THE PHOSPHOMONOESTERASE SYSTEM OF HUMAN SEMINAL FLUID AND RED CELLS

A THESIS

PRESENTED TO

THE FACULTY OF GRADUATE STUDIES

UNIVERSITY OF MANITOBA

IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE
MASTER OF SCIENCE

BY
GEORGE STUART WIBERG
APRIL 1951

ACKNOWLEDGMENTS

I should like to express my gratitude to Dr. F.D. White and to Dr. G.E. Delory for their kind assistance and helpful criticism, their ready counsel and encouragement.

TABLE OF CONTENTS

PART II

THE PHOSPHOMONOESTERASE SYSTEM OF HUMAN SEMINAL FLUID AND RED CELLS

SECTION

SECTION

III - EXPERIMENTAL WORK

SECTION

LIST OF TABLES

LIST OF FIGURES

PREFACE

The work reported here fell into two parts:-

(1) an examination of seminal fluid for hydrolytic action on esters other than those of phosphoric acid, and,

(2) the determination of the rates of hydrolysis of various phosphate esters, with special reference to the hexose phosphates, by human seminal fluid and red cell phosphatases.

Previous studies on seminal fluid phosphatase have revealed that it showed a distinct preference for certain substrates, the most marked being for phenyl phosphate. This lead to considerations as to whether some additional enzymes were present. Experiments were devised to detect the presence of these enzymes. This aspect will be dealt with in Part I.

Phosphatases are widely distributed throughout animal tissues, yet their physiological role is poorly understood. The hexose phosphates are also widely distributed throughout the body, and the possibility exists that they might

serve as natural substrates for the phosphatases. Martland and Robison (1927), postulated a mechanism for ossification involving alkaline bone phosphatase and hexose phosphate esters. It was felt that the acid phosphatases might similarly have some metabolic relationship with these substances. The action of the acid phosphatases of seminal fluid and red cells upon the hexose phosphates is studied in Part II.

PART I

I. THE HYDROLYTIC ACTION OF SEMINAL FLUID ON ESTERS OTHER THAN THOSE OF PHOSPHORIC ACID

1. Introduction

The phosphatase of seminal fluid shows a marked preference for certain substrates over others, e.g., phenyl phosphate is invariably hydrolyzed to a much greater extent than other phosphate esters. This could be due to the presence of a phenolase which hydrolyzed the phenolic linkage rather than the phosphate linkage in the substrate. This point does not seem to have been investigated previously, indeed it was found that there was a general dearth of information on the behaviour of seminal fluid towards non-phosphoric esters.

Accordingly it was decided to test this experimentally to determine if seminal fluid possessed any phenolase activity. It was also decided to see if seminal fluid displayed any general hydrolytic activity towards ethyl butyrate.

2. Phenolase Activity

Preliminary Observations

Provided a phenolase was present, seminal fluid would be expected to hydrolyze other phenyl esters besides phenyl phosphate. Therefore if some other phenyl ester be substituted as substrate, and evidence of its cleavage demonstrated to a comparable extent to that of phenyl phosphate, this would be proof that a phenolase was responsible for the pronounced hydrolysis of phenyl phosphate.

Method

(a) Principle

The method of Huggins and Smith (1947) for the estimation of phenylsulphatase activity was used. In this method disodium phenyl phosphate is replaced by the potassium salt of para-nitrophenyl sulphate as substrate. If a phenolase was present, it would liberate the p-nitrophenol from -p-nitrophenyl sulphate as well as the phenol from phenyl phosphate since the enzyme would be working on the phenol-inorganic acid linkage. The p-nitrophenol liberated was estimated colorimetrically. In neutral or acid solutions, p-nitrophenol is colourless, but in alkaline media p-nitrophenol develops a yellow colour which can be estimated quantitatively.

Rat liver homogenate, known to contain a phenylsulphatase was used to check the validity of the method.

P-nitrophenyl sulphate was not available commercially and had to be prepared in the laboratory. Details of this preparation are given in the next sub-section.

(b) Preparation of Potassium p-nitrophenyl sulphate

47 ml. of dimethylaniline were added to 50 ml. of carbon disulphide in a 500 ml. suction flask, then placed in an ice bath under the hood. 9.1 ml. of chlorosulphonic acid were added dropwise, and then 13.9 gm. of p-nitrophenol were added rapidly. The mixture was stirred for one hour and then permitted to stand overnight. 100 ml. of 0.4 M potassium hydroxide were added which brought about the immediate formation of bright yellow crystals. After thorough stirring, the flask was placed on a water bath at about 80° C. and most of the carbon disulphide removed by evaporation in vacuo. Excess dimethylaniline was removed by centrifugation, and the residual yellow mass was dried in vacuo for twenty-four hours. The crude product was recrystallized four times from 80% ethanol. The dry compound was stored at 0° C. to minimize decomposition.

(c) Reagents

0.5 N Acetate Buffer

63.97 gm. sodium acetate were dissolved in distilled water, 1.70 ml. glacial acetic acid added and the mixture diluted to 1 litre. The pH was adjusted on the pH meter to 5.80.

0.005 M Potassium paranitrophenyl sulphate

0.1285 gm. potassium p-nitrophenyl sulphate was dissolved in distilled water and made up to 100 ml.

1.0 N NaOH

P-nitrophenol Stock Solution

P-nitrophenol (Eastman Kodak) was recrystallized twice from water, and 100 mg. were dissolved in 100 ml. water.

Enzyme solutions

Seminal Fluid 1:25 dilution in water.

Rat Liver Homogenate - The liver was removed from a freshly sacrificed white rat, weighed rapidly, and then hogogenized with five times its weight of ice water in a Waring Blendor for 3 minutes. The homogenate was then centrifuged for 10 minutes and the supernatant liquid collected.

(d) Procedure

Each determination was run in duplicate with blanks. 3 ml. of buffer, 1 ml. of enzyme solution, and a small crystal of thymol (to inhibit bacterial growth) were added to a test tube, which was then allowed to equilibrate for 15 minutes in a constant temperature oven at 37° C. 1 ml. of the substrate was then pipetted into each of the experimental tubes. The tubes were then shaken and stoppered. After incubating for eighteen hours, 5 ml. of 1.0 N NaOH were added to all tubes. This pH was sufficiently alkaline to kill the enzyme activity. 1 ml. of the substrate solution was then added to the blanks, and the contents of the tubes were mixed by inverting several times.

The solutions were read in a Coleman Junior Spectrophotometer at 420 millimicrons against a standard containing 10 micrograms of p-nitrophenol in 10 ml. 0.5 N NaOH and the amount of free p-nitrophenol estimated.

Results

The arbitrary unit of phenylsulphatase activity is defined as the amount of enzyme which produces a colour equivalent of 10 micrograms of p-nitrophenol in a volume of 10 ml. 0.5 N NaOH, in 18 hours at 37° C., in acetate buffer at pH 5.8, the substrate being 0.005 molar.

Results of a series of experiments for both seminal fluid and rat liver homogenate are given in Table I. A comparison of the amount of phenol liberated from phenyl phosphate to the amount of p-nitrophenol liberated from p-nitrophenyl sulphate by weight for some typical experiments is given in Table II.

Discussion

Seminal fluid did show some phenylsulphatase activity. As might be expected, it was not a constant value but ranged from 0.146 to 0.75 phenylsulphatase units, for the samples investigated. Rat liver homogenate displayed a much higher phenylsulphatase activity. This latter points to the validity of the method in estimating phenolase activity.

The amount of hydrolysis by seminal fluid was very small considering that the time of incubation was eighteen hours and the enzyme concentration was 1:25. Experience has shown that under similar conditions,

0.05 molar phenyl phosphate would be fully hydrolyzed.

Table II shows that considerably more phenol by weight is liberated from phenyl phosphate than p-nitrophenol from p-nitrophenyl sulphate, even with the time of hydrolysis and the enzymic concentration of the latter being greatly in excess of that employed with respect to phenyl phosphate.

Delory and King (1939), Andersch and Szczpinski, (1947) and Delory (1948) have established that p-nitrophenyl phosphate is hydrolyzed to a slightly greater extent than phenyl phosphate for a large number of phosphatases including the prostatic enzyme. The ratio of the phosphate liberated from the p-nitrophenyl ester to that from the phenyl ester is not a constant but varies slightly with the conditions of the test. In the great majority of phosphatases examined, including prostatic phosphatase, it was of the order of 1.15.

The above factors would suggest that the pronounced hydrolysis of phenyl phosphate, in distinction to the other phosphate esters, is not the result of a phenolase being present in seminal fluid. Although seminal fluid has been found to possess some phenolase activity, it is not sufficient to markedly affect the hydrolysis of phenyl phosphate.

TABLE I

PHENYLSULPHATASE ACTIVITY OF SEMINAL FLUID AND RAT LIVER HOMOGENATE

Sample	Micrograms of P-nitrophenol Liberated	Phenylsulphatase Units
(a) Seminal Fluid		
1.	1.46	0.146
2.	2.2	0.22
3.	2.4	0.24
4.	7.5	0.75
(b) Rat Liver Homogenate		
1.	62.5	6.25

TABLE II

COMPARISON BY WEIGHT OF PHENOL LIBERATED FROM PHENOL PHOSPHATE TO THAT OF PARANITROPHENOL FROM PARANITROPHENOL SULPHATE

Mg. phenol liberated per 100 ml. enzyme	Mg. p-nitrophenol liberated per 100 ml enzyme
92.4	0.240
81.9	0.220
89.95	0.146
62.22	0.750
49.50	
16.82	

3. Action of Seminal Fluid upon Ethyl Butyrate

Preliminary Remarks

Ethyl butyrate has been commonly used as a substrate for investigating the hydrolytic activity of what might be called the "non-specific" esterases. It has also been used in the investigation of lipases where it sometimes serves as a substrate, in lieu of the triglycerides. It was felt that ethyl butyrate would fulfill the substrate requirements in investigating seminal fluid for the presence of any enzyme which might exhibit a general hydrolytic effect, i.e. an unlimited esterase. Similarly, if lipases were present, it was felt that the ethyl butyrate would as a consequence show some degree of hydrolysis.

Method in Principle

(a) The method used was one developed for the estimation of lipase activity by this department (White, 1950). In this method the substrate is homogenized with the buffer using a hand homogenizer. The enzyme is added to buffered substrate and the mixture shaken. It is allowed to incubate for a peridd of thirty minutes at a temperature of 37° C., and then added to ice water to diminish enzyme activity. Blanks are run at the same time, the enzyme solution being added at the end of the period of incubation. The tests and blanks are titrated

to the original pH with standard alkali. The difference in ml. alkali between that required for the test and that for the blank will give the measure of enzyme activity.

