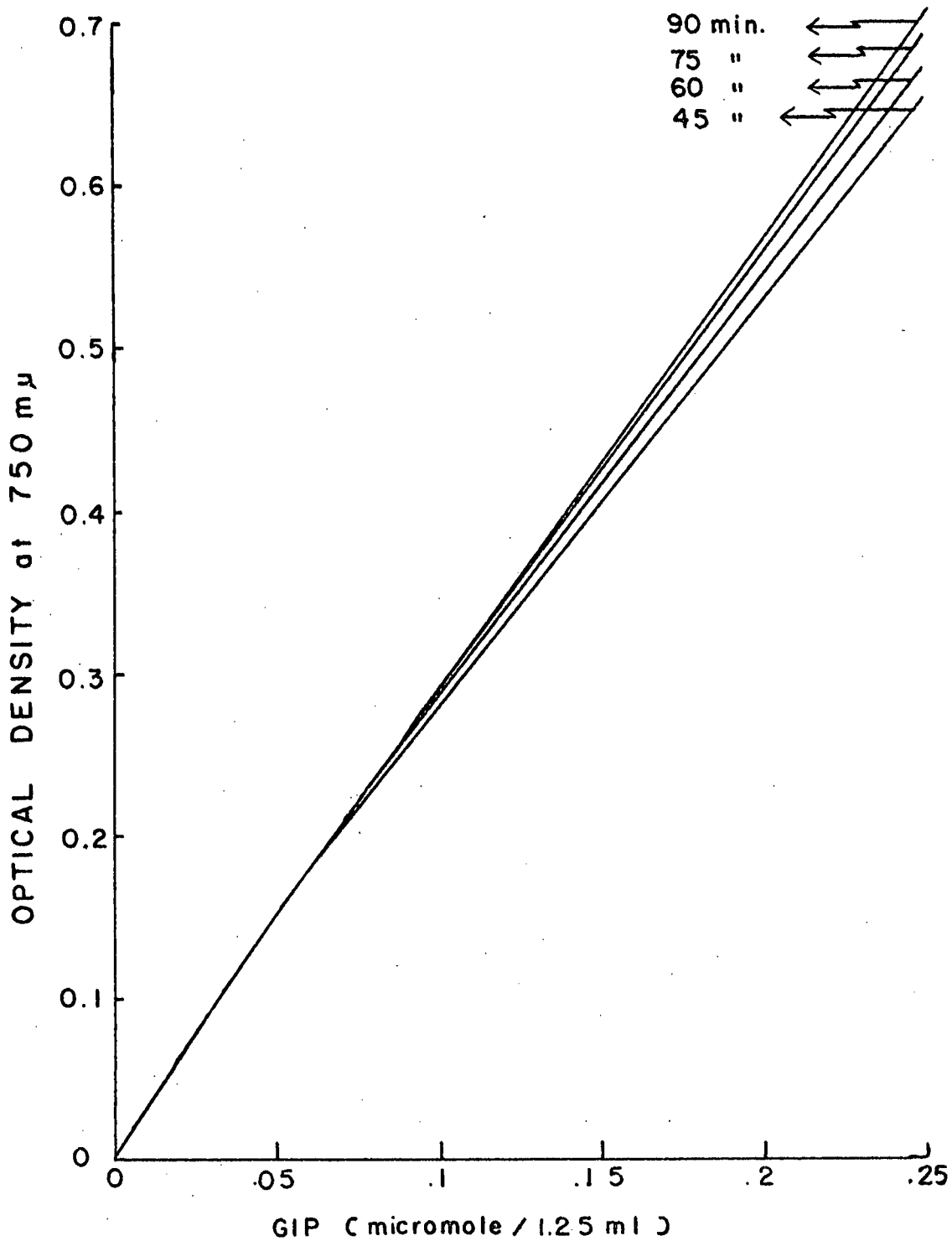


Fig. 4. G1P determination. Orthophosphate liberated after 7 minutes at 100°C in 1N HCl and determined by Gomori's method.

The reaction was stopped by adding an equal volume of 0.2N perchloric acid. The deproteinized mixture was left at room temperature for 45 minutes, under which condition the phosphorus at C-1 position of RlP is completely hydrolysed. During this time, the precipitated proteins were removed by contrifugation at low speed. The labile phosphorus, which is a measure of RlP for the same reason that it is for GlP, was determined by Gomori's method (1942). A standard curve, very similar to that used for GlP determination, is shown on Fig. 5.

Determination of protein: Two methods were used to determine protein concentration. The Biuret method, according to Kingsley (1939) as modified by Snow (1949) was used for higher concentrations of proteins. The reagent is prepared by dissolving 180 g. of NaOH and 2 g. of cupric sulfate in two separate flasks (approx. 400 ml each). When the solution of NaOH is cooled, the two solutions are mixed together and diluted to one liter. An aliquot (containing from 0.01 to 1.2 mg. of protein nitrogen) is diluted to 5 ml with distilled water; 5 ml of the reagent are then added and the contents of the tube are mixed thoroughly. After 25 minutes, readings are taken at 540 mu. In order to prepare a standard curve, the exact amount of protein is determined by a micro-Kjeldalh digestion and distillation.(1)

The method of Lowry et al (1951) was used when the amount of protein to be estimated was very low. Two reagents, A and B, are prepared as follows: Reagent A is prepared by dissolving 2 g. of Na_2CO_3 in 100 ml of 0.01N NaOH; reagent B is prepared by dissolving 0.5 g. of $CuSO_4.5H_2O$ in 100 ml of 1% sodium tartrate. Both reagents are very stable at room

(1) I wish to thank Dr. H.L.A. Tarr for preparing the standard curve.

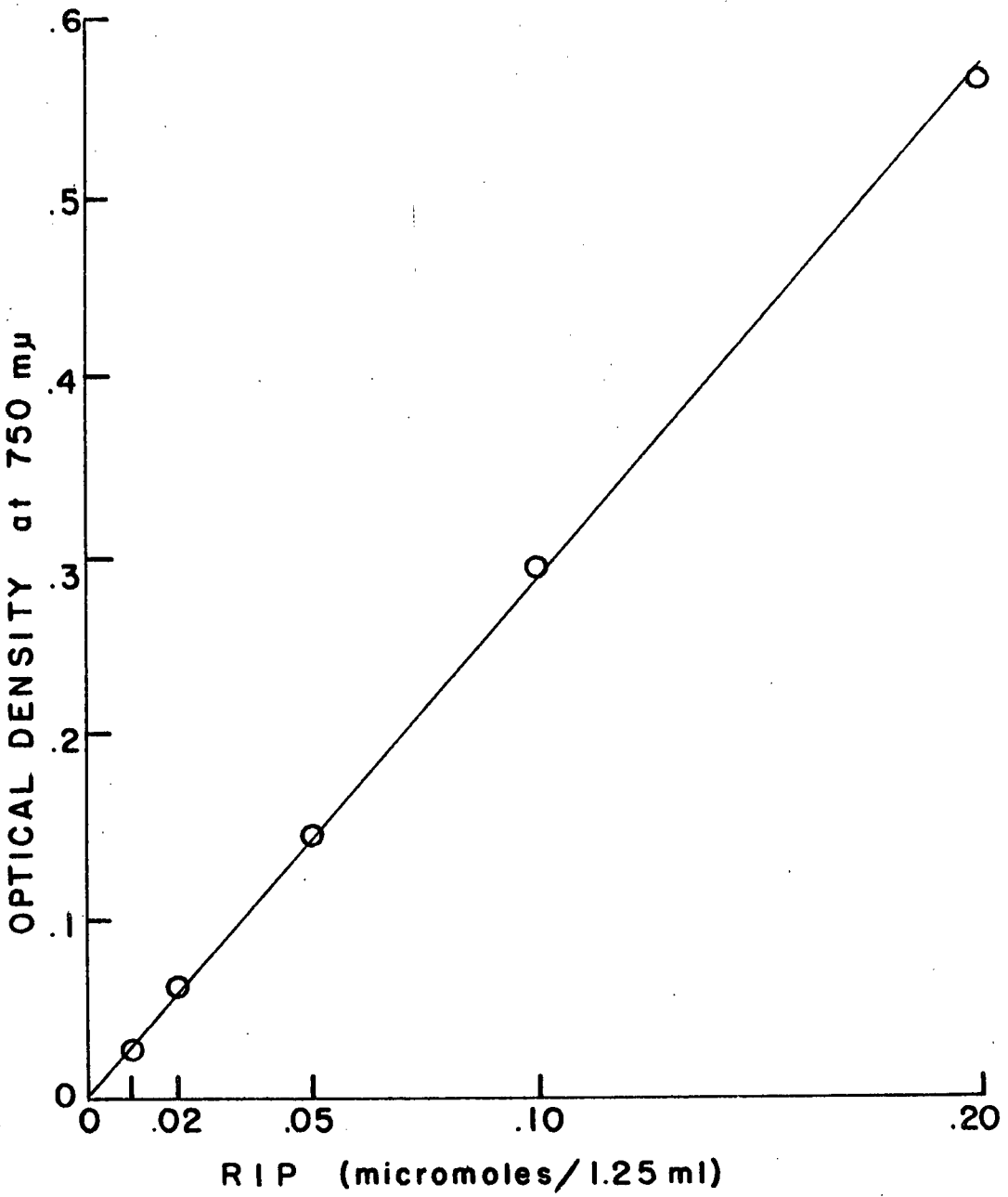


Fig. 5. RlP determination. Orthophosphate liberated after 45 minutes at 20-22°C and determined by Gomori's procedure.

temperature when kept separately. 50 Millilitres of A is mixed with 1 ml of B. This mixture must be prepared daily. 2.5 Millilitres of this mixture is pipetted into a test tube. The aliquot of protein-containing solution (usually 0.1 ml) is then added and the volume is made up to 3.0 ml with distilled water. The solution is allowed to stand at room temperature for at least 10 minutes. 0.25 Millilitres of the Phenol Reagent (Folin Ciocalteau) 1N in H_2SO_4, is then added with constant shaking using a vibrator. The optical density is read after at least 30 minutes at 750 mμ.(2)

EXPERIMENTAL

A) Phosphoglucomutase

The activity was first detected by paper chromatography. There was good evidence for disappearance of G 1P and appearance of G6P.

Purification by ammonium sulfate precipitation: Fractionation of proteins is often achieved by using neutral salts as precipitating agents. Ammonium sulfate is one of the most useful salts for this technique. The general procedure can be summarized as follows:

The protein solution is adjusted to, say, pH 7.0 and is often chilled to 0°C. Ammonium sulfate, either solid or dissolved in water as a saturated solution, is added slowly to give a certain percentage of saturation. If some proteins have precipitated, they are removed by centrifugation or filtration. More ammonium sulfate is then added to the supernatant liquid to increase the concentration. Another portion

(2) I wish to thank Dr. Neil Tomlinson for preparing the standard curve.

of precipitated protein is removed as before. Thus by small increments in the salt concentration, one may obtain several protein fractions. But these fractions almost invariably contain a number of different proteins.

When solid ammonium sulfate is added, one can easily calculate the amount to be used, knowing that the solubility of this salt is 70.6 g. per 100 ml at 0°C. In order to find the exact amount of salt to be added to go from one concentration to another, one may use the nomogram given by Dixon (1953).

When a saturated solution is used, the following equation, developed during our experiments, can be used to find the volume of saturated solution to be added.

$$x = \frac{A\,(F - H)}{1 - F}$$

where x = volume of saturated ammonium sulfate solution to be added

A = initial volume

F = final concentration

H = initial concentration

Using this equation, the calculation of the change in volume is obviously very easily done.

In a typical experiment, 10 ml of a crude water extract from lingcod muscle was fractionated in an attempt to purify PGM. The precipitate of each fraction was dissolved in 1 ml of distilled water. The enzyme was assayed in the presence of cysteine (0.025M) and Mg^{++} as $MgSO_4$ (0.001M).

Results are given in Table I.

Table I. Partial purification of PGM from a crude water extract of a frozen lingcod muscle using ammonium sulfate precipitation.

	Specific activity units(1)	Total protein nitrogen	Total activity (units)
Crude extract heated at 55°C	5.9	5.4 mg.	32.7
0.0-0.5 amm. sulf. precipitate	0.0	1.6 "	0.0
0.5-0.6 " " "	1.9	0.7 "	1.33
0.6-0.7 " " "	17.4	0.58 "	10.08
0.7-0.8 " " "	6.0	0.48 "	2.88

(1) The specific activity is expressed as units per mg of protein N. The unit is defined as 1 micromole of G1P converted to G6P per minute, at 30° C.

Purification by heating: A separate experiment was done to find the effect of heating on the destruction of PGM, to see if this enzyme could be partially purified by this treatment. Unless otherwise stated, all steps were carried out at 0-3° C.

Lingcod muscle (136 g.), excised from a living fish and frozen at -20°C for two days, was chopped rapidly and blended in an ordinary type of Waring blender with 408 ml of cold distilled water. This was then centrifuged at 0°C at 8000 x G for 15 minutes. The supernatant solution was saturated with ammonium sulfate at pH 7.0. The precipitated protein was collected by centrifuging 15 minutes at 8000 x G. The precipitate was dissolved in a small amount of water and dialysed for 20 hours at

0°C against several changes of distilled water. Some denatured protein precipitated during dialysis and was removed by centrifuging 10 minutes at 8000 x G. The supernatant liquid (approximately 250 ml) was freeze dried. The residue was dissolved in Tris-HCl buffer to give 100 ml of a solution 0.01M in respect to the buffer and at pH 7.0. As the pH had decreased during lyophylization, it was necessary to add cautiously small amounts of 0.1N NaOH to attain the above pH value. Insoluble protein was removed by centrifugation at 8000 x G, and the supernatant liquid was kept frozen at -20° C. It remained active for several months under these conditions.

Lyophylised extract (30 ml) was heated rapidly to 56° C with stirring and promptly cooled in an ice-water bath. The coagulated protein was removed by centrifuging at 8000 x G for 10 minutes. The supernatant liquid was similarly treated at a higher temperature, and this was repeated 4 times at increasing temperatures. The enzyme was then assayed in each fraction in the presence of Mg^{++} (0.001M) and cysteine (0.01M). Results of this experiment are given in Table II.

The fact that the total activity was slightly higher in step 2 than in step 1 might be due to the removal of an inhibitory substance (e.g., a protein) by ammonium sulfate precipitation. However, the difference is slight and is certainly not significant. The results show that fish muscle PGM is as stable to heat as the rabbit muscle enzyme as prepared by Najjar (1948). It also suggests that it has a relatively low molecular weight, which would probably be comparable with 74,000 of the rabbit muscle PGM (Keller et al, 1956).

Table II. Purification of PGM by heating a crude water extract of lingcod muscle.