Method in Detail

Reagents

Calcium Acetate - Veronal Buffer pH 9.5

10 gm. calcium acetate and 5 gm. veronal were dissolved in distilled water and made up to 1 litre. Final pH was adjusted electrometrically to pH 9.5.

Redistilled Ethyl Butyrate

0.05 N NaOH

Procedure

2.0 ml. ethyl butyrate were added to 8.0 ml. distilled water in a test tube and the mixture shaken. It was then homogenized with 90 ml. buffer, by passing three times through a hand homogenizer, the test tube being rinsed several times with the homogenate to ensure that all the ethyl butyrate was taken up.

20 ml. of the above solution was pipetted into a test tube, which was placed in a water bath at 37° C. and allowed to equilibrate for 10 minutes. At the end of this period, 1 ml. of the enzyme solution, which had likewise been allowed to equilibrate for a similar time,

was added to the homogenized mixture. This solution was incubated for thirty minutes. At the end of the period of incubation, the mixture was transferred to a 250 ml. beaker containing 80 ml. of ice water.

The solution was titrated to the original pH using a Beckman Model G pH meter. The beaker was placed on an electromagnetic stirrer, and the large electrodes of the pH meter were placed below the level of the solution.

The validity of the method was checked by using rat serum, which was known to contain both lipase and pseudocholinesterase. The serum was obtained from a freshly sacrificed white rat and was used undiluted.

Results

Table III shows the degree of hydrolysis of ethyl butyrate by seminal fluid (in dilutions of 1:10, 1:50 and 1:100). The activity of rat serum in this respect is also shown in the same Table.

Discussion

As the results in Table III show, seminal fluid has no hydrolytic effect on ethyl butyrate. It seems therefore, that the enzyme responsible for the hydrolysis of phenyl phosphate and other phosphoric esters is a true phosphatase, its lipase and "non-specific esterase" activity being negligible.

TABLE III

ACTION OF SEMINAL FLUID AND RAT SERUM UPON ETHYL BUTYRATE

Dilution	No. of Ml. 0.05N NaOH used in test	No. of ml. 0.05N NaOH used in blank
(a) Seminal Fluid		
1:10	0.88	0.89
1:50	0.88	0.88
1:100	0.88	0.88
(b) Rat Serum		
Undiluted	2.00	1.20
Undiluted	2.07	1.30

4. Summary

Seminal fluid was tested for phenolase activity. Four different samples of seminal fluid were found to possess phenolase activity ranging from 0.146 to 0.75 arbitrary units.

Considerably more phenol was liberated from phenyl phosphate by seminal fluid than p-nitrophenol from p-nitrophenyl sulphate, even when the latter had a longer period of incubation and less dilute enzyme solution.

The hydrolytic action of seminal fluid upon ethyl butyrate was investigated. No hydrolytic action was found.

The hydrolytic action of seminal fluid towards phenyl phosphate does not appear to be caused by a phenolase.

PART II

THE PHOSPHOMONOESTERASE SYSTEM OF HUMAN SEMINAL FLUID AND RED BLOOD CELLS

SECTION

I. HISTORICAL

1. Early Work on the Phosphatases

Phosphatases are enzymes which catalyze the hydrolysis of phosphate esters yielding inorganic phosphate and what might be called in a very general sense, the alcohol.

The first phosphatase was discovered in 1907 by Suzuki, Yosimura and Takaishi. They found an enzyme in wheat and rice bran capable of decomposing phytin (the calcium magnesium salt of inositol hexosephosphoric ester), with the liberation of inorganic phosphate. Subsequently a great many phosphatases have been found in both the plant and animal kingdom.

McCollum and Hart (1908) demonstrated the presence of an enzyme in calf liver and blood that would hydrolyze phytin. Plasmata derived from various organs of the dog was found by Levene and Medigreceanu (1911) to hydrolyze nucleotides with the liberation of free phosphate; intestinal mucosa and kidney producing the most active extracts. Grosser and Hussler (1912), found an enzyme that would hydrolyze glycerophosphate widely

distributed in animal organisms.

Much of the early work on phosphatases has been reviewed by Kay (1932).

The first phosphatases that were discovered all showed their optimum activity in the alkaline range from pH 8 - 10, although it was later shown that the optimum depended upon the conditions of hydrolysis as well as the substrate. (King and Delory 1939).

In 1931, Roche demonstrated the existence of a phosphatase in red blood cells which had a pH optimum on the acid side of neutrality. Subsequently other acid phosphatases have been discovered, amongst which may be mentioned yeast acid phosphatase, possessing a pH optimum of 4.0 with phenyl phosphate, and the acid phosphatases of liver, kidney and spleen. Demuth in 1925 found an acid phosphatase in human urine, which Kutscher and Wolbergs (1935) traced to the prostate gland. This phosphatase displayed a pH optimum of 5.1 for phenyl phosphate and a plateau for beta-glycerophosphate ranging from pH 4.0 - 5.1. This phosphatase was later found to be present in the seminal fluid.

Classification of the Phosphatases

The following scheme has been put forward by Folley and Kay (1936) for the classification of the phosphatases.

Class of Enzyme	Type of Substrate Hydrolyzed	Products
A. Phosphomonoesterases	Monoesters of orthophosphoric acid	R.OH and H_3PO_4
B. Phosphodiesterases	Diesters of orthophosphoric acid	R_1OH and $R_2-H_2PO_4$
C. Pyrophosphatases	Salts and diesters of pyrophosphoric acid	$R_1-H_2PO_4$ and $R_2-H_2PO_4$
D. Metaphosphatases	Salts of metaphosphoric acid	Salts of orthophosphoric acid
E. Phosphoamidases	N-substituted amide phosphoric acids	$R.NH_2$ and H_3PO_4

The phosphomonoesterases have been further subdivided according to the following table:

Subclass	Approx optimum pH	Relative rates of hydrolysis with with alpha and betaglycerophosphate	Effect of Mg at optimum pH
A1	9-10	beta greater than alpha	Activation
A2	4.5-5.5	beta greater than alpha	No activation
A3	3-4	beta greater than alpha	Activation with alpha but not with beta
A4	6	alpha greater than beta	Activation

Recent work has emphasized certain difficulties in these classifications. For example, both seminal fluid and red cell phosphatases exhibit widely different pH optima for different substrates and under different conditions of incubation. As a result, these classifications need revision if they are to continue in the literature. For clinical convenience, it has proved advantageous to divide the phosphatases into two main groups, the acid and the alkaline phosphatases, the former exhibiting their optimum activity in the acid range, and the latter on the alkaline side of neutrality.

2. Seminal Fluid Phosphatase

In 1925, Demuth found a phosphatase present in human urine. This was extensively investigated by Wolbergs in 1935, and he reported, amongst other data, that it possessed a pH optimum between 3 and 4, acted more strongly on beta- than alphaglycerophosphate, and was not activated by magnesium ions. Urine phosphatase was found to be present to a greater extent in males than in females. Kutscher set forth to trace this down, and later in the same year, Kutscher and Wolbergs (1935) found the prostate gland to be very rich in an acid phosphatase. It possessed a pH optimum between 4 and 6.5, acted more strongly on beta- than alphaglycerophosphate, and was not activated by magnesium. On evidence such as this, the source of the male urinary phosphatase was tentatively tied up with the prostate gland. At the same time, Kutscher and Wolbergs (1935), were able to show that the seminal fluid was enriched with this enzyme during ejaculation.

Subsequent to these discoveries, considerable work has been done on the phosphatase of the prostate and seminal fluid, the most noteworthy contributors being the King school in London and the Gutmans with their collaborators in New York.

Gutman, Sproule and Gutman (1936), observed that bones which were the sites of metastases from carcinoma of the prostate had greatly increased acid phosphatase activity, in addition to the normal increase of alkaline phosphatase arising from the laying down of new bone salt. These findings were confirmed by Barringer and Woodward (1938), who stated:-
"prostatic cancer retains the ability to produce large amounts of acid phosphatase, which is characteristic of the normal adult prostate, and that distant metastases shares this ability with the primary tumor."

Gutman and Gutman (1938) and Robinson, Gutman and Gutman (1939) demonstrated that normal serum contained an acid phosphatase. The activity of the normal serum was low, but the serum phosphatase of patients with metastasizing prostatic carcinoma was usually raised.

This fact is used nowadays in the diagnosis of carcinoma of the prostate. Barringer and Woodward (1938), noted that in certain cases, other than prostatic cancer, elevated serum acid phosphatase levels were encountered. The source of this normal serum acid phosphatase has still to be established. It is generally believed to originate from the tissue phosphatases and also the red cells.

Various methods have been proposed for the differentiation of the so-called normal serum acid phosphatase and that present in the serum from prostatic metastases. Herbert (1946), devised a technique using absolute alcohol, which he claimed would inactivate the prostatic phosphatase but not the normal serum acid phosphatase. Abul-Fadl and King (1948), developed a method using formalin. The formalin inhibited the serum acid phosphatase but not the prostatic enzyme. This subject has been reviewed by King and Delory (1948).

Physiological Role

In addition to the clinical aspects of prostatic phosphatase mentioned above, there has been a certain amount of work done upon its physiological role, particularly with reference to its presence in the seminal fluid. Gutman and Gutman (1941), investigated the seminal fluid of sterile males for phosphatase activity, and found that it was of the same level as that of normal men. Delory (1947), conducted an investigation into the phosphatase activity of the seminal fluid in connection with the number of spermatozoa and their motility. He did not discover any correlation between these factors.

Lundquist (1946) reported that the function of seminal fluid phosphatase is to hydrolyze phosphorylcholine. Stored seminal fluid, he stated, was rich in both choline and inorganic phosphate, whereas freshly ejeculated semen is poor in both these substances, but rich in phosphorylcholine. The pH optimum of this reaction he found to be identical with the pH of the vaginal tract. It is not certain what would be accomplished by the splitting of phosphorylcholine, for it is not a compound containing high energy phosphate bonds.