	Volume (ml)	Total protein nitrogen (mg)	Specific activity (units)(1)	Total units
1- Crude water extract	380	2413	1.33	3,140
2- Extract sat'd with ammonium sulfate, dialysed and centrifuged	250	962	3.63	3,480
3- Lyophylised, centrifuged	100	465	6.88	3,210
4- Heated at 56°C	99	262	10.20	2,675
5- " " 60°C	99	208	8.51	1,770
6- " " 64°C	99	182	8.27	1,500
7- " " 68°C	99	150	5.17	724

(1) Specific activity is expressed as units per mg. of prot. N. A unit is defined as 1 micromole of G1P converted to G6P per minute, at 30°C.

Purification by chromatography on DEAE-cellulose: The relatively new method for fractionating proteins using diethylaminoethyl cellulose (DEAE-cellulose) was used quite successfully. The procedure used is described below. The apparatus is illustrated in Fig. 6.

DEAE-cellulose was suspended in 0.01N NaOH and washed with water on a Büchner funnel with Whatman No. 1 filter paper until the pH of the effluent was about 8.0.

A portion of the suspension (13 g. dry weight) was equilibrated with Tris-HCl buffer (0.01M) at pH 7.0 and then packed in a refrigerated column

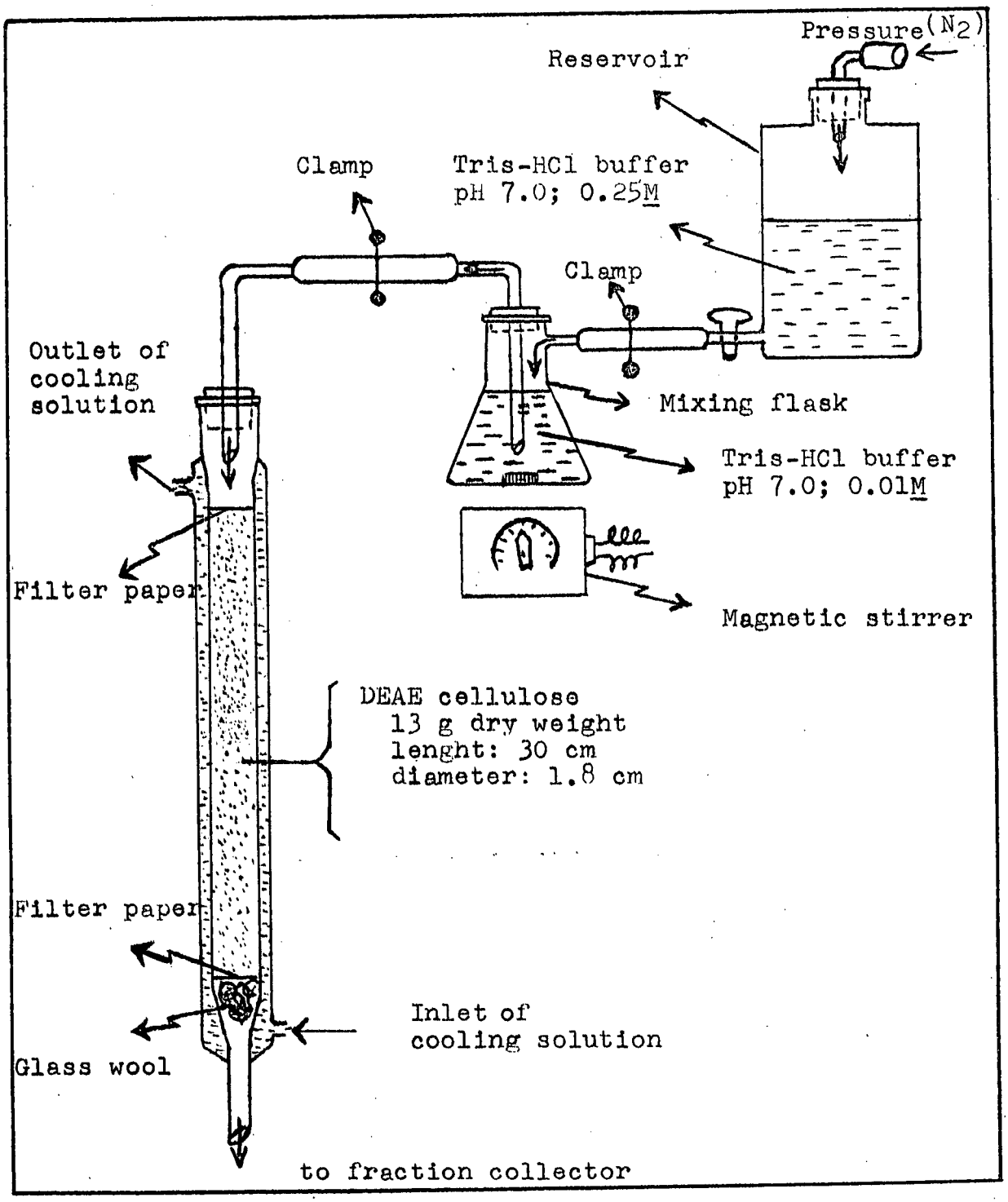


Fig. 6. Apparatus used for chromatography of glycolytic enzymes on DEAE-cellulose.

(1°C), 1.8 cm in diameter and 30 cm in height. A crude extract from ling-cod muscle, which had been saturated with ammonium sulfate, dialysed, heated to 55° C and lyophylised (1 ml containing 33 mg. of protein) was then absorbed on the column and eluted with gradient elution. The gradient was obtained by means of a single 250 ml mixing flask and a reservoir. Initially the mixing flask was filled with Tris-HCl buffer (0.01M, pH 7.0) and the reservoir contained Tris-HCl buffer (0.25M, pH 7.0). A slight pressure from a nitrogen cylinder was applied to maintain a flow rate of about 0.6-1.0 ml per minute. Fractions of 6 ml were collected using a fraction collector (Gilson Medical Electronics, Madison, U.S.A.).

The separation of the different proteins into several peaks, as shown in Fig. 7, was due to the increasing concentration of the buffer, one protein being more easily eluted from the DEAE-cellulose than another. It is presently very difficult to establish any relation between the nature of a protein and the concentration of the buffer by which this protein is removed from the DEAE-cellulose. However, it was interesting to establish the curve showing the increase in eluting buffer concentration obtained by the apparatus shown in Fig. 6.

The theoretical curve is obtained by applying the following equation (1), which establishes the concentration of the buffer after a given volume has been added to the mixing flask:-

$$C = (C_2 - C_1)e^{-\frac{v}{\bar{V}}} + C$$

where:

C = concentration of the buffer at a given time

(1) I wish to thank Dr. G .L. Pickard, Professor of Physics and Director of the Institute of Oceanography, University of British Columbia, for evolving this equation.

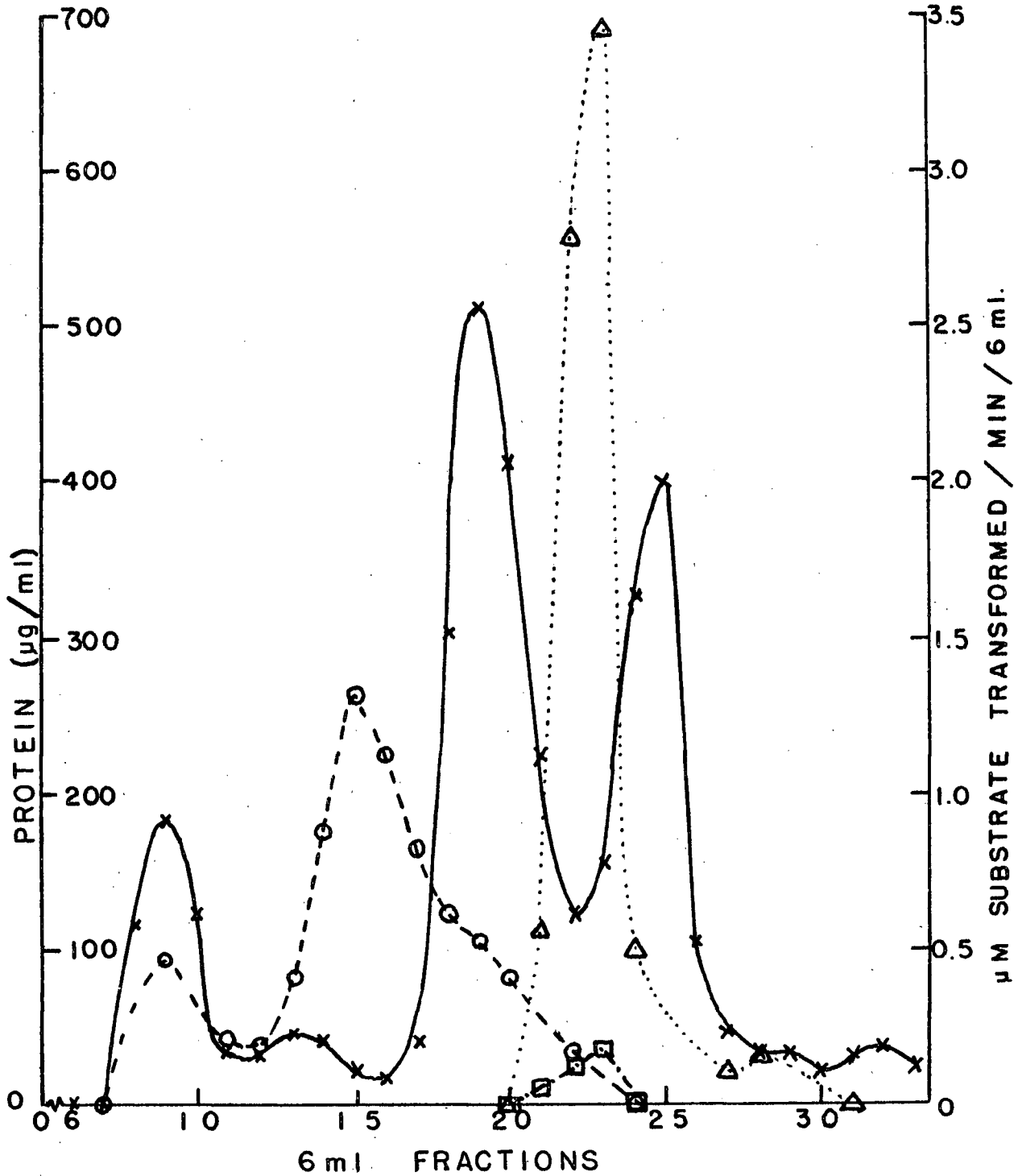


Fig. 7. DEAE-cellulose column. Elution diagram showing protein concentration (×——×), phosphoglucose isomerase (○- - -○), phosphoglucomutase (△·····△) and phosphoribomutase (□-·-·-□). Mixing flask: 250 ml of Tris-HCl, 0.01M, pH 7.0. Reservoir: Tris-HCl, 0.25M, pH 7.0.

C_1 = concentration of the buffer in the reservoir (0.25M)

C_2 = concentration of the buffer in the mixing flask (0.01M)

V = constant volume of the mixing flask (250 ml)

v = volume added to the mixing flask.

e = base of natural log = 2.718...

Different curves can be obtained, by varying C_1 or C_2, as shown on Figs. 8 and 9. By using these, or similar curves, it is possible to predict approximately the location of a given protein when the same protein is fractionated using a different gradient eluting buffer.

Each 6 ml fraction was assayed for protein and for the various enzymes. It is clearly shown in Fig. 7 that PGM can be obtained practically free from PGI. On the other hand, PRM was found to be eluted in practically the same tubes as PGM. This does not necessarily mean that these two enzymes, catalysing different reactions, are identical. This will be discussed later.

The tubes showing the greatest activity were pooled in one batch and preserved at 0°C. The enzyme was not stable under these conditions and was completely destroyed by freezing. Many enzymes often become unstable following purification. As indicated later, it was found that a crude extract, having a very active PGM, could be diluted a few hundred times in a solution of albumin without losing too much activity.

Equilibrium of the reaction: A crude extract of muscle, saturated with ammonium sulfate and dialysed for two days against distilled water, was used as enzyme, since purified preparations lost activity rapidly at 0°C and were therefore very inconvenient to use. The extract, diluted

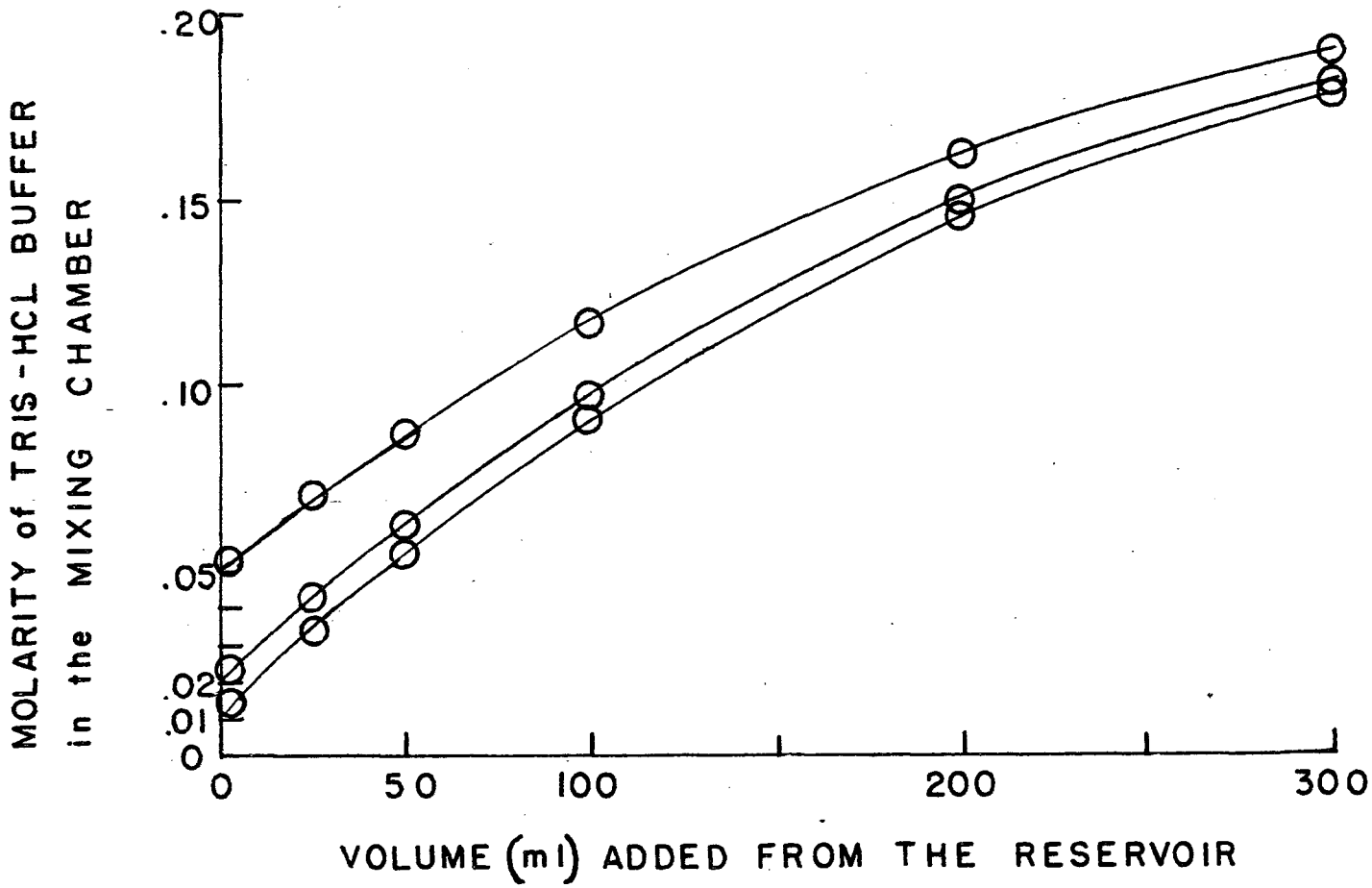


Fig. 8. Concentration of gradient eluting buffers obtained in a mixing chamber containing 250 ml of buffers 0.01M, 0.02M, 0.05M respectively and a reservoir containing the same buffer 0.25M.

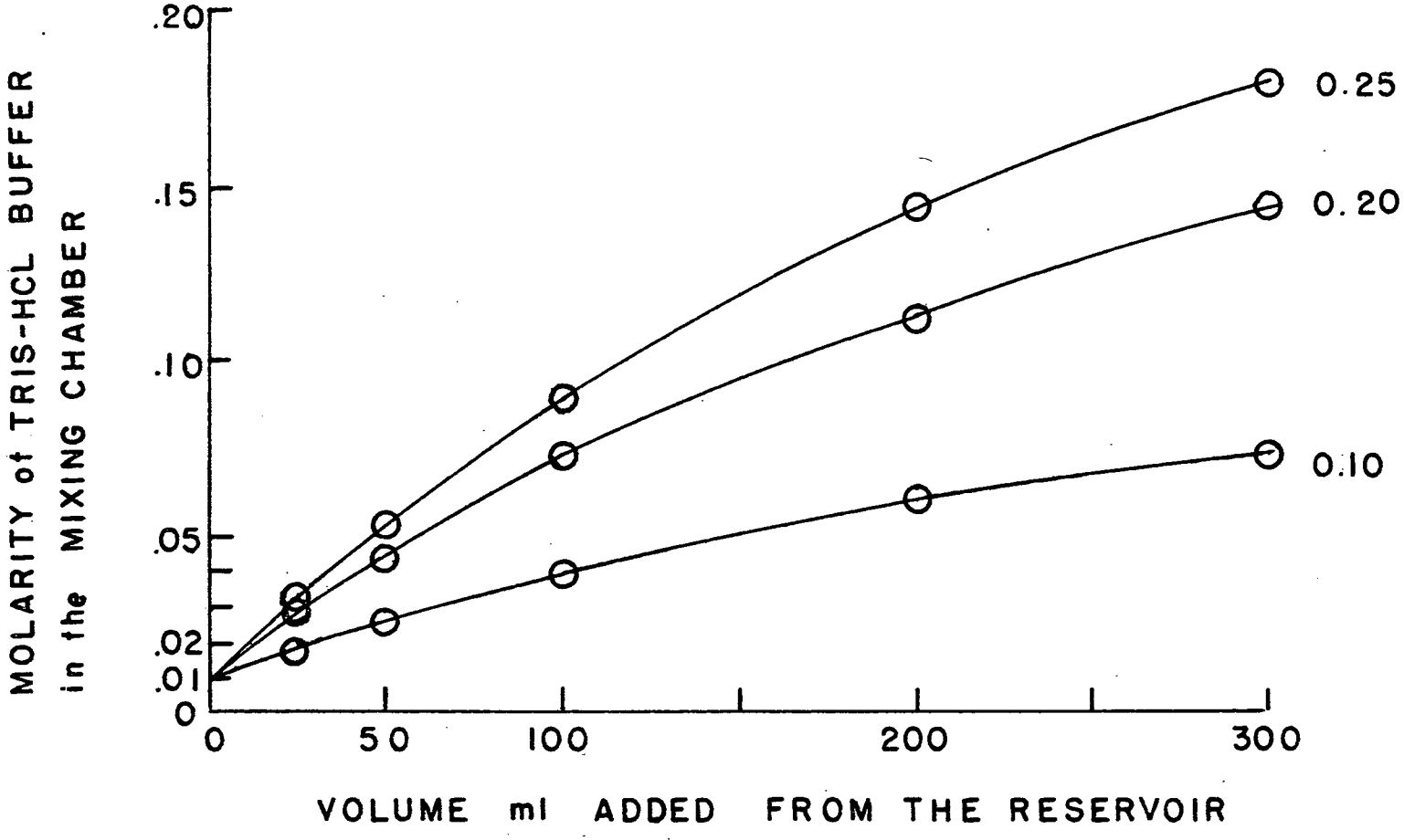


Fig. 9. Concentration of gradient eluting buffers obtained in a mixing chamber containing 250 ml of buffer 0.01M and a reservoir containing the same type of buffer in 0.10M, 0.20M or 0.25M concentrations.

200-fold in albumin (0.1%) was added to substrate kept at 0°C in a series of tubes. Sulfuric acid (2N) was added to one tube while still at 0°C, the other tubes being transferred to a water bath at 30°C. The reaction was stopped in each tube after different time intervals. These experiments were carried out in duplicate with practically no difference between the two series, so that the results could be plotted as the average (Fig. 10).

The results show an equilibrium of approximately 92% in favor of the stable phosphate ester (G6P). This is a little lower than the findings of previous workers. A very precise analysis by Colowick and Sutherland (1942) gave 94.5% in favor of the stable ester. The same figure was found by Najjar (1948), while Ramasarma (1954) found 95% when working with green gram (Phaseolus radiotus). Creasey and Gray (1951) found 97.5% when working on brown adipose tissues of rats.

The deviation of the value found in the present work from the values reported by others probably falls within the experimental error of the method. It is possible that some fructose-6-phosphate (F6P) may have been formed from G 6P by the action of PGI since a crude enzyme extract was used. F6P is somewhat more labile than G6P and it might be partially hydrolysed under the conditions for complete hydrolysis of G1P.

Effect of pH: The conditions used were the same as above. The pH of the reaction mixture was adjusted to different values with cautious addition of traces of 2N NaOH. The pH of the reaction mixture was checked in small beakers, using twice the volume for the reaction.

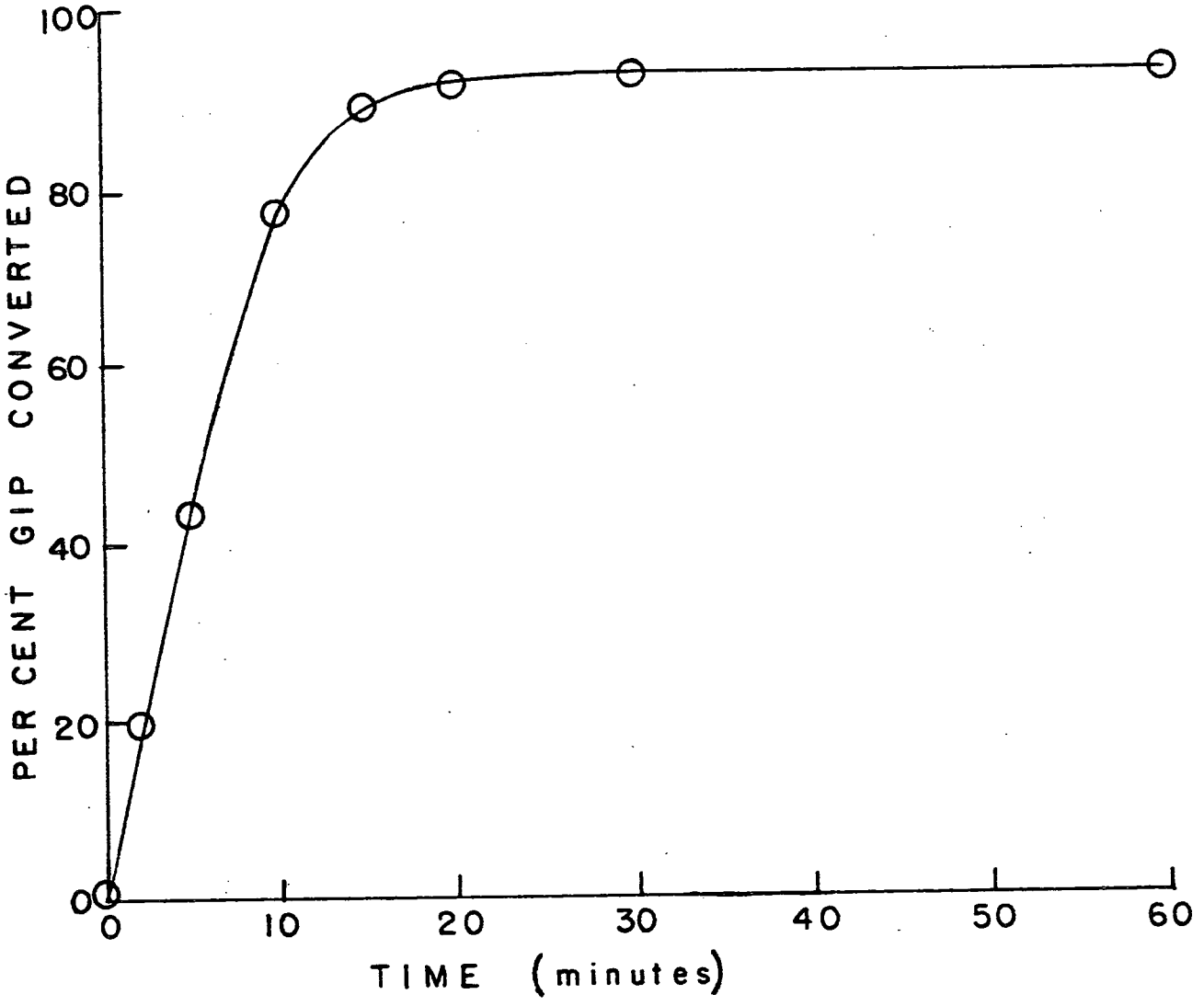


Fig. 10. Time curve of phosphoglucomutase at 30°C, pH 7.35, 0.001M Mg^{++}, 0.01M cysteine. 4.68 µg of protein per ml.

An optimum pH of approximately 7.5 was found, as shown on Fig. 11. This value agrees well with the results of different workers who studied rabbit muscle PGM (Stickland, 1949; Jogannathan and Luck, 1949; Najjar, 1948).

Effect of Mg^{++}: $MgCl_2$ was used as source of Mg^{++} in this portion of the work. The enzyme used in this case was prepared by chromatography on DEAE-cellulose. The results, as shown on Fig. 12, are in agreement with previous findings. Mg^{++} was found essential, or at least a very strong activator, of PGM by Cori et al (1938b), Kendal and Stickland (1938), Stickland (1949), Bolotina (1954), Ramasarna (1954) and Guarino (1955). The present findings correspond almost exactly to the results of Najjar (1948) with crystalline PGM of rabbit muscle.

Effect of cysteine: The effect of cysteine was studied under the same conditions as Mg^{++}, except that the amount of cysteine was varied, Mg^{++} having a final concentration of 0.001M. Fig. 13 shows that optimum activity is obtained when the cysteine concentration is 0.01M. The cysteine requirement of rabbit muscle PGM was studied by Najjar (1948) with results similar to these given above. Requirment for cysteine was also demonstrated by Sutherland (1949). However, Ramasarma et al (1954) could not find any activation of this enzyme by cysteine when working with a partially purified enzyme from green gram.

Effect of some sugar diphosphates: The effect of the sugar diphosphates, $G16P_2$, $R15P_2$ and $DR15P_2$, was studied using a crude watery lingcod extract, saturated with ammonium sulfate, dialysed for two days and then lyophylized as usual. Barium ions were removed from the $G16P_2$ by treatment

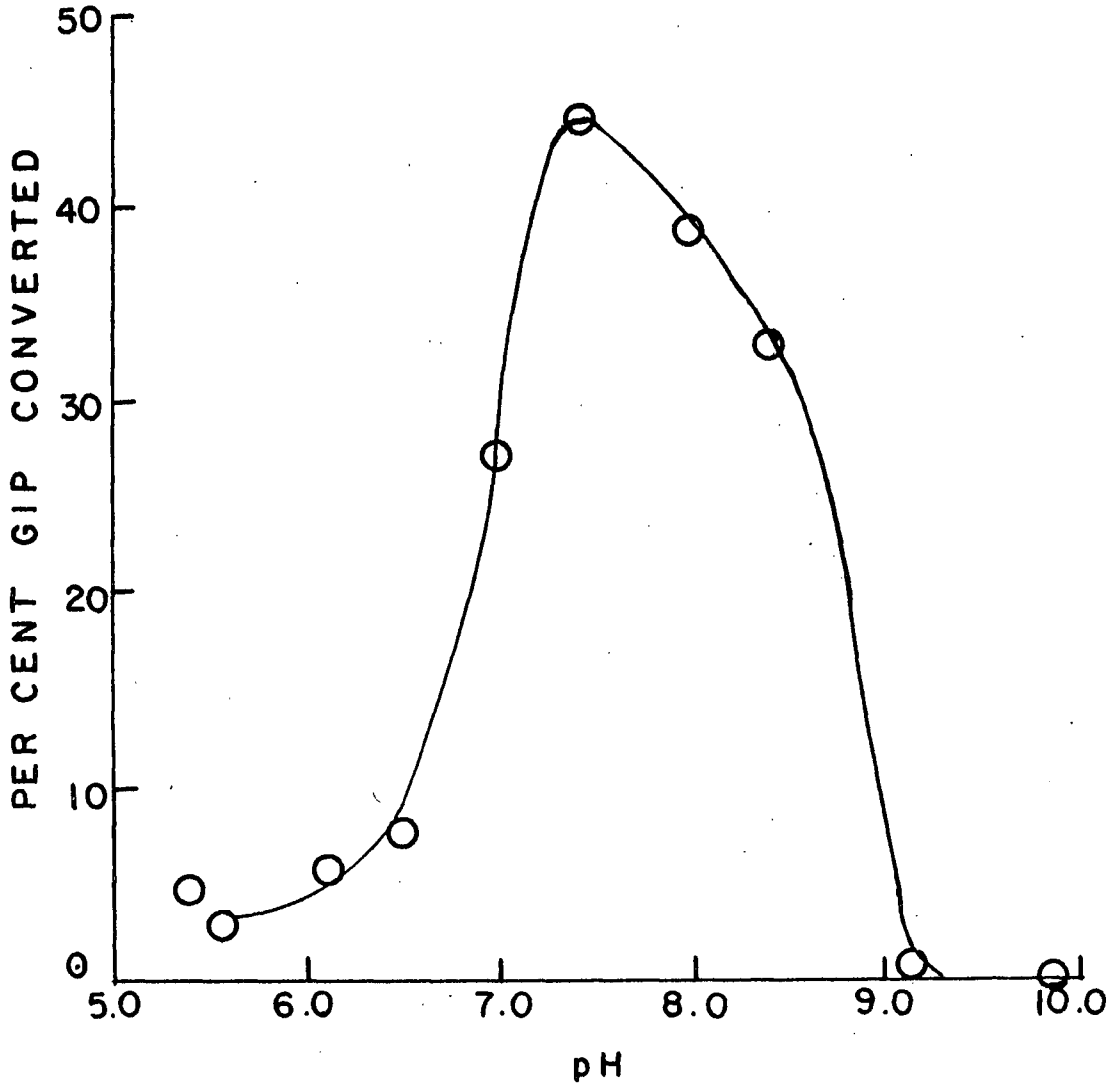


Fig. 11. The effect of pH on the activity of phosphoglucomutase at 30°C, 0.001M Mg^{++}, and 0.01M cysteine. Time of the reaction: 6 minutes. 1.18 µg of protein was used in a final volume of 0.25 ml for each tube.