Lardy and Phillips (1941) reported that seminal fluid phosphatase might have the function of a lecithinase, since they found a decrease in lipoid phosphorus and an increase in ester phosphorus in semen which had been allowed to stand. MacLeod (1939), assigns some glycolytic function to this enzyme. Here one thing is of interest, Mann (1948), was able to demonstrate the presence of fructose in seminal fluid, and further that sperm cells were able to metabolize fructose, glucose and mannose. This ability to metabolize fructose is not found in many tissues. Mann further points out, that it is difficult to follow any glycolytic pathway in human seminal fluid, since the seminal plasma is so rich in phosphatase, that the substrates of the primary glycolytic enzymes, (the hexose phosphates,) are rapidly hydrolyzed. Reis (1940) found that human seminal plasma had a phosphatase activity towards certain nucleotides.

Nucleotides attacked included inosinic acid, adenylic acid and yeast adenine nucleotide. He was able to show that among the seminal phosphatases, there was one specifically concerned with the hydrolysis of adenylic acid which he called "5-nucleotidase". This enzyme was completely inactive towards A.T.P. and adenine nucleotide. Using bull semen, Mann (1945), was able to confirm the existence of a highly active 5-nucleotidase. With bull semen, he found the activity could be expressed by the ratio:

$$\frac{\text{moles of phosphorus split from adenylic acid}}{\text{moles of phosphorus split from betaphosphoglycerol}} = \frac{300}{1}$$

Abul-Fadl and King (1949) have made a detailed study of the acid phosphatases of seminal fluid and red cells with respect to kinetics, pH optima, and the effect of metallic and acidic ions. Although they accumulated considerable data, they did not postulate any physiological mechanism for seminal fluid phosphatase.

3. Red Cell Phosphatase

The presence of a phosphatase in the red cells of mammalian blood was first demonstrated by Martland, Hansman and Robison in 1924, and confirmed by Demuth in 1925. Roche (1931) published the first detailed study of this enzyme using the red cells of cattle and the white rat. He found that it had an optimum lying between pH 6.0 and 6.8, and that it acted more energetically upon the alpha- than upon the betaglycerophosphate. The hydrolysis was inhibited in the presence of inorganic phosphate or glycerol. Roche also stated that this enzyme possessed the ability to synthesize phosphoric esters from inorganic phosphate and the various alcohols, e.g., glycol, glycerol and hexoses. In 1942, Roche, van-Thoai and Badouin claimed to have discovered two distinct acid phosphatases in the red cells of cattle and rats, these possessing pH optima of 4.0-4.2 and 5.4, respectively. The beef phosphatase hydrolyzed the alpha-glycerophosphate isomer to the greater extent. The phosphatase with the optimum at pH 4.0 was inhibited by magnesium ions, whereas that with an optimum at 5.4 was activated. Ascorbic acid likewise inhibited the enzyme with the more acid pH optimum and activated the other. Both were activated by glutathione and cysteine. Cystine, sodium sulphite and ferrous sulphate were found to inhibit the phosphatase with the lower pH optimum, but had no marked effect on the other phosphatase.

These workers, Roche et al, further reported the presence of two pyrophosphatases, distinct from the phosphomonoesterases, in the red cells of cattle. These pyrophosphatases could be separated from the phosphomonoesterases by adsorption and elution from kaolin at pH 5.0. Both the pyrophosphatases were activated by magnesium, and to an even greater extent by ascorbic acid.

Behrendt (1943) found a phosphatase in washed human red cells. Its presence was confirmed by King Wood and Delory in 1945. They found it to be optimally active at pH 4.8 - 5.2. It was inhibited by magnesium, fluoride, prolonged treatment with ethanol, and was not appreciably effected by glycine or ascorbic acid. Abul-Fadl and King (1949) later reported a confirmation of the work of Roche that red cell phosphatase possessed two pH optima, one between 4.0 - 4.3, and the other between 5.0 and 5.5. The latter workers found this red cell enzyme hydrolyzed alpha-glycerophosphate more rapidly than betaglycerophosphate, and that the effect of magnesium was negligible. Calcium, manganese and zinc showed varying degrees of inhibition, while cobalt and nickel inhibited in certain cases and not in others. Copper ions had a marked inhibitory effect even at relatively low concentrations (0.0002 M), iron inhibited at a stronger concentration. Glycine caused inhibition while alanine was without effect. Glutathione and cysteine by themselves possessed no marked effect. Cyanide (0.01 M) showed slight activation.

In the present work, the presence of a second pH optimum for red cell phosphatase was found only occasionally. Most of the blood samples were obtained from surplus blood from routine tests performed in the Winnipeg General Hospital Biochemical Laboratory, and this blood was generally collected early in the morning, and held several hours at room temperature before release. Since Abul-Fadl and King (1949) noted that the more acid enzyme was destroyed by standing for a few hours at room temperature, it could well be that the second peak was destroyed before the blood samples were obtained.

SECTION II - ANALYTICAL PROCEDURES

1. Estimation of Phosphatase Activity

(a) Early Work

In a problem of this nature, it is essential that some basis be established for the comparison of the activity of the enzyme towards different substrates.

The first widely adopted for the estimation of phosphatase activity in blood plasma was that of Jenner and Kay (1932). It consisted of incubating the enzyme in a solution of sodium beta-glycerophosphate with a glycine buffer. The enzyme activity was destroyed and the liberated inorganic phosphate determined. The phosphatase activity was defined as the number of mg. of phosphorus liberated per 100 ml. plasma.

This method was modified by King and Armstrong (1934). They replaced the sodium beta-glycerophosphate with disodium phenyl phosphate as the substrate, and measured the amount of phenol liberated after the method of Folin and Ciocalteau (1927). In this method a veronal buffer was employed. The phosphatase activity was expressed as units per 100 ml. serum and is numerically equal to the number of mg. phenol that would be set free from the phenyl phosphate under standard conditions.

Delory and King (1945) have made a further modifcation of this method. A sodium carbonate - sodium bicarbonate buffer is used for the alkaline phosphatase estimation, and a sodium citrate - citric acid buffer is used for estimation of acid phosphatase activity.

In the United States the method of Bodansky (1933) is generally used. This method employs sodium betaglycerophosphate as the substrate, and measures the inorganic phosphate liberated. Since phenol is approximately three times the molecular weight of phosphorus, sometimes Bodansky units are converted into King-Armstrong units by multiplying by three. The rates of hydrolysis of phenyl phosphate and betaglycerophosphate are not the same under all conditions and this conversion should be reviewed with reserve.

As it was the intention to test other phosphate esters besides phenyl phosphate, and compare the degree of hydrolysis, obviously only the liberated phosphate could be measured since it was the only common hydrolytic product. It was therefore decided that the phosphatase activity would be expressed as the number of mg. of phosphorus liberated per 100 ml. of enzyme solution per unit of time.

It was also decided to use phenyl phosphate as a standard substrate and compare all the other phosphoric esters to it. A ratio would be established with phenyl phosphate equal to 1. It is often difficult to obtain from biological substances, enzyme preparations whose activity

is of a constant magnitude, e.g., considerable variation has been noted in the phosphatase activity of seminal fluid. With phenyl phosphate being employed as a standard substrate, and its degree of hydrolysis being taken as unity, it was hoped that this trouble would be obviated.

(b) Determination of Inorganic Phosphate as an Index of Phosphatase Activity

For the determination of phosphatase activity, it was necessary to measure the inorganic phosphate set free without hydrolyzing the ester phosphorus. Many methods have been proposed for the determination of inorganic phosphate, and the method of choice would possess the advantages of simplicity, sensitivity, rapidity and accuracy.

Gravimetric Methods. - Gravimetric methods depend upon the precipitation of phosphorus as magnesium ammonium phosphate, which can be weighed as such, or else converted to magnesium pyrophosphate prior to weighing by ignition. Such methods however are not suitable to the present problem, for they are laborious and time consuming and not readily applicable to the estimation of very small amounts of inorganic phosphate.

Colorimetric Methods - Most of the colorimetric methods in use are modifications of the original method of Bell and Doisy (1920). The basis of this method is that ammonium molybdate and inorganic phosphate interact to form phosphomolybdic acid, which is then reduced to a lower valence oxide of molybdenum by stannous chloride. This "molybdenum oxide" has a blue colour, the intensity of which is proportional to the amount of inorganic phosphate originally present.

Fiske and SubbaRow (1925) modified this method by replacing the stannous chloride with 1-amino-2-naphthol-4-sulphonic acid in a sodium sulphite - sodium bisulphite solution (A.N.S. reagent) as the reducing agent. King (1947) changed the final pH of the Fiske and SubbaRow procedure. Berenblum and Chain (1938) tested the effectiveness of a series of reducing agents including A.N.S., potassium iodide, stannous chloride, etc. They devised a method in which the phosphomolybdic acid was extracted with iso-butyl alcohol and reduced with stannous chloride. Allen (1940) substituted amidol for A.N.S. Waygood made a critical study of all these methods in 1948, and finally adopted ascorbic acid as the reducing agent.

These various methods were suitably investigated to see which held the most promise for the present study. It was found that the rate of colour development in the Fiske and SubbaRow method was not uniform from sample to sample, and that considerable time was required before the maximum depth of colour was obtained. Also traces of iron, sometimes present in the trichloracetic acid, produced what Fiske and SubbaRow called a "clock" reaction. This was a very slow deepening of the blue colour, and was independent of the concentration of the inorganic phosphate. Obviously the Fiske and SubbaRow method was not suitable for the present task.

The King method gave a stable colour, but its pH was such that it hydrolyzed the labile phosphate esters, particularly the glucose-1-phosphate. The technique of Berenblum and Chain involved the extraction of phosphomolybdic acid in separatory funnels. This method was cumbersome and unsuited to routine use. The method of Allen gave stable colours for a period of from three to thirty minutes.

Waygood found that there were two pH ranges suitable for the estimation of inorganic phosphate. These were from 0.5 to 1.5 and from 3.1 to 4.5. At the upper limits of the second range, hydrolysis of labile phosphate esters would be negligible. Unfortunately Waygood's method was too sensitive. When the concentration of inorganic phosphorus exceeded 30 micrograms, considerable time was required to attain full colour development.

Finally it was decided to test a modification of the King procedure. In this modification, double quantities of both ammonium molybdate and A.N.S. reagent were used. It was hoped that the excess of molybdate would bring about a rapid formation of the phosphomolybdic acid, and that the excess of ANS reagent would fully reduce the phosphomolybdic acid before the hydrolysis of the labile phosphate esters became significant. Such was found to be the case upon testing, and hence this modification of King's method for the determination of inorganic phosphate was adopted for estimating phosphatase activity.