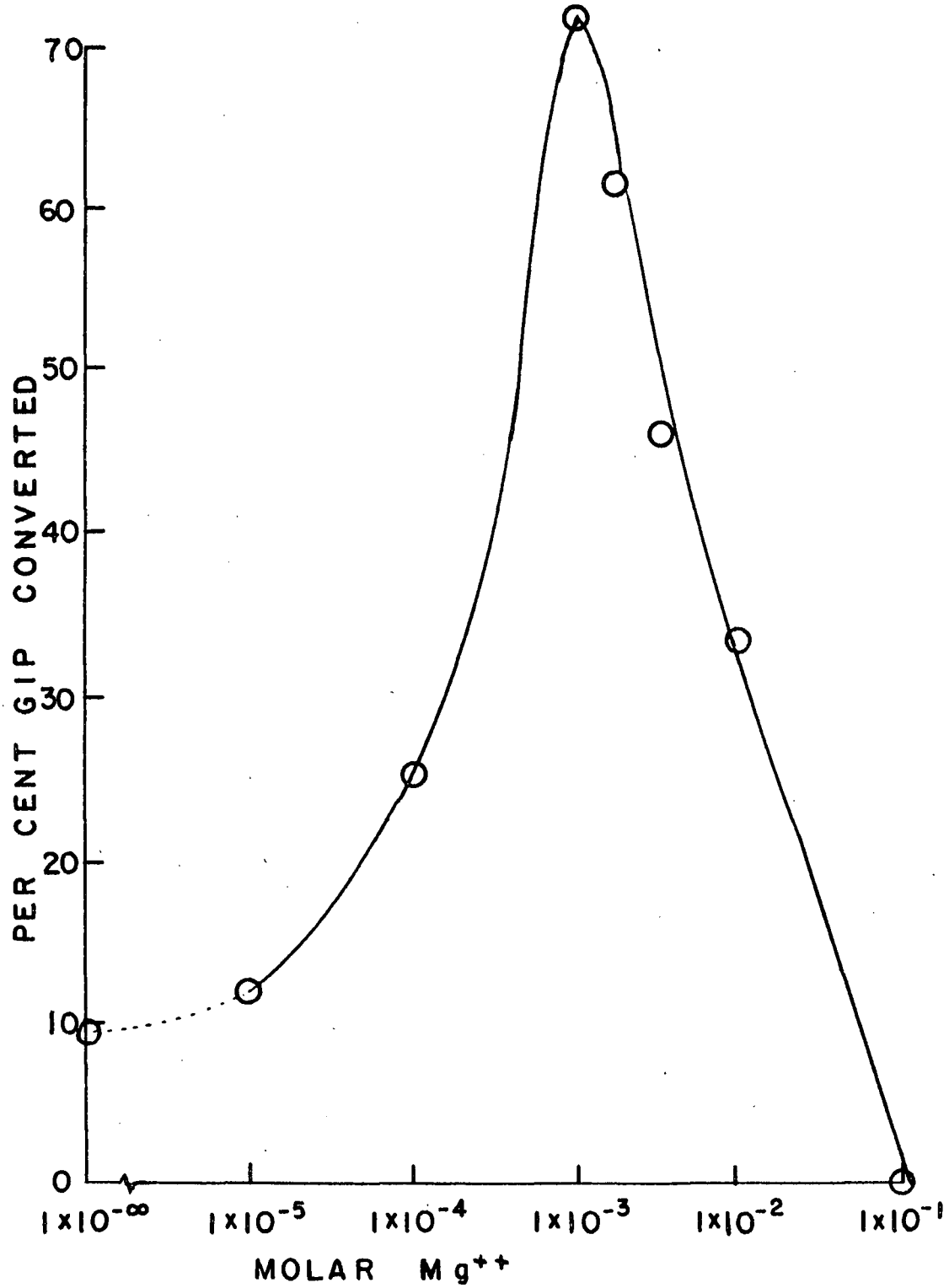


Fig. 12. The effect of Mg^{++} concentration on the activity of phosphoglucomutase at 30°C, pH 7.26 and 0.01M cysteine. 17.6 µg of protein, eluted from a DEAE-cellulose column, were used in final volume of 0.14 ml. Time of the reaction: 20 minutes.

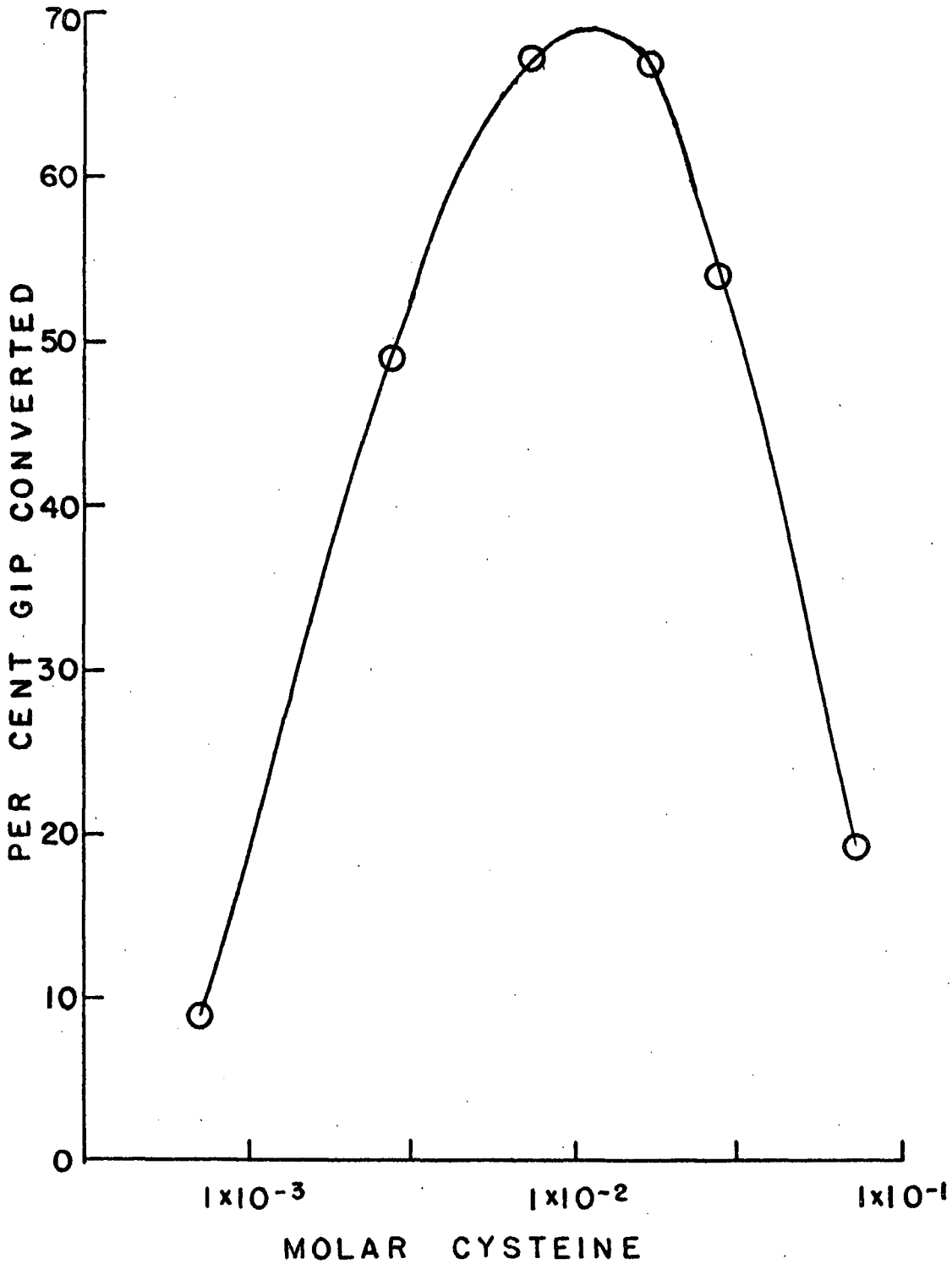


Fig. 13. The effect of cysteine concentration on the activity of phosphoglucomutase at 30°C, pH 7.26 and 0.001M Mg^{++}. 17.6 µg of protein, eluted from a DEAE-cellulose column, were used in a final volume of 0.14 ml. Time of the reaction: 20 minutes.

with Dowex 50 x H^+ resin, while both pentose diphosphates were used as the tetrahexylammonium salts.

It is seen from Fig. 14 that none of these diphosphates activated PGM and that all were inhibitory at the higher concentrations. These results were rather unexpected since phosphoglucomutase is usually considered to require $G1,6P_2$ as an essential co-factor. It is now well established, at least for the mammalian muscle enzyme, that $G1,6P_2$ is an intermediate for the converions of G1P to G6P, according to the following diagram:

Glucose-1-phosphate + phosphoenzyme

⇅

Glucose-1, 6-diphosphate + dephosphoenzyme

⇅

Glucose-6-phosphate + phosphoenzyme

Thus phosphorus would pass from the phosphoenzyme to G1P to give $G1,6P_2$, the enzyme being dephosphorylated by this process. It would be rephosphorylated by picking up phosphorus from the C-1 position of $G\ 1,6P_2$, to yield G6P. $G1,6P_2$ acts as an activator for this reaction but only when the enzyme has been previously dephosphorylated.

The requirement for a diphosphate ester was first suspected by Kendal and Stickland (1938). It was then well established by Caputto et al (1948), Leloir et al (1948), Cardini et al (1949) and Sutherland et al (1949). A detailed study of this mechanism was recently made by Najjar and McCoy (1958).

With the fish muscle enzyme no requirement for $G1,6P_2$ was found when

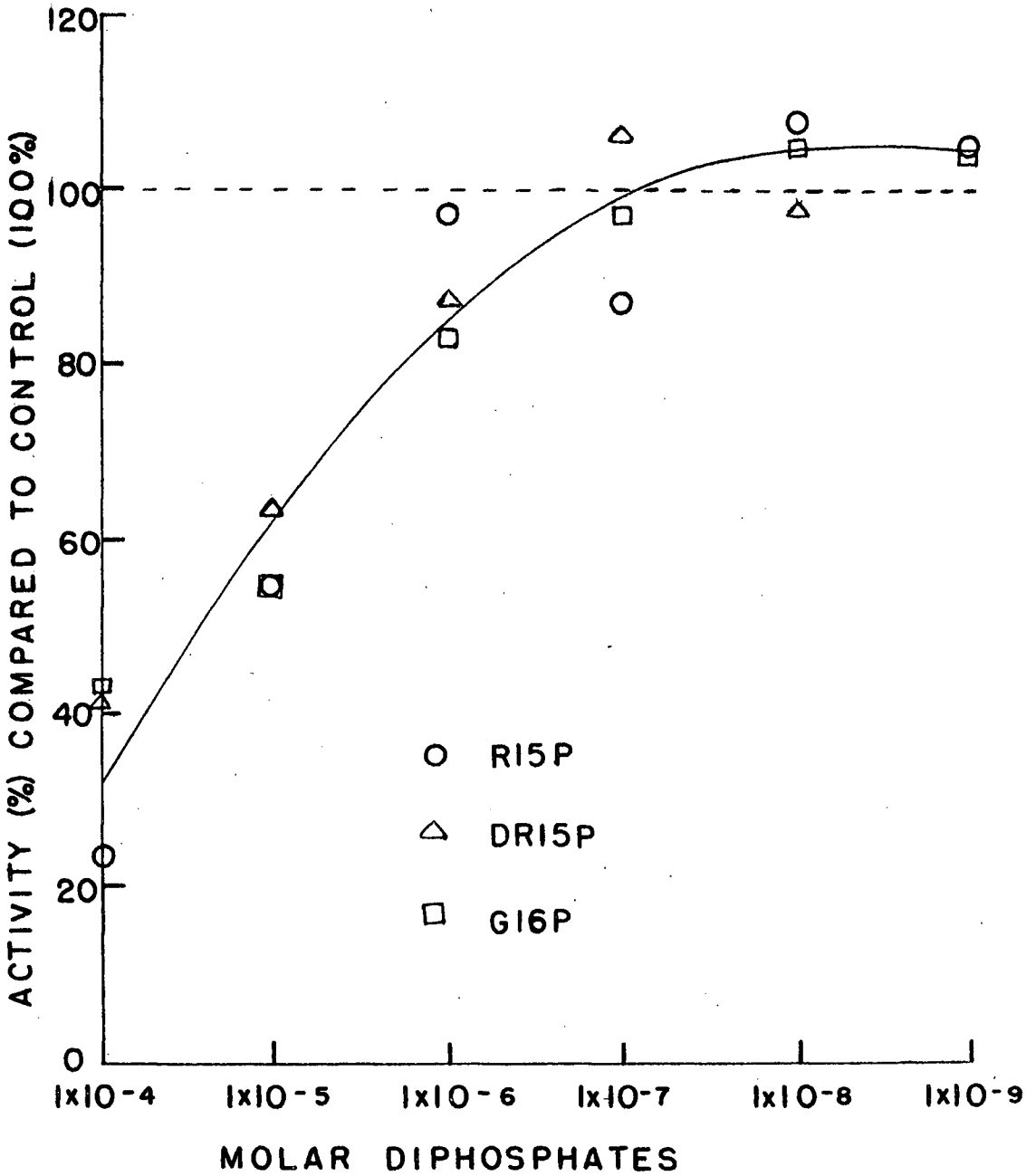


Fig. 14. The effect of some sugar diphosphates on the activity of phosphoglucomutase at 30°C, pH 7.3, 0.01M cysteine, and 0.001M Mg^{++}. Approximately 1 µg of protein, from a crude water extract, dialysed and lyophylised, was used in a final volume of 0.25 ml. Time of the reaction: 7 min. Control (100%) had no diphosphate ester.

using several different preparations, even one which was partially purified by the DEAE-cellulose column method. Two attempts were made to remove possible phosphorus from the fish enzyme, employing the method of Najjar and Pullman (1954).

A crude water extract of lingcod muscle, previously saturated with ammonium sulfate, dialysed and lyophylised, was incubated with an excess of G1P, followed by prolonged dialysis against Tris-HCl buffer (pH 7.0, 0.01M). This incubation was done under conditions similar to those used for the enzyme assay. This treatment was repeated three times, but in no case could activation of the enzyme by G16P_2 or by two pentose diphosphates be demonstrated. This treatment resulted, in one particular case, in a loss of approximately 95% of the total activity.

Although these results might correspond to the reality, they are not sufficient to conclude that PGM from lingcod muscle is not activated by G16P_2. It is possible that enzyme, working on the same principle as the mammalian enzyme, i.e., oscillating over a state of phosphoenzyme and dephosphoenzyme, has a different manner of binding the phosphorus which would not be removable by the method that we have used. It is also possible that there was a trace of G 16P_2 in the preparation of G1P that we have used. The fact that 8-hydroxyquinoline acts as an activator (Fig. 15) could indicate that G 16P_2 was in fact present. It will be seen later, for instance, that the PRM reaction is inhibited by this compound, inhibition that is counteracted by the diphosphates.

When studying the effect of 8-hydroxyquinoline, it was found that this compound could be dissolved in pure anhydrous ethanol, this latter

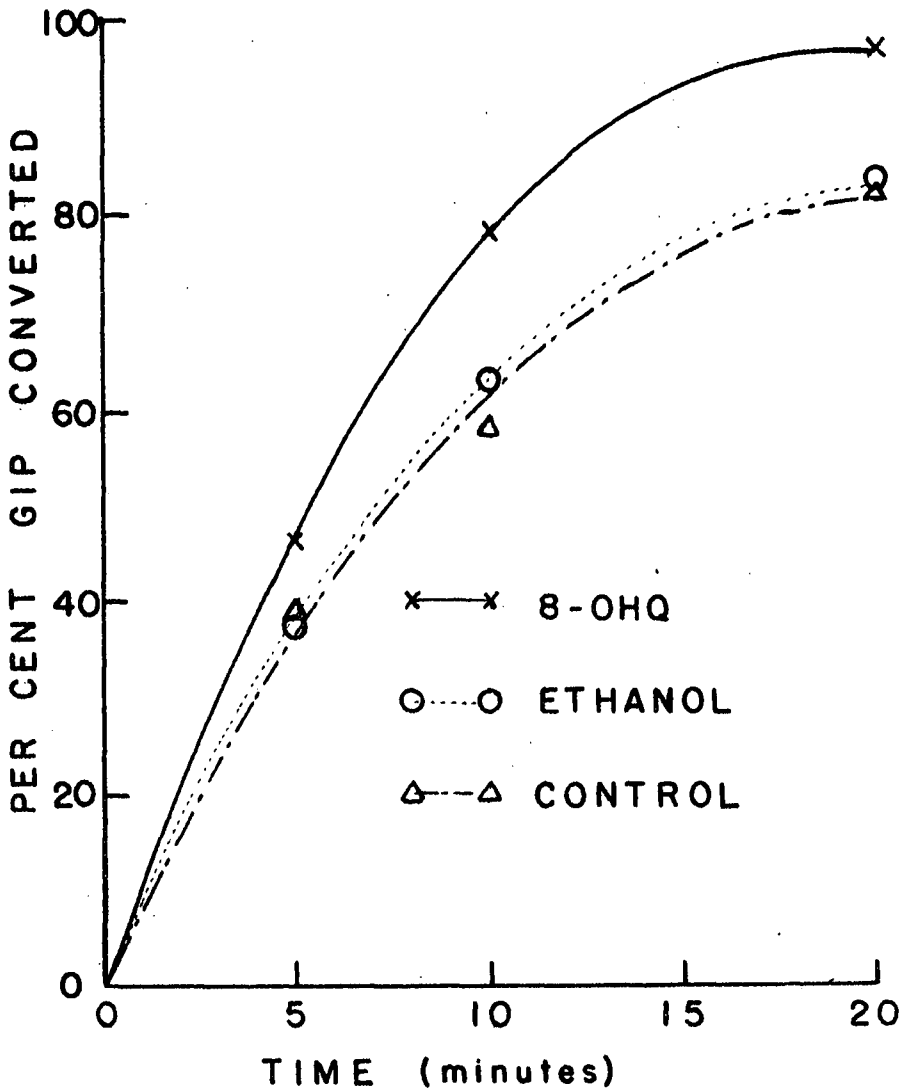


Fig. 15. The effect of 8-hydroxyquinoline (0.001M in ethanol) on the activity of phosphoglucomutase at 30°C, pH 7.3, 0.01M cysteine and 0.001M Mg^{++}. 230 µg of protein, from a crude water extract, dialysed and lyophylised, were used in a final volume of 5.0 ml.

compound being harmless to enzyme activity as shown in Fig. 15.

B) Phosphoribomutase

Equilibrium of the reaction: Fig. 16 shows that an equilibrium is attained when at least 85% of R1P has been converted to R5P. This is in good accord with Klenow's (1953) findings.

Effect of pH: The effect of pH was studied in the presence of $R1,5P_2$. The pH was adjusted to different values with cautious addition of traces of 2N HCl and 2N NaOH, the substrate being approximately neutral when prepared. The final pH was checked in small beakers with twice the volume used for the reaction.

Fig. 17 shows a wide range of pH for the optimum activity, between 7.0 and 9.0. This compares with 7.5 as found by Klenow (1953) but differs from the findings of Guarino and Sable (1955) who give a pH optimum range of 6.7 to 7.2, for bovine uterus enzyme.

Effect of Mg^{++}: A crude water extract, saturated with ammonium sulfate, dialysed and lyophylised, was used for this experiment. The results, as shown on Fig. 18, indicate that no significant activation, if any, was obtained by the addition of Mg^{++}, but that a very strong inhibition took place when higher concentrations were used. Similar findings were obtained by Guarino and Sable (1955 , 1956), although Klenow (1953) found activation by this ion.

Effect of cysteine: The preparation was the same as that used for the effect of Mg^{++}. As the presence of cysteine interferes slightly (2 or 3%) with the determination of phosphorus by Gomori's method, a control was run

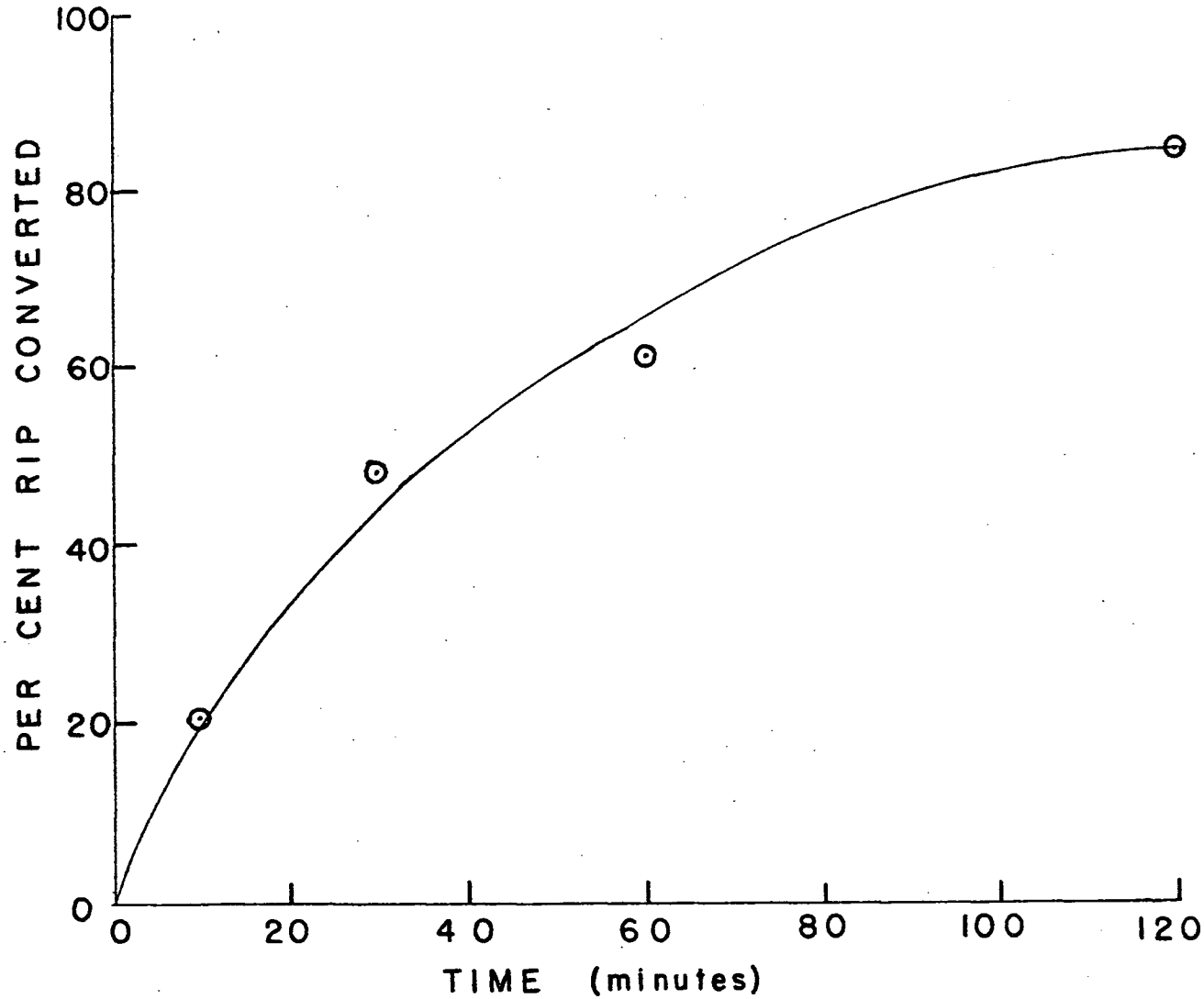


Fig. 16. Time curve of phosphoribomutase activity at 30°C, pH 7.26. 6313 µg of protein, from a crude water extract, saturated with ammonium sulfate, dialysed and lyophylised, were used in a final volume of 2.25 ml.

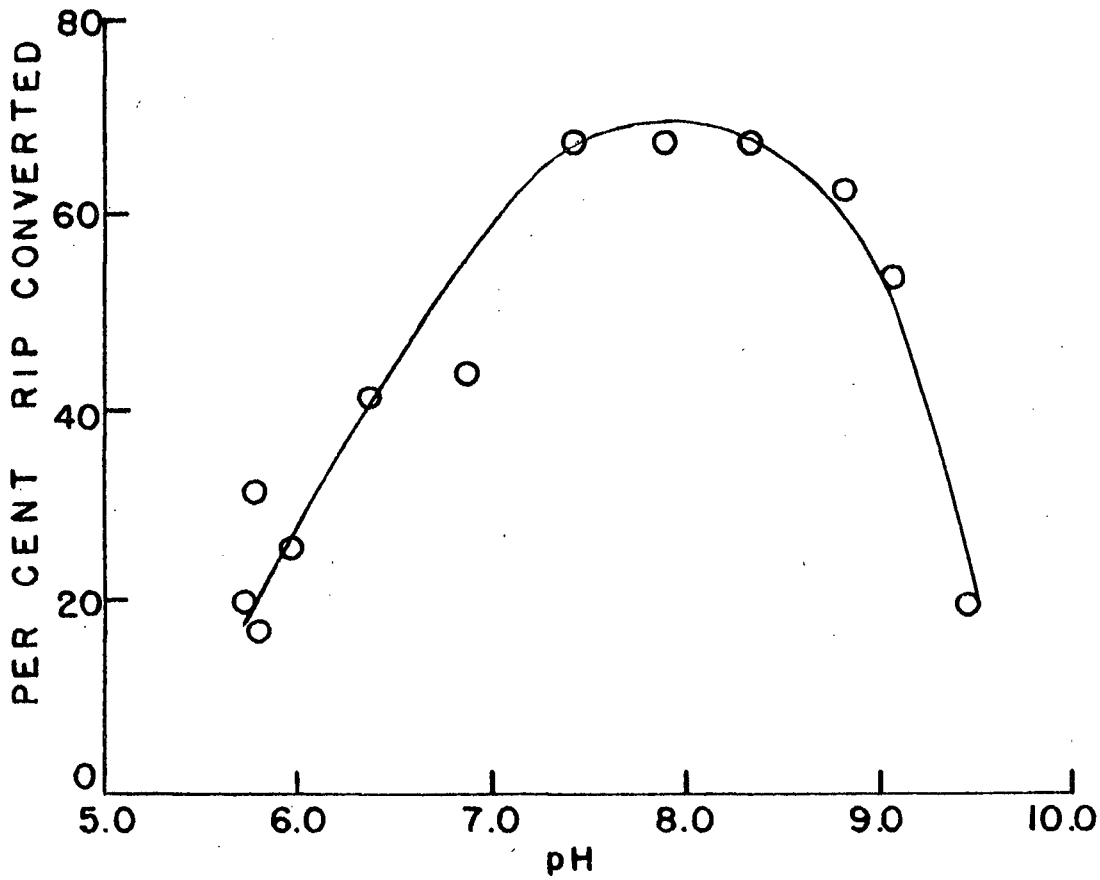


Fig. 17. The effect of pH on the activity of phosphoribomutase at 30°C, 1×10^{-6} M $R15P_2$. 1063 µg of protein, from a crude water extract, saturated with ammonium sulfate, dialysed and lyophylised, were used in a final volume of .375 ml. Time of the reaction: 40 minutes.