(c) Choice of Buffer

Three buffer systems were available covering the acid range to be surveyed; viz., phosphate buffer, citrate buffer and acetate buffer. Phosphate buffer was rejected for obvious reasons. Citrate buffer in strong concentration, interfered with the colour development in phosphate determinations. Acetate buffer was free from this objection and hence was selected.

(d) Conditions of Incubation

The temperature of incubation was 37° C.

The time of incubation was 30 minutes for seminal fluid phosphatase, and 1 hour for red cell phosphatase.

(c) Method in Detail

Reagents

20% Trichloracetic Acid - 20 gm. of the acid are dissolved in water and made up to 100 ml. When 1 - 2 ml. are treated with 10% potassium thiocyanate solution, no more than a just perceptible pink colour should develop.

Acid Ammonium Molybdate - 5 gm. ammonium molybdate are dissolved in 80 ml. water containing 15 ml. concentrated sulphuric acid, and when cool, made up to 100 ml.

A.N.S. Reagent - 0.2 gm 1-2-4 aminonaphtholsulphonic acid, 12 gm. sodium metabisulphite and 2.4 gm. anhydrous sodium sulphite are dissolved in water and made up to 100 ml. This solution is allowed to stand overnight, and if not clear, it is filtered before use. This reagent is not stable and should be replaced every two weeks.

Standard Phosphorus Solution

Stock Solution - 2.194 gm. potassium dihydrogen phosphate are dissolved in 500 ml. water. This contains 1 mg. P per ml.

Dilute Standard Solution - 1 ml. of the stock solution is diluted with water to 100 ml. This contains 0.01 mg. P per ml. Both the Stock and the Dilute Standard Solutions should be treated with 1 - 2 drops chloroform to inhibit bacterial growth.

Acetate Buffers - 0.2 molar acetate buffers were prepared, according to the method of Cole (1920), covering the acid range pH 3.5 - 7.0 by suitable fractions of a pH unit. Final pH was adjusted electrometrically. A few drops of chloroform were added to prevent bacterial growth.

Substrates - 0.01 molar solutions were prepared of the following substances:

Disodium phenyl phosphate

Sodium alphaglycerophosphate

Sodium betaglycerophosphate

Glucose-1-phosphate (K salt)

Glucose-6-phosphate (Na salt)

Fructose-6-phosphate (Na salt)

Fructose-1, 6-diphosphate (Na salt)

The last three hexose phosphates were available commercially only in the form of their calcium or barium salts. Calcium and barium ions have been shown to exert an activating effect on acid phosphatases, so they were converted into the sodium derivatives. This was accomplished by adding a stoichiometric quantity of dry sodium carbonate before making up to the mark with water. The insoluble barium or calcium carbonate was removed by filtration. A few drops of chloroform were added to inhibit bacterial growth.

Enzyme Solutions

Seminal Fluid - Fresh seminal fluid was kindly supplied by Dr. David Swartz from samples submitted to his Sterility Clinic in Winnipeg. The seminal fluid was diluted from 1:500 to 1:2000 in physiological saline, depending upon its phosphatase activity. The degree of dilution was determined by making preliminary trials with a 1:500 dilution, and increasing the dilution until a suitable degree of hydrolysis was obtained.

Red Cells - Fresh oxalated whole blood was centrifuged and the plasma discarded. It was washed three times with physiological saline, centrifuged and the supernatant layer removed, including the buffy layer of leucocytes. The red cells were diluted 1:10 with water and shaken to ensure haemolysis.

Procedure

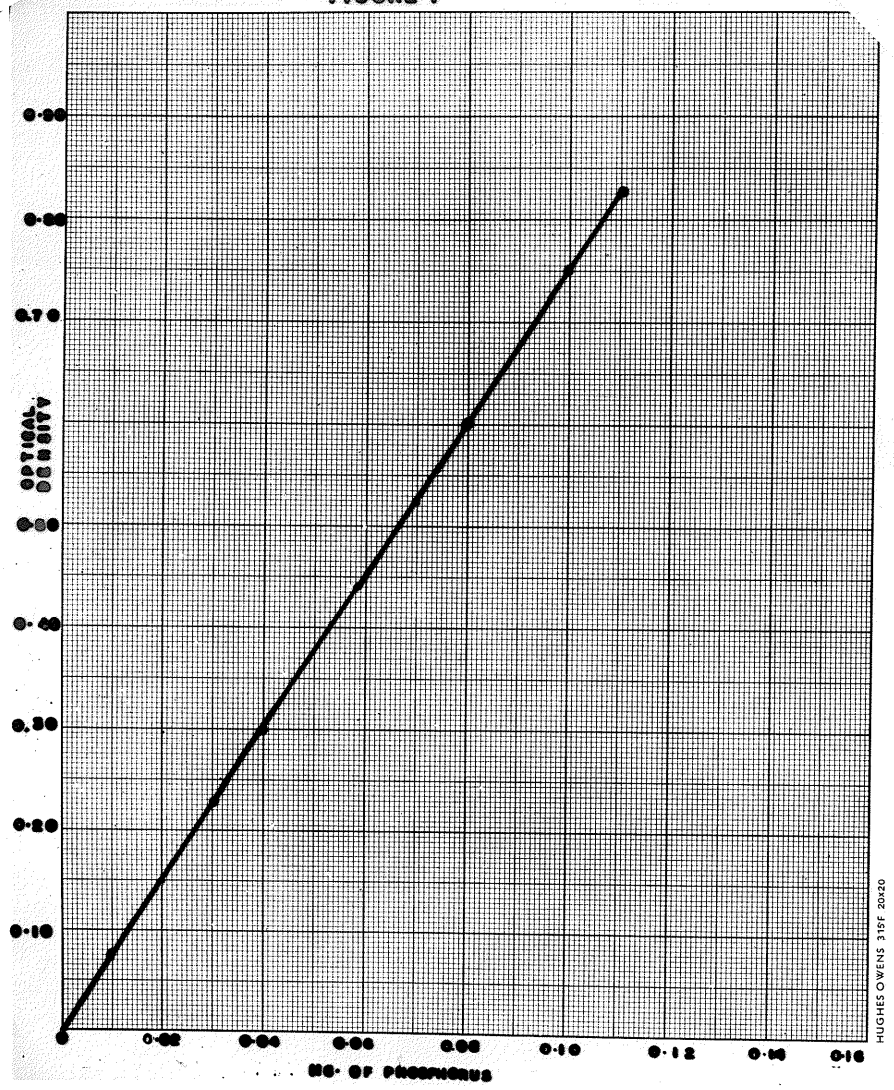
The tests and the blanks were run in duplicate. 2 ml. of the buffer and 2 ml. of the substrate were each added to a test tube, which was then allowed to equilibrate at 37° C. for 10 minutes. A volume of enzyme solution sufficient for all the tests and blanks was equilibrated at the same time. When the 10 minutes had elapsed, a stopwatch was started, and 2 ml. of the enzyme solution were added to each of the tests at thirty second intervals. All the test tubes were then stoppered, shaken, and then incubated for the exact period as noted on page 33. At the end of the period of incubation, 1 ml. of trichloracetic acid was added to each tube of the tests at thirty second intervals, and then to the blanks. 2 ml. of enzyme solution were now added to the blanks. After this, all the solutions were filtered.

A suitable aliquot of the filtrate, (1 ml. in the case of phenyl phosphate and 2 ml. for the other phosphate esters), was transferred to a 15 ml. volumetric flask, 2 ml. of acid ammonium molybdate added, and then 0.5 ml. of A.N.S. reagent. The solutions were made up to the mark with water. At the same time, a standard was prepared, containing 5 ml. of the dilute standard in lieu of the incubated solution. This was equal to 50 micrograms of inorganic phosphorus. The colours were read exactly 10 minutes after the addition of the A.N.S. reagent, using a Coleman Junior Spectrophotometer set at 650 millimicrons, and the inorganic phosphate calculated.

Reliability of Method

A series of tests were performed in which the optical density was found for known concentrations of inorganic phosphate. A graph in which the optical density was plotted against the phosphorus concentration was prepared and is shown as Fig. 1 on page 39. A straight line is produced, which signifies that the method follows Beer's Law, and hence is suitable for the determination of inorganic phosphate, and therefore the estimation of phosphatase activity.

FIGURE 1



Relationship between the optical density and the inorganic phosphorus concentration using the modified King method.

SECTION III - EXPERIMENTAL WORK

1. Introductory

The experimental work shall be reported in several subsections. It is proposed to present together, the results obtained from the seminal fluid phosphatase and the red cell phosphatase rather than in separate sections, since this will facilitate comparison and contrast of these respective enzymes.

2. Determination of the pH optima for Seminal Fluid and Red Cell Phosphatases

Introductory

In studying enzyme reactions, either the physiological pH, an arbitrary pH or the optimum pH may be employed. The physiological pH is often difficult to detect, and there is no assurance that the pH actually found is the true physiological pH, since the introduction of extraneous material or the removal from natural surroundings could have significant effects. An arbitrarily selected pH has certain inherent dangers, especially when it is used with different substrates.

If two substrates possess pH optima widely separated, and the arbitrary pH chosen lies much closer to one than to the other, one is apt to attribute an enzymic preference for one substrate over the other which may not be the case. At the optimum pH, the enzyme is acting to the fullest extent possible under the experimental conditions, which makes comparisons valid.

The pH optimum is actually a function of several factors, including purity of the enzyme preparation, substrate and substrate concentration, buffer, time and temperature of incubation. Any change in these factors can result in a shift of the pH optimum. Accordingly, if the term pH optimum is to be meaningful, it must be rigidly defined as to enzyme concentration, substrate concentration, buffer, time and temperature of incubation.

Method

The procedure followed was the same as that given on page 36, but with the following exceptions. Tests were run in triplicate rather than in duplicate. At the half-period of incubation, this third test tube was removed and its pH determined. This constituted the pH of the test. A series of buffers, ranging from pH 3.5 to 7.0 and differing by 0.5 pH units, were employed. The amount of hydrolysis in each case was noted. A new series of buffers, differing

by 0.2 pH units but confined to the area of maximum hydrolysis, were substituted and the test repeated, as above. The amount of free inorganic phosphate was then calculated, and that pH showing maximum hydrolysis was considered to be the pH optimum. pH optima were determined for phenyl phosphate, the two glycerophosphate isomers, and the hexose phosphates, using both seminal fluid and red cell phosphatases.