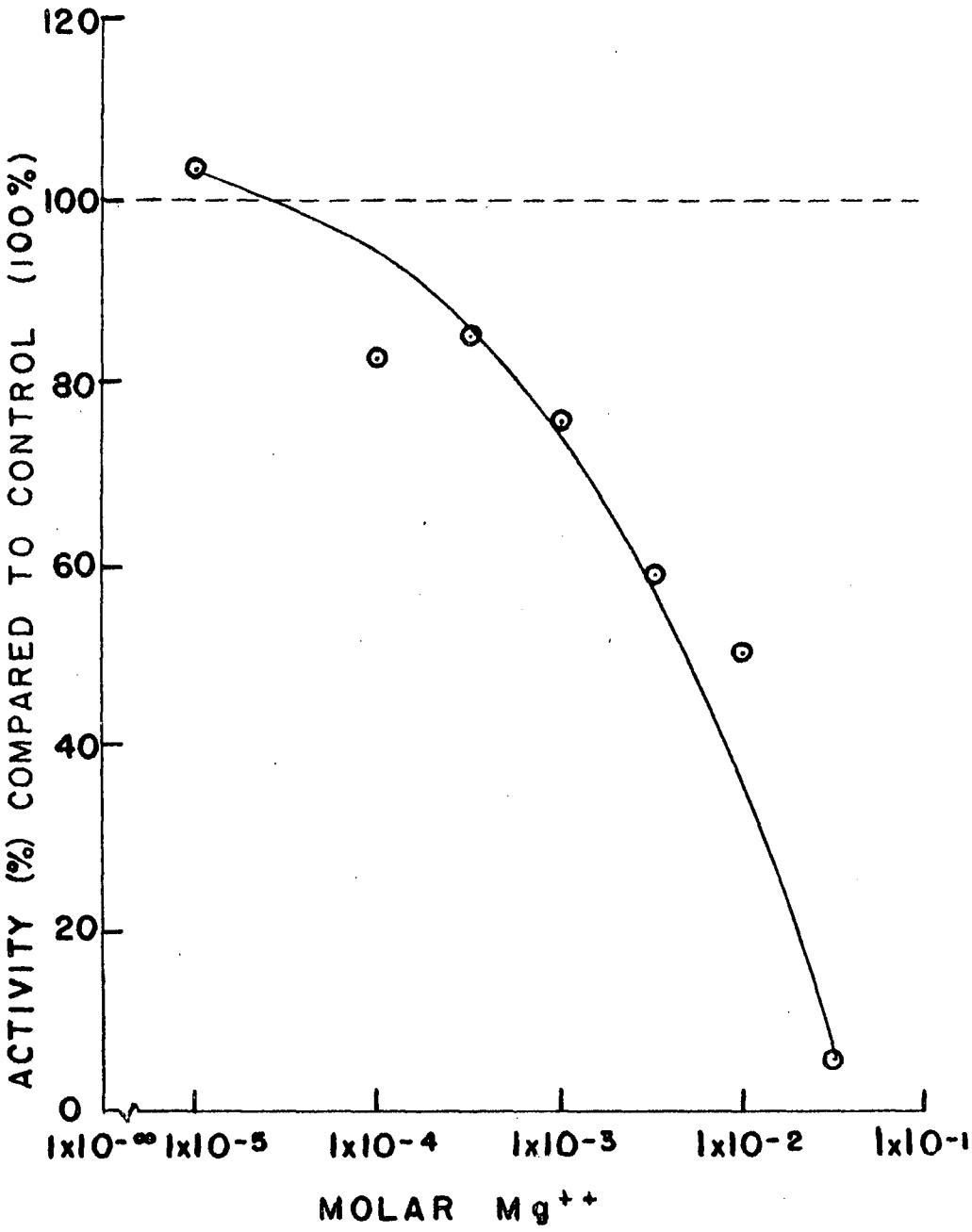


Fig. 18. The effect of Mg^{++} concentration on the activity of phosphoribomutase at 30°C, pH 7.2. 1063 µg of protein, from a crude water extract, saturated with ammonium sulfate, dialysed and lyophylised, were used in a final volume of 0.375 ml. Time of the reaction: 30 minutes. Control (100%) had no Mg^{++} added.

for each concentration of cysteine. Fig. 19 shows that there was no enhancement of activity. A strong inhibition was observed when greater concentrations of cysteine were used. This compares well with the findings of Guarino and Sable (1956) who worked with bovine uterus, although the same authors (1955) have found activation by cysteine when working with human blood cells.

Effect of some sugar diphosphates: As the mechanism involved in the reaction catalysed by PRM is generally claimed to be similar to that of the PGM reaction, an investigation was made to see if the three sugar diphosphates ($G16P_2$, $R15P_2$, $DR15P_2$) could activate the PRM reaction. The enzyme was found to be activated by these three diphosphates. Figs. 20, 21 and 22 show a significant increase in all cases. Fig. 21 shows that 8-hydroxyquinoline has a strong inhibitory action but that this inhibition is almost completely counteracted by the three diphosphates. $G16P_2$ restores the activity of the enzyme to the level of the control (i.e. without $G16P_2$ or 8-hydroxyquinoline), while Klenow (1953) has found that $G16P_2$, in the presence of 8-hydroxyquinoline, restored activity to a value slightly higher than that of the control.

The present findings also indicate that $R15P_2$ and $DR15P_2$ would be more effective than $G16P_2$ in counteracting the inhibition by 8-hydroxyquinoline.

Klenow (1953) did not attempt to explain the effect of 8-hydroxyquinoline, and no explanation appears possible at present. It would be of great interest to investigate this phemomenon more thoroughly.

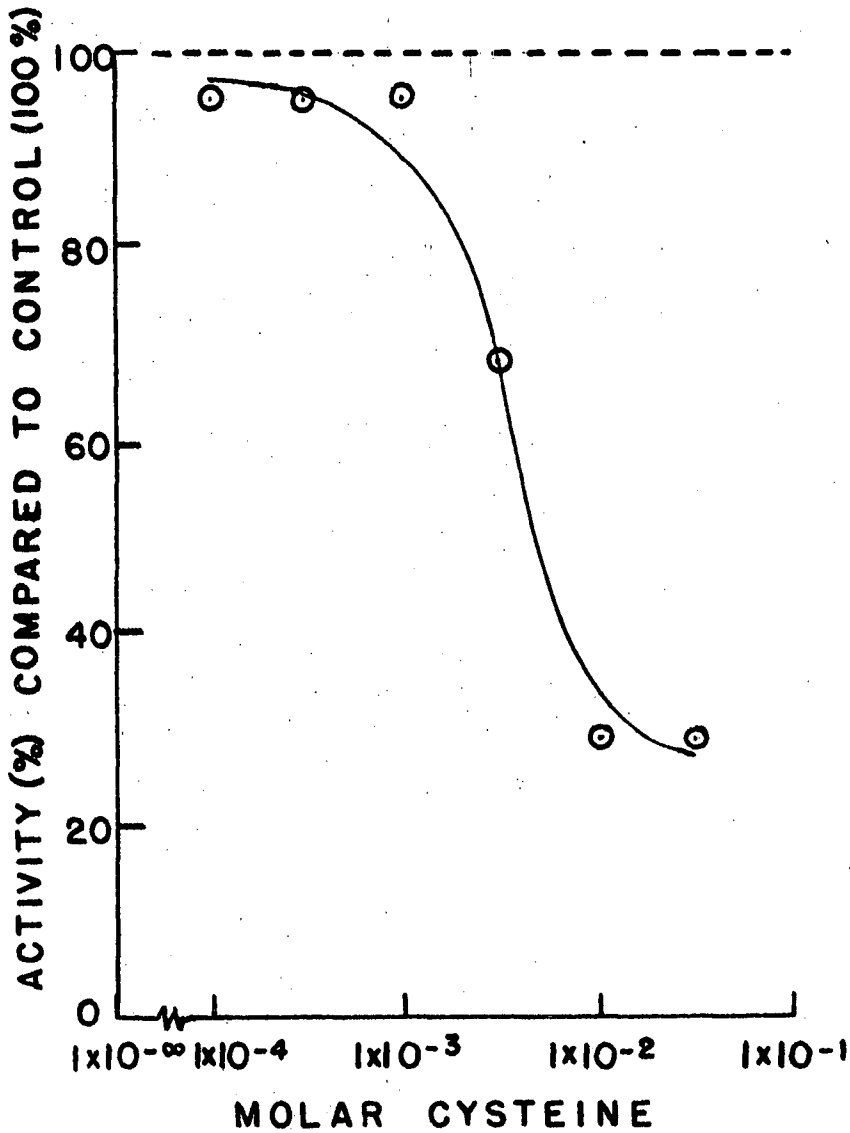


Fig. 19. The effect of cysteine concentration on the activity of phosphoribomutase at 30°C, pH 7.2. 1063 µg of protein, from a crude water extract, saturated with ammonium sulfate, dialysed and lyophylised, were used in a final volume of 0.375 ml. Time of the reaction: 30 minutes. Control (100%) had no cysteine added.

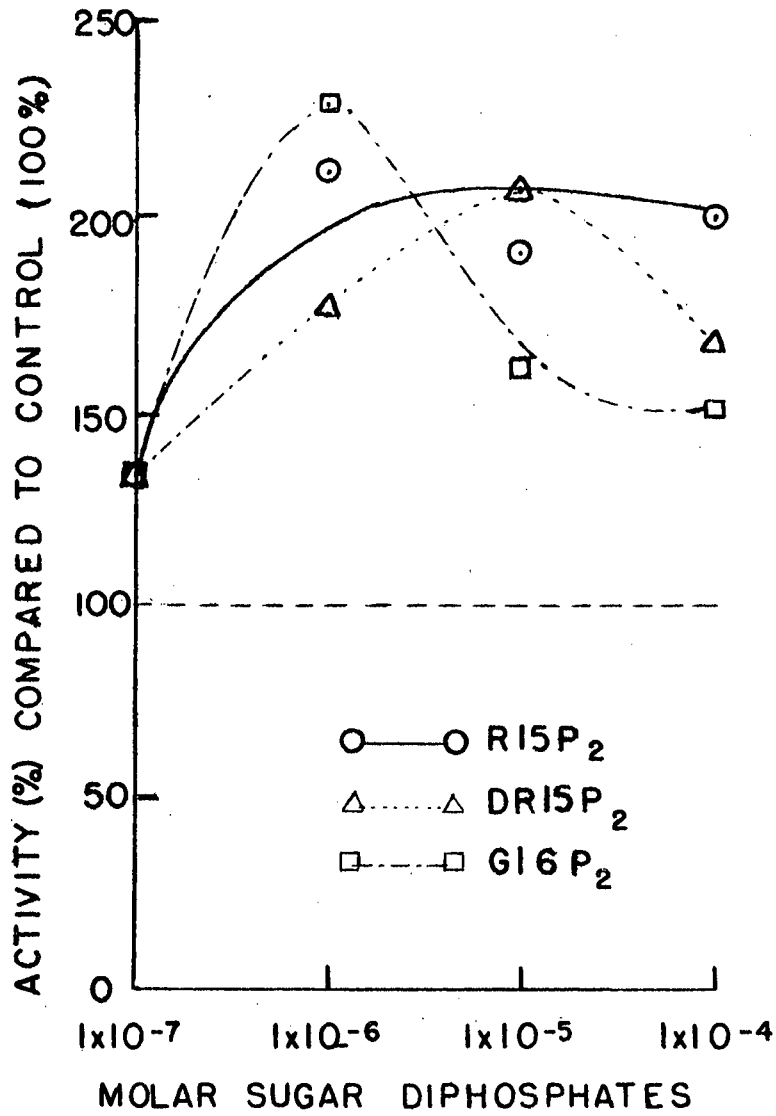


Fig. 20. The effect of sugar diphosphates concentration on the activity of phosphoribomutase at 30°C, pH 7.2. 1063 µg of protein, from a crude water extract, saturated with ammonium sulfate, dialysed and lyophylised, were used in a final volume of 0.375 ml. Time of the reaction: 30 minutes. Control (100%) had no sugar diphosphate added.

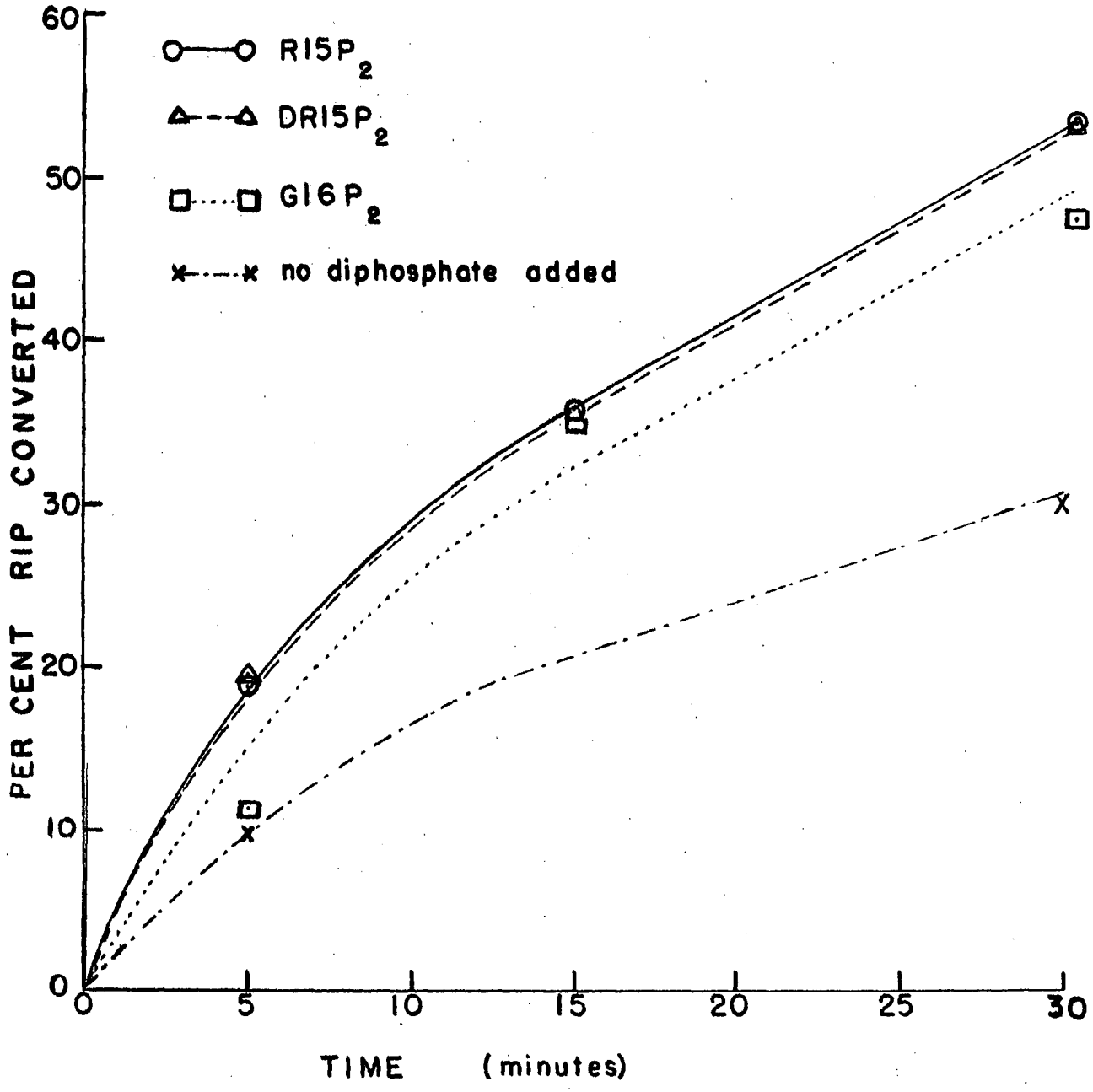


Fig. 21. The effect of sugar diphosphates (1×10^{-5}M) on the activity of phosphoribomutase at 30°C, pH 7.15. 10,400 µg of protein, from a crude water extract, saturated with ammonium sulfate, dialysed and lyophylised, were used in a final volume of 1.875 ml.