Results

The pH optimum for each substrate investigated for both seminal fluid phosphatase and red cell phosphatase is given in Table IV on page 43.

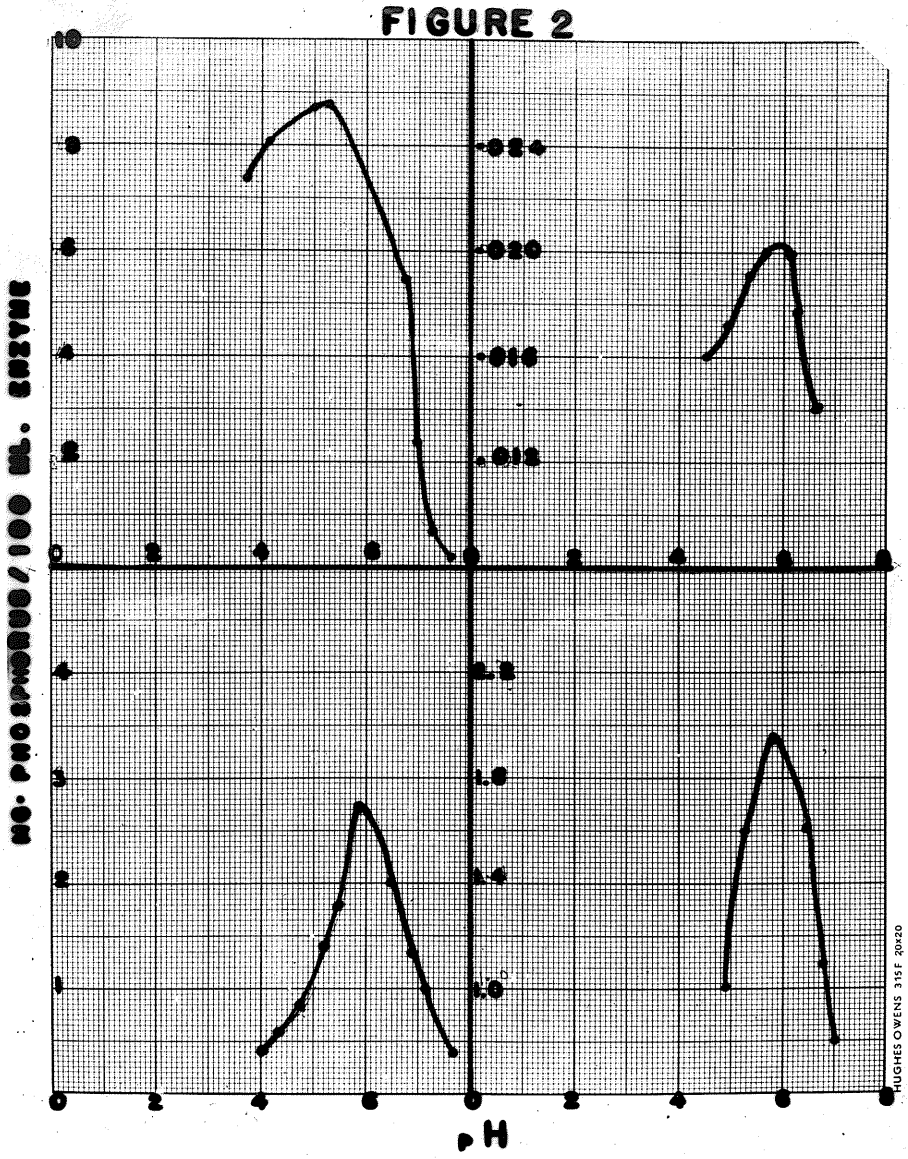
pH activity curves for both enzymes covering all the substrates investigated are given in Figs. 2 - 5 on pages 44 - 47 inclusive.

TABLE IV

pH OPTIMA OF VARIOUS PHOSPHATE ESTERS WITH SEMINAL FLUID AND RED CELL PHOSPHATASE

Substrate	Seminal fluid Phosphatase pH optima	Red cell Phosphatase pH optima
Phenyl phosphate	5.21	5.80
Sodium alpha-glycerophosphate	5.80	5.6-5.8
Sodium beta-glycerophosphate	5.50	6.30
Glucose-1-phosphate	4.88	6.00
Glucose-6-phosphate	4.90	5.01
Fructose-6-phosphate	5.51	5.82
Hexose diphosphate	5.70	6.5-6.8

FIGURE 2



pH Activity Curves

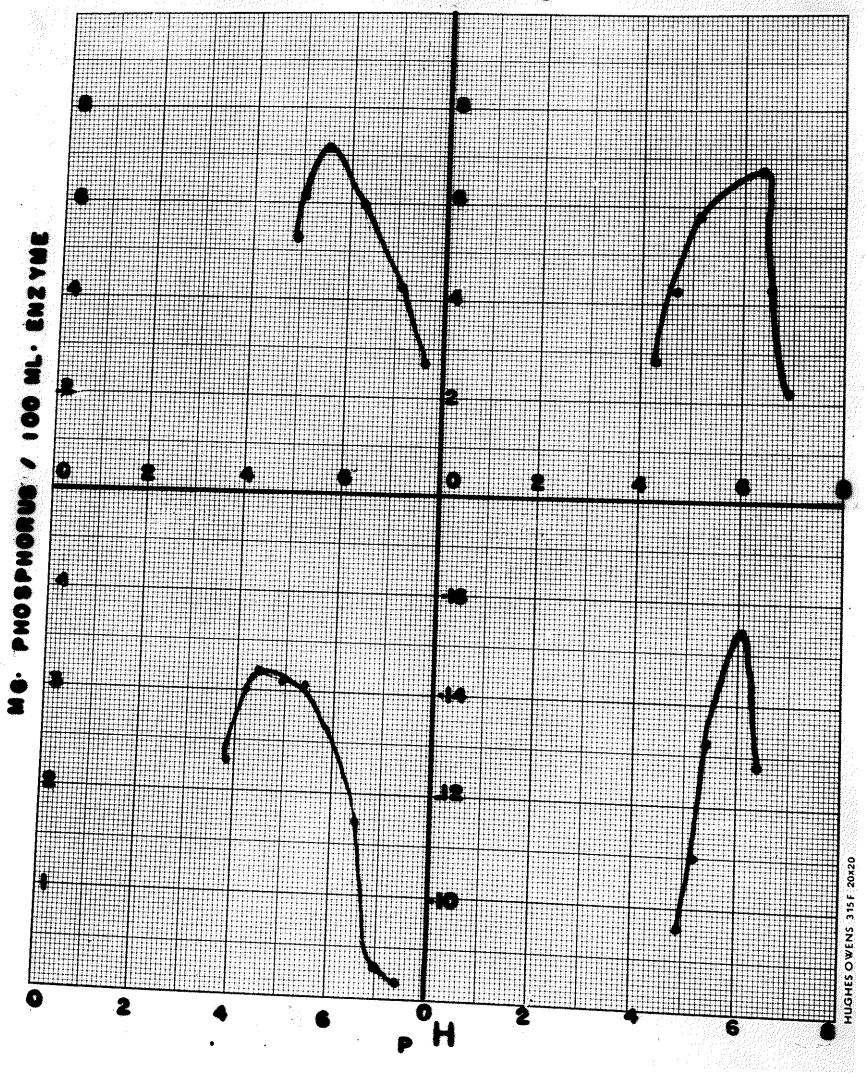
(a) Phenyl phosphate with seminal fluid phosphatase

(b) Phenyl phosphate with red cell phosphatase

(c) Sodium alpha-glycerophosphate with seminal fluid phosphatase

(d) Sodium alpha-glycerophosphate with red cell phosphatase

FIGURE 3



pH Activity Curves

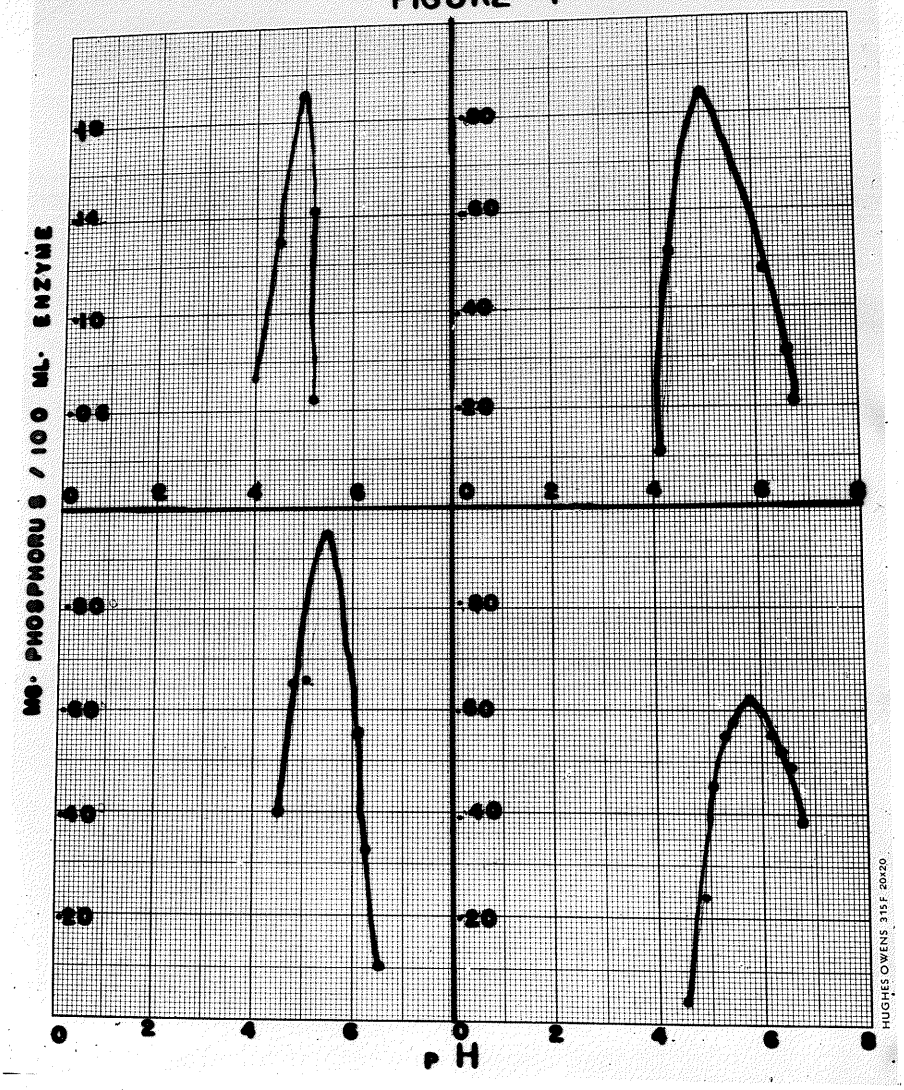
(a) Na betaglycerophosphate and seminal fluid phosphatase