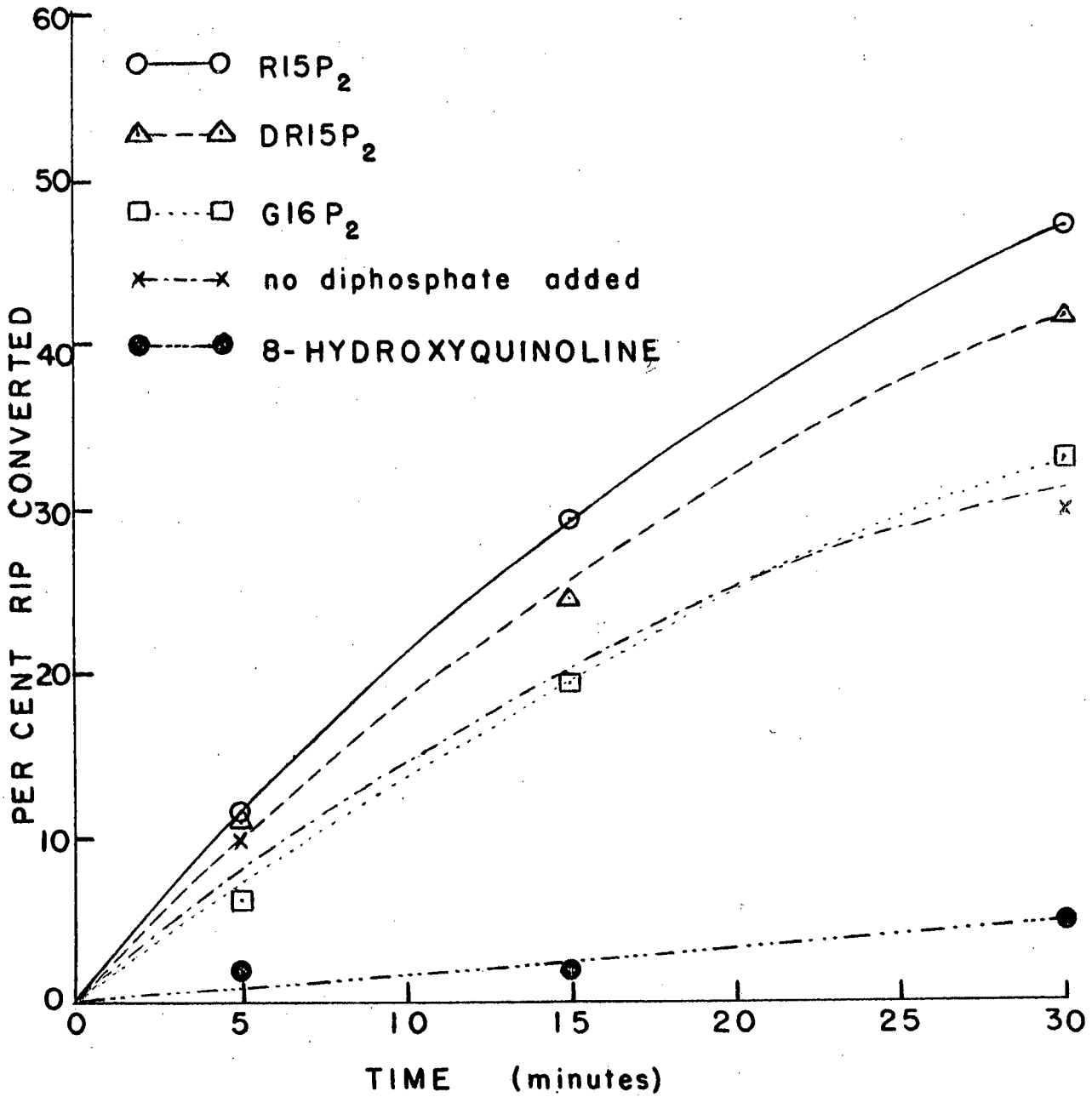


Fig. 22. The effect of sugar diphosphates (1×10^{-5}M) on the activity of phosphoribomutase in the presence of 8-hydroxyquinoline (1×10^{-3}M) at 30°C, pH 7.15. 10,400 µg of protein, from a crude water extract, saturated with ammonium sulfate, dialysed and lyophylised, were used in a final volume of 1.875 ml.

DISCUSSION

Although PRM and PGM were previously found in lingcod muscle (Tarr, 1957, 1958a), there have not been any data on their properties in fish muscle.

According to the present findings, these two enzymes have many points of resemblance with the corresponding mammalian enzymes. PGM apparently has the same properties in both groups with regard to equilibrium, optimum pH, and requirement for Mg^{++} and cysteine. However, $G16P_2$ did not activate this enzyme in lingcod muscle. There could be many reasons for this. If this is an actual non-requirement for $G16P_2$ by PGM, it means that the PGM reaction in fish muscle is not explicable by the same mechanism as it is in mammals.

The properties of PRM in mammalian muscle are not too well established and it is therefore difficult to compare the mammalian and fish enzyme. However, the present results, when compared to the limited observations by other workers, would indicate that fish and mammalian PRM are very similar.

The activation of PRM by $G16P_2$, $R15P_2$ and $DR16P_2$ is a good indication of the similarity of this enzyme in mammals and fish, although the effect of $DR15P_2$, a compound for which there is no known biochemical function at the present time, was observed for the first time.

If the properties of PGM and PRM are compared in the light of the above work, it looks very much as if these two enzymes are not identical in fish. Thus, it was observed that 8-hydroxyquinoline activated PGM and inhibits PRM. PGM, but not PRM, was greatly activated by cysteine and

Mg^{++}. The ratio of the activity of these two enzymes was found to vary considerably. When partially purified on a DEAE-cellulose column, they showed a ratio of approximately 200:1 in favor of PGM, while a crude water extract, saturated with ammonium sulfate, dialysed and lyophylised, showed a ratio of 1600:1. These figures are on a protein basis. However, these figures are subject to discussion, since the assays cannot be carried out under the same conditions, one requiring Mg^{++} and cysteine and the other one not.

The argument in favor of the identity of these two enzymes would be supported by the fact that both enzymes were eluted simultaneously from a DEAE-cellulose column.

IV. PHOSPHOGLUCOSE ISOMERASE

INTRODUCTION

The isomerisation of G6P to F6P was shown by Lohmann (1933) to be catalysed by an enzyme which he called phosphohexoisomerase. It was later called phosphoglucose isomerase (PGI) when it was found that the conversion of F6P to mannose-6-phosphate (M6P) is catalysed by a completely different enzyme (Gottschalk, 1947; Slein, 1950).

PGI is probably very widely distributed in nature. It was found to be present in yeast, muscle and other mammalian tissues by Lohmann (1933). Tsuboi et al (1958) found it in human erythrocytes. Bodanski (1953) found it in serum of several mammals. Hanes (1940) found it in plants. Somers and Cosby (1945) found it in pea meal. It was shown to be present in Aspergillus niger by Singh (1959) and in Penicillium chrysogenum by Sih (1956). A tunicate, Molgula, commonly called sea-

squirt, was found to contain it (Sable et al, 1953).

The present chapter reports the study of PGI in lingcod muscle.

MATERIALS

Fructose was purchased from Merck and Co., Ltd.

Fructose-6-phosphate (F6P), as barium salt, was obtained from Schwarz, Inc., Mount Vernon, N.Y. The barium ions were removed by passage through a small column of Dowex x H^+ resin.

Resorcinol was obtained from Coleman and Bell Co.

Maleic anhydryde was obtained from the Fisher Scientific Co.

Some materials which were listed for the study of PGM and PRM were also used for the present experiment.

METHODS

Enzyme assay for PGI: Unless otherwise stated, the following conditions were used.

Tris-maleate buffer was used in most cases with a final concentration of 0.025 to 0.035M at pH 8.2. G6P was used as the substrate with a final concentration of 2.5×10^{-3}M. The enzyme, diluted in H_2O when necessary, was added after a preincubation of 5 minutes of the substrate at 30°C. The reaction was stopped by adding an equal volume of 2N hydrochloric acid in order to have a final concentration of 1N hydrochloric acid. Determination of F6P formed could be carried out directly on the deproteinized reaction mixture without centrifugation

or filtration since the concentration of the protein was very low in all cases. A control (with H_2O instead of enzyme) and a blank (with H_2O instead of G 6P) were run at the same time.

1) Qualitative detection of F6P formed: The appearance of F6P was checked by paper chromatography, using the same procedure as that used for detection of G1P and G6P (Page 13).

2) Quantitative estimation of F6P formed: The method of Roe (1933) was used throughout this experiment. A preliminary test showed that very precise results could be obtained with a final volume of 1.25 ml instead of 6.0 as suggested. Into a small test tube (8mm x 75mm), an aliquot of 0.25 ml was pipetted, followed by 0.25 ml of resorcinol (0.1% in ethanol) and 0.75 ml of HCl (30%). The tube was well shaken and immersed in a water bath at 80°C for 8 minutes . It was then cooled under cold running water. The intensity of the color was read immediately at 490 mu.

3) Standard curve for fructose: 2.5 mg of fructose were dissolved in a final volume of 50 ml of H_2O. A total of 10 different aliquots were used to prepare the curve shown on Fig. 23.

4) Standard curve for F6P: 13.62 mg of F6P (barium salt; M.W.: 395.5) were dissolved in 6.19 ml of H_2O. This corresponds to 1 mg of fructose per ml. The barium ions were removed by passage through a small column of Dowex x H^+ resin. A total of 10 different aliquots were used to prepare the curve shown on Fig. 24. In this case, the values obtained must be multiplied by 1.44 in order to determine the amount of F6P. Fig. 24, when compared with 23, shows that the fructose of F6P responds to about 60.5% of the theoretical of the fructose test. This accords with the observations of Umbreit et al (1951).

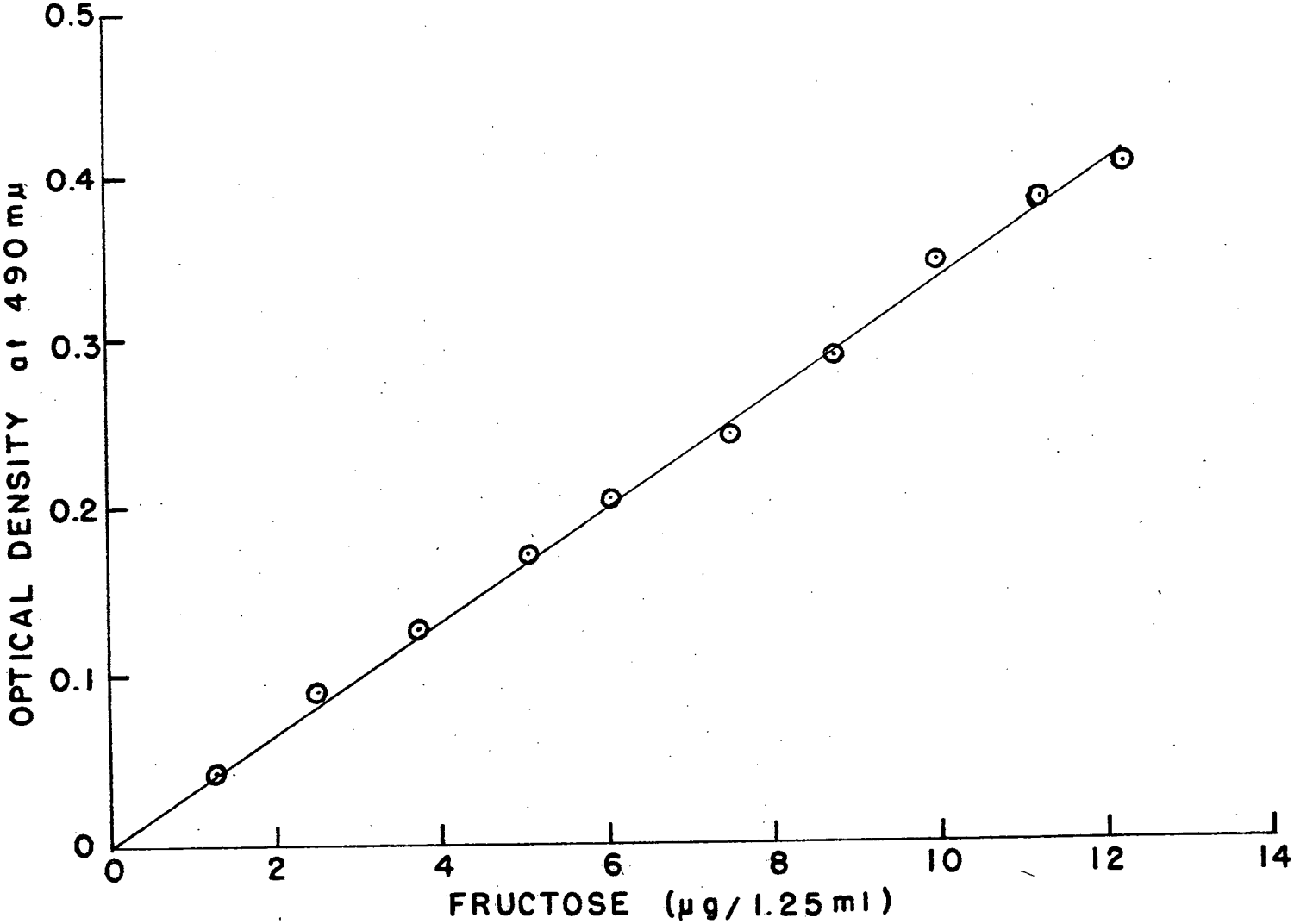


Fig. 23. Fructose determination by Roe's method.

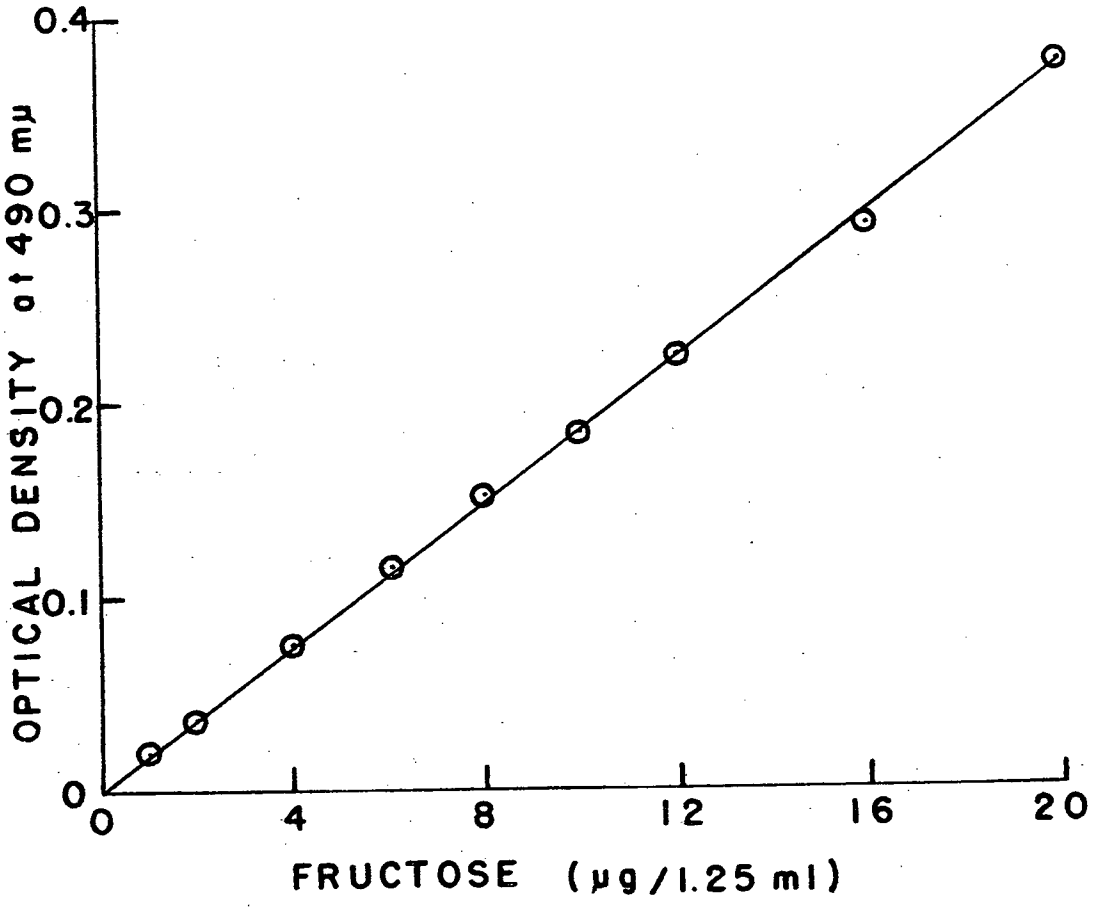


Fig. 24. Fructose-6-phosphate determination by Roe's method.