(b) Na betaglycerophosphate and red cell phosphatase

(c) Glucose-1-phosphate and seminal fluid phosphatase

(d) Glucose-1-phosphatase and red cell phosphatase

FIGURE 4

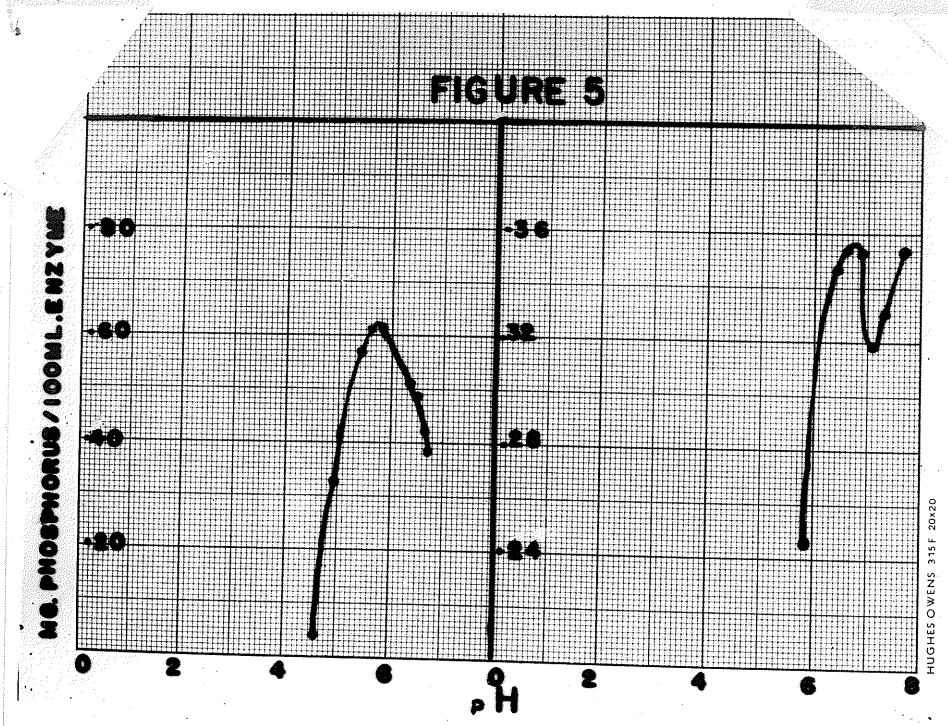


(a) Glucose-6-phosphate and seminal fluid phosphatase

(b) Glucose-6-phosphate and red cell phosphatase

(c) Fructose-6-phosphate and seminal fluid phosphatase

(d) Fructose-6-phosphate and red cell phosphatase



pH Activity Curves

(a) Fructose-1, 6-diphosphate and seminal fluid phosphatase

(b) Fructose-1, 6-diphosphate and red cell phosphatase

3. Rates of Hydrolysis

Introductory

The rates of hydrolysis of phenyl phosphate, the glycerophosphates, and the hexose phosphates were determined both for seminal fluid and red cell phosphatase. These results will be expressed as the ratio of inorganic phosphorus liberated from each ester to that liberated from phenyl phosphate under similar test conditions.

Method

The method employed followed that given on page 36. Each substrate was investigated separately. It was buffered at its optimum pH. Simultaneously, phenyl phosphate, also buffered at its optimum pH, was incubated with the same enzyme preparation. The amount of liberated phosphorus was estimated, and then converted into mg. liberated per 100 ml. enzyme. This result was divided by the amount which was liberated from phenyl phosphate per 100 ml. enzyme under conditions, of a similar nature.

Results

(i) Hydrolysis of Glycerophosphates

Table V gives the amount of hydrolysis of the glycerophosphates by seminal fluid phosphatase and red cell phosphatase.

(ii) Hydrolysis of Hexose Phosphates

The hydrolysis of the hexose phosphates by seminal fluid and red cell phosphatases is shown in Table VI.

Discussion

Table V shows that seminal fluid phosphatase hydrolyzes the beta isomer of glycerophosphoric acid to the greater extent, whereas red cell phosphatase acts more strongly upon the alphaglycerophosphate. This fact has been confirmed many times by previous workers, as noted in the Historical Introduction.

Table V also shows that phenyl phosphate is more fully hydrolyzed than either of the glycerophosphates for both enzymes. Also it is evident that seminal fluid phosphatase has a stronger hydrolytic effect on the glycerophosphates than the red cell enzyme, for the alphaglycerophosphate is hydrolyzed 8 times as much and the beta isomer 70 times as much with seminal fluid phosphatase.

Another interesting difference in the hydrolysis of the glycerophosphates, is that the alpha- and beta-glycerophosphates are hydrolyzed to more or less the same extent by seminal fluid phosphatase, whereas red cell phosphatase attacks the alphaglycerophosphate almost 7 times as much as it does the betaglycerophosphate.

Before discussing the hexose phosphates in detail, the case of fructose-1,6-diphosphate requires special attention. Seminal fluid and red cell phosphatases are able to hydrolyze both carbon 1 and carbon 6 phosphate linkages in the monophosphates, since both glucose-1-phosphate and glucose-6-phosphate liberate inorganic phosphorus. Therefore it is reasonable to assume that both linkages in the hexose diphosphate molecule are attacked, and that two phosphate radicals are set free. Accordingly if one is to give comparable results the degree of hydrolysis of the fructose-1,6-diphosphate should be divided by two.

Table VI shows that hexose diphosphate is attacked to the greater extent by both seminal fluid and red cell phosphatases, however as mentioned in the preceding paragraph this value must be divided by two to place it in its proper perspective.

Table VI shows that there is a definite gradation in the degree of hydrolysis of the hexose phosphates by seminal fluid. This gradation, in descending order is as follows:

glucose-1-phosphate

fructose-6-phosphate

fructose-1,6-diphosphate

glucose-6-phosphate.

The red cell phosphatase, on the other hand does not show such a pronounced gradation. Glucose-6-phosphate and fructose-6-phosphate are hydrolyzed to the same extent, and somewhat more than the hexose diphosphate. Glucose-1-phosphate is hydrolyzed to the least extent. An interesting point in this regard is the complete reversal of the position of glucose-1-phosphate, from being the most hydrolyzed with seminal fluid phosphatase to the least attacked with the red cell enzyme. One obvious deduction is that the phosphatases of seminal fluid and red cells are not the same.

Table VI also indicates that seminal fluid phosphatase hydrolyzes the hexose phosphates to a greater extent than does its red cell counterpart. The hydrolysis of glucose-1-phosphate is 20 times greater, glucose-6-phosphate twice as great and the fructose phosphates both four times as great. This fact also suggests the dissimilarity of the enzyme systems.

TABLE V

DEGREE OF HYDROLYSIS OF ALPHA AND BETA GLYCEROPHOSPHATES WITH SEMINAL FLUID AND RED CELL PHOSPHATASES

Substrate	Seminal fluid Phosphatase	Red cell Phosphatase
Phenyl phosphate	(1.000)	(1.000)
Sodium alpha-glycerophosphate	0.370	0.045
Sodium beta-glycerophosphate	0.480	0.007

Note: Results are expressed as percentages of the phenyl phosphate hydrolysis.

TABLE VI

DEGREE OF HYDROLYSIS OF THE HEXOSE PHOSPHATES WITH SEMINAL FLUID AND RED CELL PHOSPHATASE

Substrate	Seminal fluid Phosphatase	Red cell Phosphatase
Phenyl phosphate	(1.000)	(1.000)
Glucose-1-phosphate	0.317	0.015
Glucose-6-phosphate	0.139	0.062
Fructose-6-phosphate	0.282	0.069
Fructose-1,6-diphosphate	0.166	0.044

Notes: Results are expressed as percentages of the phenyl phosphate hydrolysis.

For comparison, the values of the fructose-1,6-diphosphate hydrolysis have been halved.

4. Effect of Dialysis

In the work mentioned so far, the amount of inorganic phosphate liberated by the action of a preparation of red cells or seminal fluid has been taken as a measure of the phosphatase activity. This is justified when phenyl phosphate is the substrate, but with the hexose phosphates other factors must be considered. Suppose, for example, a mixture of phosphatase and hexokinase be incubated with glucose-6-phosphate (all necessary activating substances being present), then, while inorganic phosphate will be liberated by the action of the phosphatase, there will be an uptake of phosphate due to the synthesis of glucose-6-phosphate. In the circumstances a falsely low measure of the phosphatase activity will be recorded. Similar considerations apply to other glycolytic enzymes which may coexist with the phosphatase, such as phosphorylase, phosphotriose isomerase, etc.

Attempts were therefore made to measure the true phosphatase activity by taking advantage of the fact that the glycolytic enzymes mentioned above are inactivated by dialysis, while seminal fluid phosphatase is said to be unaffected, Abul-Fadl and King (1949). In addition, it was also desired to establish whether dialysis would have any effect on the degree of hydrolysis of the various substrates.

Method

The method was unchanged insofar as the incubation and estimation of inorganic phosphate was concerned. However, both undialyzed and dialyzed enzyme solutions were used. Dialyzed solutions of the enzymes were prepared by placing the normal enzyme preparation in a cellophane dialyzing tube and dialyzing overnight against running water. Upon occasion, the length of the period of dialysis was increased, but these special cases will be mentioned individually in the discussion.

Results

The hydrolysis of the hexose phosphates by dialyzed and undialyzed seminal fluid phosphatase was measured. The results of these experiments are given in Table VII.

The effect of dialysis upon the hydrolysis of phenyl phosphate by red cell phosphatase is given in Table VIII.

The degree of hydrolysis of the glycerophosphates and the hexose phosphates, relative to phenyl phosphate, by red cell phosphatase before and after dialysis, is given in Table IX.

Discussion

The typical results presented in Table VII show that overnight dialysis has very little effect on seminal fluid phosphatase. This is in marked contrast to the findings with red cell phosphatase which are presented in Table VIII. The actual fall in the red cell phosphatase level was, not unexpectedly, variable. On the whole activity decreased with increasing time of dialysis, and the activity could be completely removed with prolonged dialysis.

A complicating factor is that the activity of the red cell phosphatase gradually goes off on standing, even in the ice chest. For example one sample of red cell phosphatase lost 45% of its original activity in five days standing at 0° C. This factor is no doubt involved in the loss of activity with dialysis, but the velocity of the loss of activity with dialysis is too great to be due to this factor alone.

This fact was emphasized by the following experiment. 50 ml. of red cell phosphatase solution were prepared, and 25 ml. of this solution were dialyzed overnight in a cellophane tube, while the remaining 25 ml. were kept in a test tube, and placed in the same beaker in which the dialyzing membrane was suspended. Both enzyme preparations were tested for phosphatase activity the next day. The preparation which had been dialyzed overnight showed a 23% loss of activity, yet the conditions of storage were practically the same.

Since there was such considerable variation in the loss of activity upon dialysis, no table of "typical" results could be prepared.

Table VIII shows that this loss of activity is not restored by magnesium ions nor the addition of boiled blood. Many alkaline phosphatases contain magnesium as a prosthetic group, and are activated by its presence. However such is not the case with red cell phosphatase. Boiled whole blood was used in case some metal or organic group was present as a prosthetic group. Since the activity was not restored one may conclude, that if a prosthetic group is being removed by dialysis, it must be a heat labile compound, or on the other hand an irreversible inactivation is brought about by dialysis. The length of dialysis was 48 hours and the only substrate investigated was phenyl phosphate.

A series of incubations was carried out with dialyzed and undialyzed red cell phosphatase to see if any substrate was particularly affected. As Table IX shows, the loss of activity had no effect upon the relative degree of hydrolysis. It must be emphasized that in these experiments the amount of hydrolysis with the dialyzed enzyme was less in every instance than that obtained with the undialyzed enzyme. In these experiments the length of dialysis was overnight. Another interesting fact is that the same order of substrate preference prevails after dialysis.

This could mean that a single phosphatase is acting upon all the different substrates. As it undergoes an irreversible inactivation, only a portion of the substrate will be hydrolyzed, compared to that which was hydrolyzed before. If several phosphatases were present and different ones were were responsible for attacking different substrates, it would not be unreasonable to expect some variation in the velocities of inactivation, and hence in the degree of hydrolysis, by dialyzed and undialyzed enzyme preparations.

TABLE VII

EFFECT OF DIALYSIS UPON THE HYDROLYTIC ACTIVITY OF SEMINAL FLUID PHOSPHATASE

Substrate	Undialyzed Enzyme	Dialyzed Enzyme
Phenyl phosphate	1.000	1.000
Glucose-1-phosphate	0.317	0.352
Glucose-6-phosphate	0.133	0.135
Fructose-6-phosphate	0.300	0.289
Hexose diphosphate	0.350	0.348

TABLE VIII

THE HYDROLYSIS OF PHENYL PHOSPHATE BY DIALYZED AND UNDIALYZED RED CELL PHOSPHATASE AND THE EFFECT OF THE ADDITION OF MAGNESIUM AND OF BOILED WHOLE BLOOD TO THE DIALYZED ENZYME

Undialyzed Red Cell Phosphatase	Dialyzed Red Cell Phosphatase	Dialyzed Red Cell Phosphatase plus 0.01 molar Magnesium	Dialyzed Red Cell Phosphatase plus Boiled Whole Blood
15 mg.P liberated per 100 ml. enzyme	1.57 mg.P liberated per 100 ml. enzyme	1.57 mg.P liberated per 100 ml. enzyme	1.62 mg.P liberated per 100 ml. enzyme

TABLE IX

A COMPARISON OF THE DEGREE OF HYDROLYSIS OF PHOSPHATE ESTERS BY RED CELL PHOSPHATASE BEFORE AND AFTER DIALYSIS

Substrate	Undialyzed Red cell Phosphatase	Dialyzed Red cell Phosphatase
Phenyl phosphate	(1.000)	(1.000)
Alpha-glycerophosphate	0.070	0.080
Beta-glycerophosphate	0.002	0.001
Glucose-1-phosphate	0.012	0.017
Glucose-6-phosphate	0.058	0.062
Fructose-6-phosphate	0.032	0.033

Note: Results are expressed as percentages of the phenyl phosphate hydrolysis under the respective conditions of dialyzed and undialyzed enzyme.

5. Effect of Cyanide

Introductory

The addition of cyanide has been found to inhibit many enzyme reactions, the most marked inhibition being on those enzymes containing iron and copper. Roche (1946) and Abul-Fadl and King (1949) investigated the effect of 0.01 molar cyanide upon different phosphatases. In those alkaline phosphatases which contained magnesium as a prosthetic group, they found inhibition, but the acid phosphatases were not significantly affected.

It was decided to test the effect of 0.01 and 0.1 molar cyanide upon the acid phosphatases of semen and red cells. The so-called "respiratory enzymes" are inhibited by cyanide and it was desired to ascertain if their inhibition would have any effect upon the degree of hydrolysis of phosphate esters by the acid phosphatases.

Method

0.04 and 0.4 molar sodium cyanide were prepared. 2 ml. of cyanide was added to the substrate-buffer mixture before equilibration. This gave final concentration of 0.01 and 0.1M cyanide. Other tests were run simultaneously in which the cyanide was replaced by 2 ml. of water. Other details of the method remained unchanged.

Results

The effects of cyanide upon seminal fluid phosphatase and red cell phosphatase and their action upon different substrates are shown in Table X. Seminal fluid phosphatase was investigated in 0.01 and 0.1 molar concentrations, but the red cell phosphatase was investigated in the stronger concentration only. In the Table, the word "nil" indicates the complete absence of enzymic activity.

Discussion

Table X shows that 0.01 molar cyanide has little effect upon the hydrolysis of the phosphate esters apart from the hexose diphosphate. The hydrolysis of hexose diphosphate is activated one-and-one-half times. It is interesting to note that Gomori (1943) found a specific hexose diphosphatase in intestinal mucosa, which, he reported, was activated by cyanide.

Table X also shows that in every instance, phosphatase activity was completely inhibited by the presence of 0.1 molar cyanide. Therefore it seems probable that there is some critical concentration at which the enzymic activity is completely destroyed. Roche (1946) used the fact that acid phosphatase activity was not destroyed by 0.01 molar cyanide to lend credence to the belief that metallic prosthetic groups are absent.

TABLE X

EFFECT OF CYANIDE ON THE HYDROLYSIS OF PHOSPHATE ESTERS BY SEMINAL FLUID PHOSPHATASE AND RED CELL PHOSPHATASE

Substrate	Seminal Fluid Phosphatase			Red Cell Phosphatase	
	Normal	0.01 Molar Cyanide	0.1 Molar Cyanide	Normal	0.1 Molar Cyanide
phenyl phosphate	1.000	0.90	nil	1.0	nil
glucose-1-phosphate	0.317	0.304	nil	0.015	nil
glucose-6-phosphate	0.139	0.157	nil	0.062	nil
fructose-6-phosphate	0.282	0.261	nil	0.069	nil
hexose diphosphate	0.333	0.451	nil	0.087	nil

6. Interconversion of the Glucose Phosphates

Introductory

Broh-Kahn, Mirsky, Persutti and Brand (1948), studied the action of liver phosphatase on hexose phosphates. They reported that when glucose-1-phosphate was incubated with liver slices, it was rapidly converted into glucose 6 phosphate. The glucose-6-phosphate was then converted into free phosphate and glucose. The latter reaction proceeded at a much slower velocity.

Since seminal fluid and red cell phosphatases have both been assigned some glycolytic activity by various workers, (as noted in the historical sections on these enzymes), it seemed important to determine whether glycolytic enzymes were affecting the results of the phosphatase studies. Glycolytic enzymes could affect the amount of phosphate being liberated and hence alter the value of the phosphatase activity as determined.

Experimental

The quantitative determination of the glucose phosphates in the presence of each other is based upon their different rates of hydrolysis in 1 N HCl at 100° C. Glucose-1-phosphate is labile and is fully hydrolyzed

by 1 N HCl at 100° C. within 7 minutes, while glucose-6-phosphate is not attacked under the same conditions. The total phosphate in any mixture can be estimated after a preliminary "wet ashing" with 70% perchloric acid until the organic matter has been destroyed. The inorganic or unbound phosphate can be estimated in the usual way, i.e, the free phosphate in the blank.

It was established qualitatively with Seliwanoff's reagent that no fructose was formed when either glucose-1-phosphate or glucose-6-phosphate was incubated with seminal fluid or red cell phosphatases.

Therefore the distribution of the total phosphate would be as follows:

total phosphate = glucose-1-phosphate plus glucose-6-phosphate plus inorganic phosphate.

If, P_t represents the total phosphate,

P_7 represents the phosphate after hydrolysis with 1 N HCl at 100° C. for 7 minutes, and,

P_0 represents the inorganic phosphate,

then: glucose-1-phosphate = $P_7 - P_0$, and,

glucose-6-phosphate = $P_t - P_7$.

Method in Detail

Total phosphate was determined as follows. 1 ml aliquots of the incubated and blank enzyme solutions were transferred to 15 ml. volumetric flasks. 1.2 ml. of 70% perchloric acid were added to the flasks, which were then heated on a hot plate until the dark colour disappeared. Sometimes it was necessary to add 1 - 2 drops 30% hydrogen peroxide to facilitate the oxidation of the organic matter. Upon completion of the oxidation, the phosphate was estimated in the usual manner.

Inorganic phosphate was estimated by measuring the amount of free phosphate in the incubated and blank enzyme solutions.

In estimating the P_7, 1 ml. of the filtrate was transferred to a 15 ml. volumetric flask, and then 1 ml. of 2 N HCl added. This mixture was placed in a beaker of boiling water for exactly 7 minutes, cooled, and the inorganic phosphate estimated.

Results

The results of a series of tests in which either glucose-1-phosphate or glucose-6-phosphate were incubated with seminal fluid and red cell phosphatases are given in Table XI.

The difference between the P_7 and P_0 values will give the amount of glucose-1-phosphate present. The difference between the P_t and the P_7 values will give the amount of glucose-6-phosphate present. If any glucose-6-phosphate can be located in those tests where glucose-1-phosphate was incubated, this will be evidence of phosphoglucomutase activity. Conversely, the presence of glucose-1-phosphate in those mixtures whose original substrate was glucose-6-phosphate would similarly indicate glucomutase activity.