Determination of protein: This was done as previously described for the study of PGM and PRM (page 17).

EXPERIMENTAL

Purification by ammonium sulfate precipitation: The fractions, as prepared when fractionating PGM (see page 20) were tested for PGI activity. The reaction mixture contained G6P as substrate with a final concentration of 2×10^{-2}M. After the reaction was stopped by adding acid, the solution was readjusted to neutrality. The presence of F6P was verified by paper chromatography. The lower fraction (0.3-0.65 ammonium sulfate precipitate) showed a strong activity. There was a trace of activity in the 0.65-0.7 fraction while there was no detectable activity above 0.7 of saturation.

Slein (1955) found that the maximum activity was obtained at 0.55-0.65 saturation with ammonium sulfate when purifying this enzyme from rabbit muscle. Working on Aspergillus niger, Singh (1959) found that the maximum activity was obtained at 0.58-0.72 saturation with ammonium sulfate.

Purification by heating: A crude water extract, precipitated with ammonium sulfate (up to 0.8 saturation) and dialysed, was used for this experiment. The procedure followed was the same as that used for the study of PGM (page 20), except that the temperatures were different. The reaction was carried out at 26°C and pH 7.0. The final concentration of G6P was 1.3×10^{-3}M. Results, as recorded in Table III, show the enzyme is completely destroyed between 55°C and 60°C.

Table III. Effect of heating on PGI activity.

	O.D. at 750 mu in Roe's (1933) method (see Fig. 24) Time (minutes)		
	0	10	20
Crude extract	0.013	0.096	0.137
Heated at 50°C	0.019	0.122	0.140
" " 55°C	0.016	0.090	0.122
" " 60°C	0.011	0.012	0.012
" " 65°C	0.007	0.016	0.012

Tsuboi et al (1958), studying this enzyme in human erythrocytes, found that the critical temperature was 47°C to 50°C at pH 8.5 for destruction of the enzyme previously purified 1200-fold. When preparing PGM from rabbit muscle, Stickland (1949) found that it was free of phosphohexoisomerase when the extract was heated at 52°C for 30 minutes at pH 5.0. Najjar (1948) prepared PGM from rabbit muscle completely free of PGI by ammonium sulfate precipitation and heating at 65°C. As the concentration of ammonium sulfate he used was in the range necessary to precipitate PGI, it must be concluded that PGI was completely destroyed by heating at 65°C.

Purification by chromatography on DEAE-cellulose: Details of the procedure are given in a previous section (page 22). PGI activity was determined for each tube (as plotted on Fig. 7). This graph shows the total activity for each tube and not necessarily the degree of purity.

A 27-fold purification, on a protein basis, was obtained by this method.

The tubes showing the maximum activity were pooled in one plastic bottle and kept at 0°C for several days without appreciable loss of activity. However, freezing and thawing this preparation resulted in complete destruction of the enzyme. Fig. 7 also shows that PGI can be obtained completely free of PGM since there is practically no overlapping between these two enzymes.

Equilibrium of the reaction: The equilibrium was studied on a preparation partially purified on DEAE-cellulose. The final volume of the reaction mixture was 1 ml. Aliquots (0.1 ml) were taken at different intervals of time and deproteinized as usual. F6P was then determined on each aliquot.

Fig. 25 shows an equilibrium when approximately 38% of G6P has been converted to F6P. This falls within the range obtained previously. West and Todd (1956, p. 959) give 70/30 in favor of G6P while 68/32 is given by Lardy (1949) and Slein (1955). Tsuboi et al (1958), working with human erythrocytes found an equilibrium of 60/40 in favor of G6P while Singh et al (1959) give 71/29 in favor of G6P when working on Aspergillus niger.

Effect of pH: The material used was the same as that used for studying the equilibrium. Two different buffers were used as shown in Fig. 26. Both had a final concentration of 0.035M. Buffer and substrate were mixed together and the pH adjusted to different values with cautious addition of 2N NaOH and 2N HCl. Reaction was then carried out on a final volume of 0.1 ml for each different pH value for 8 minutes.

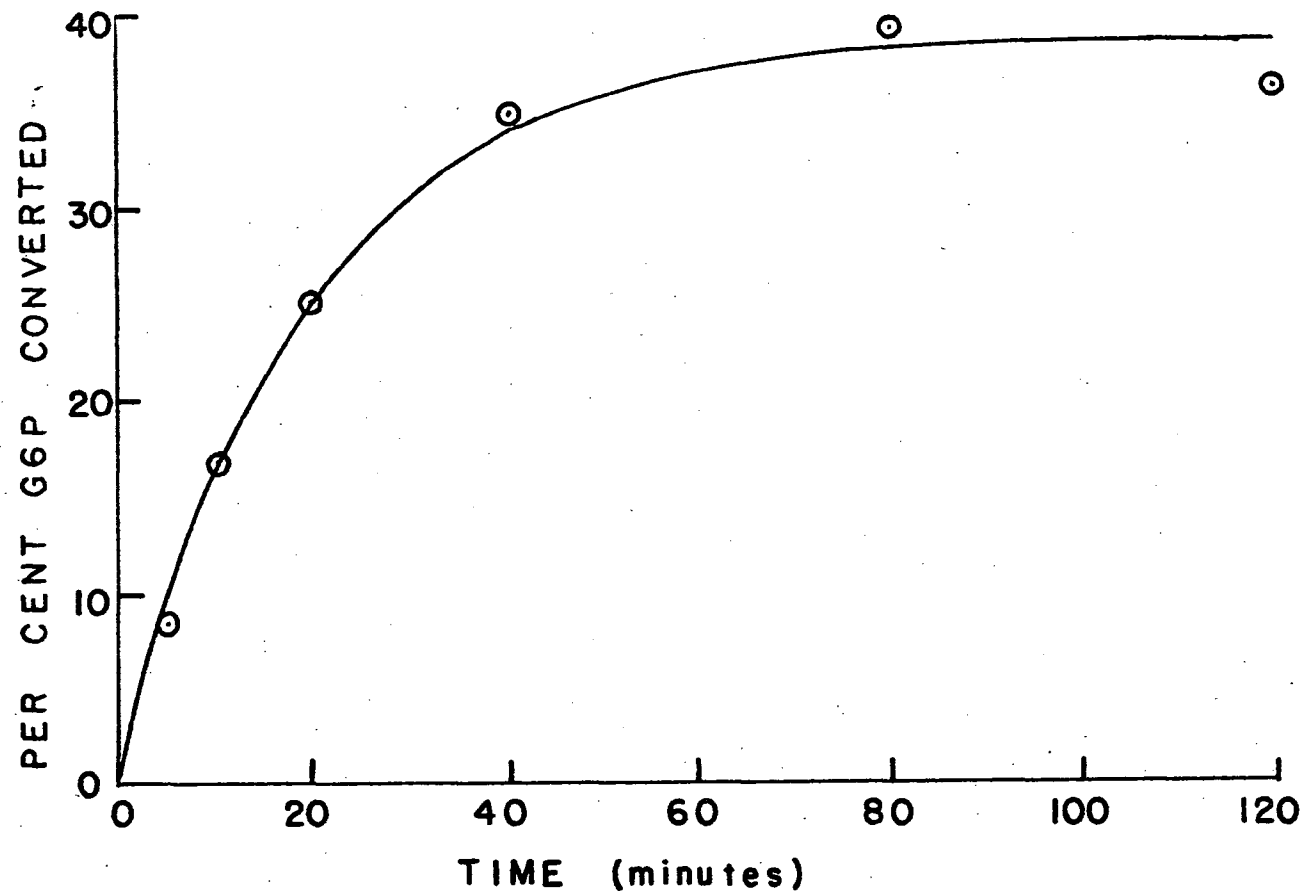


Fig. 25. Time curve of phosphoglucose isomerase activity at 30°C, pH 8.2. 12.5 µg of protein, eluted from a DEAE-cellulose column, were used in a final volume of 0.1 ml.

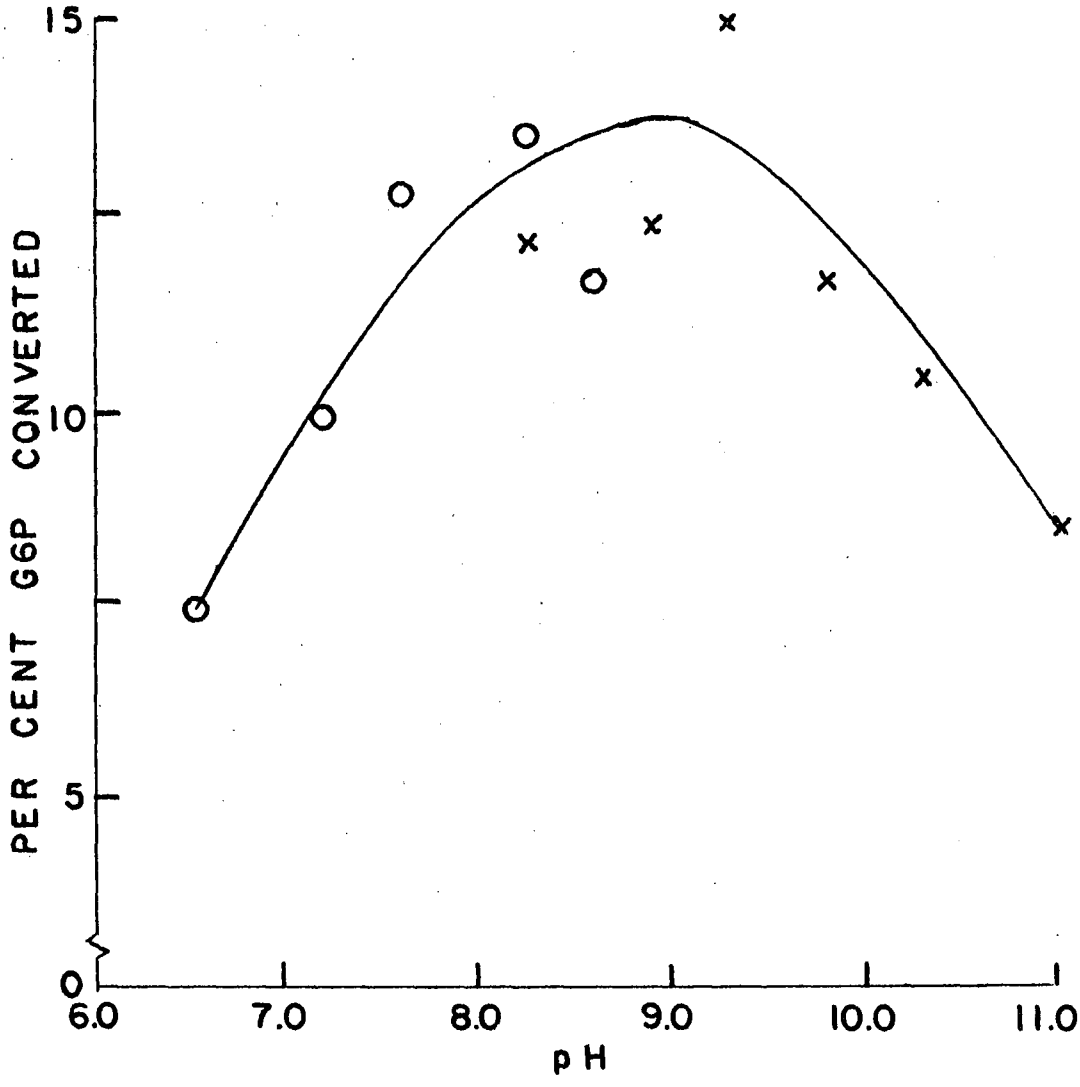


Fig. 26. The effect of pH on the activity of phosphoglucose isomerase at 30°C. Tris-maleate buffer: O—O Glycine-NaOH buffer: ×—×. 1.25 µg of protein, eluted from a DEAE-cellulose volume, were used in a final volume of 0.1 ml.

According to the present findings, there is a wide range of pH for the optimum activity, between 8.0 and 9.5. This is in the range of the values obtained by previous workers (Slein, 1955; Tsuboi et al, 1958; Singh, 1959).

DISCUSSION

The above results show that PGI is very active in muscle of lingcod. A few mμ of protein, obtained by diluting a crude extract by a few hundred times, was often adequate to complete the reaction in a relatively short period of time. The present results show that PGI from lingcod muscle is very similar in its properties to the corresponding mammalian enzyme.

Since this enzyme is relatively labile to heat, it would seem that a much better purification is obtained by chromatography on DEAE-cellulose. When so prepared, this enzyme was found to be unstable to freezing and thawing. However, it is more likely that more protein could be used to start with, so that the eluted enzyme could be easily kept in the frozen state.

V. GENERAL CONCLUSION

Considering the present results, in addition to the few results obtained by other workers, it appears highly probable that fish muscle contains the enzymes necessary for the breakdown of glycogen as they occur in mammals.

The present findings indicate that two enzymes involved in the Embden-Meyerhof pathway, PGM and PGI, and one of the "hexosemonophosphate

shunt", PRM, are present in lingcod muscle. They were found to have the same general properties as the corresponding mammalian enzymes, except that PGM was not found to require $G16P_2$ as a co-factor, unlike the mammalian enzyme.

It was not possible to compare the total activity of these enzymes in fish and mammals. The main reason was that frozen fish was used, while the study of these enzymes had been previously carried out on freshly killed mammals. However, it was observed on different crude preparations that PGI was very active and that it could be diluted several hundred times and still have high activity. Calculated on the basis of micromoles of substrate converted per unit of time per mg of protein, it was observed to be as much as 10 times more active than PGM in some cases. This observation would indicate that PGI is one of the most active glycolytic enzymes, as it is in mammals.

It was not possible to work with pure preparations of these three enzymes because of their instability. However, it is believed that the observations that were made will be of some value in any subsequent attempt to obtain greater purity.

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