If the P_t and P_7 are the same for a test, then no glucose-6-phosphate is present. If the P_7 and P_0 are the same, then glucose-1-phosphate is absent.

Discussion

Table XI shows that the P_t and P_7 are identical when glucose-1-phosphate is incubated with either seminal fluid or red cell phosphatase. This indicates that no glucose-6-phosphate is present, and that all the phosphate is present as either glucose-1-phosphate or inorganic phosphate. The P_0 is higher in the incubated sample than in the blank since it has been hydrolyzed by the phosphatase and more inorganic phosphate is present. Solutions of seminal fluid and red cells do not bring about the formation of glucose-6-phosphate from glucose-1-phosphate.

The P_7 and P_0 values for the incubated glucose-6-phosphate are the same. This indicates that only glucose-6-phosphate is present and that no conversion to glucose-1-phosphate has taken place. This observation applies to both red cell phosphatase and to seminal fluid phosphatase.

The values of the P_t, P_7, and P_0 are all slightly higher in the red cell enzyme solution used, than in the seminal fluid solution. This is due to the fact that the level of inorganic phosphate in the original seminal fluid becomes insignificant when diluted to 1:500 or greater, while that present in the red cells still remains appreciable since the dilution factor is merely 1:10.

TABLE XI

INTERCONVERSION OF GLUCOSE PHOSPHATES

Substrate	mg. P per ml. substrate				
	P_t	P_7	P_0	P_7-P_0	P_t-P_7
(a) Seminal Fluid Phosphatase					
Glucose-1-phosphate, incubated	0.29	0.29	0.008	0.282	0.000
Glucose-1-phosphate, blank	0.29	0.285	0.000	0.285	0.000
Glucose-6-phosphate, incubated	0.305	0.002	0.002	0.000	0.303
Glucose-6-phosphate, blank	0.300	0.000	0.000	0.000	0.300
(b) Red Cell Phosphatase					
Glucose-1-phosphate, incubated	0.31	0.31	0.005	0.305	0.000
Glucose-1-phosphate, blank	0.31	0.315	0.001	0.314	0.000
Glucose-6-phosphate, incubated	0.325	0.013	0.013	0.000	0.312
Glucose-6-phosphate, blank	0.320	0.001	0.001	0.000	0.319

P_t represents the totalphosphorus content.

P_7-P_0 represents the glucose-1-phosphate

P_t-P_7 represents the glucose-6-phosphate

SECTION IV

SUMMARY OF THE EXPERIMENTAL WORK ON SEMINAL FLUID AND RED CELL PHOSPHATASES

1. pH Optima

Both enzyme systems were tested to determine the pH optima with respect to phenyl phosphate, the glycerophosphates, and the hexose phosphates. pH optima were determined for the experimental conditions investigated, and are listed in Table IV on page 43.

2. Rates of Hydrolysis

The rates of hydrolysis, (with phenyl phosphate = 1) of the glycerophosphates and the hexose phosphates were studied. Seminal fluid phosphatase was found to attack beta-glycerophosphate more readily than alpha-glycerophosphate, while the opposite condition prevailed with red cell phosphatase. Seminal fluid phosphatase exhibited a definite gradation in degree of attack upon the hexose phosphates. This was, in descending order of attack, glucose-1-phosphate, fructose-6-phosphate, hexose diphosphate and glucose-6-phosphate. Seminal fluid phosphatase was also found to have a greater hydrolytic effect upon phosphate esters than the red cell phosphatase. Red cell phosphatase did not possess the same marked gradation in the degree of hydrolysis of the hexose phosphates.

3. Effect of Cyanide

The effect of cyanide on the hydrolysis of the phosphate esters was examined. The hydrolysis of fructose-1, 6-diphosphate by seminal fluid phosphatase was activated by 0.01 molar cyanide. 0.01 molar cyanide had no appreciable effect on the hydrolysis of the other hexose phosphates by the seminal fluid enzyme. 0.1 molar cyanide completely inhibited the hydrolysis of the hexose phosphates with respect to both red cell and seminal fluid phosphatase.

4. Effect of Dialysis

Both phosphatase preparations were dialyzed to see if it would make any change. Seminal fluid phosphatase was not effected by dialysis. Red cell phosphatase underwent some inactivation, which could not be restored by the addition of magnesium ions nor of boiled whole blood. The degree of inactivation was a function of the length of dialysis. Red cell phosphatase, partially inactivated by dialysis, still showed the same ratio with respect to phenyl phosphate and and the other phosphates, as existed before dialysis.

5. Interconversion of Glucose Phosphates.

Seminal fluid and red cell enzyme preparations were examined for the presence of glycolytic enzymes such as phosphoglucomutase, which can catalyze the conversion of glucose-1-phosphate into glucose-6-phosphate, or glucose-6-phosphate into glucose-1-phosphate. This reaction attains an equilibrium and its direction depends upon the initial substrate concentration. Tests in which glucose-1-phosphate was incubated, were found to contain glucose-1-phosphate after incubation. Similarly incubated samples of glucose-6-phosphate yielded only only glucose-6-phosphate, besides of course, the liberated inorganic phosphate. No interconversion of these esters took place.

6. Identity of the Enzymes

Seminal fluid phosphatase and red cell phosphatase exhibit widely different pH optima for the same substrate. They also exhibit different rates of hydrolysis for a series of substrates, and indeed possess a different order of preference. Red cell phosphatase is sensitive to dialysis while seminal fluid phosphatase remains unaffected. This is further evidence that the acid phosphatases of seminal fluid and red cells are not identical, as has been put forward by Abul-Fadl and King (1949).

BIBLIOGRAPHY

Abul-Fadl, M.A.M. and King, E.J., (1948). J. Clin. Path., 1, 80.

Abul-Fadl, M.A.M. and King, E.J., (1949). Biochem. J., 45, 51.

Andersch, M.A. and Szczpinski, A.J. (1947). Amer. J. Clin. Path., 17, 571.

Allen, R.J.L., Biochem. J., (1940), 34, 858.

Barringer, B.S. and Woodward, H.Q., (1938), Trans. Amer. Assoc. Genito-urin. Surg. 31, 363.

Behrendt, H., (1943). Proc. Soc. Exp. Biol. N.Y., 54, 268.

Bell, R.D. and Doisy, E.A., (1920). J. Biol. Chem., 44, 55.

Berenblum, J. and Chain, E., (1938). Biochem J., 32, 286 and 295.

Bodansky, A., (1933). J. Biol. Chem. 101, 93.

Broh-Kahn, R.H., Mirsky, I.A., Persutti G. and Brand, J. (1948). Archives of Biochem., 16, 87.

Cole, S.W., (1920). Practical Physiological Chemistry, Cambridge.

Delory, G.E. and King, E.J., (1943). Biochem. J., 37, 547.

Delory, G.E. and King, E.J., (1945). Biochem. J., 39, 245.

Delory, G.E., (1947). Brit. Med. J. 1, 566.

Delory, G.E., (1948). Biochem. J., 43, xiv.

Demuth, F., (1925). Biochem. Z. 159, 415.

Chr. de Duve, Berthet, J., Hers, H.G. and Dupret, L., (1949). Bull. Soc. Chim. Biol. 31, 1242.

Fiske, C.H. and SubbaRow, Y., (1925). J. Biol. Chem. 66, 375.

Folin, O. and Ciocalteau, V., (1927). J. Biol. Chem. 73, 627.

Folley and Kay H.D. (1936). Ergeb. Enzymforsch., 5, 159.

Gomori, G., (1943). J. Biol. Chem., 148, 139.

Grosser, P. and Husler, J., (1912), Biochem. Z., 39, 1.

Gutman, E.B., Sproule, E.E. and Gutman, A.B., (1936). Am. J. Cancer, 28, 485.

Gutman, A.B. and Gutman, E.B., (1938). Proc. Soc. Exp. Biol. and Med., 38, 470.

Gutman, A.B. and Gutman, E.B., (1941). Endocrinology, 28, 115.

Harrer, C.J. and King, C.G., (1941), J. Biol. Chem., 138, 111.

Herbert, F.K., (1946). Quart. J. Med., 39, 221.

Huggins, C. and Smith, D.R., (1947). J. Biol. Chem. 170, 391.

Jenner, H.D. and Kay, H.D., (1932). Brit. J. Exp. Path., 13, 22.

Kay, H.D. (1932). Physiol. Revs., 12, 384.

King, E.J. and Armstrong, A.R., (1934). Can. Med. Assoc. J. 31, 376.

King, E.J., Wood, E.J. and Delory, G.E., (1945). Biochem. J., 39, xxiv.

King, E.J., (1947). Microanalysis in Medical Biochemistry, London.

Kutscher, W., (1935). Z. Physiol. Chem. 235, 62.

Kutscher, W. and Wolbergs, H., (1935). Z. Physiol. Chem. 236, 237.

Kutscher, W. and Worner, A., (1936). Hoppe-Seyl. Z., 239, 109.

Lardy, H.A. and Phillips, P.H., (1941). Amer. J. Physiol., 133, 602.

Levene, P.A. and Medigreceanu, F., (1911). J. Biol. Chem. 9, 65 and 389.

Lundquist, F., (1946). Nature, 158, 710.

MacLeod, J., Proc. Soc. Exp. Biol., 42, 153, 1939.

Mann, T., (1945), Biochem. J., 39, 451.

Mann, T., (1946), Biochem. J., 40, 481.

Mann, T., (1949). Advances in Enzymology, 9, 329.

Martland, M., Hansman, F.S. and Robison, R., (1924) Biochem. J., 18, 1152.

Martland, M. and Robison, R., (1927)., Biochem. J., 21, 665.

McCollum, E.V. and Hart, E.B., (1908). J. Biol. Chem. 4, 497.

Reis, J., (1940), Bull. Soc. Chim. Biol. 22, 36.

Robinson, J.N., Gutman, E.B. and Gutman, A.B., (1939). J. Urol. 42, 602.

Roche, J., (1931), Biochem. J., 25, 1724.

Roche, J., Nguyen-van Thoai and Badouin, J., (1942). Bull. Soc. Chim. Biol. 24, 1249.

Roche, J., (1946), Helv. Chim. Acta., 29, 1253.

Roche, J. and Bouchilloux, S., (1950). Bull. Soc. Chim. Biol. 32, 739.

Suzuki, Yoshimura and Takaishi, (1907). Tokyo Imp. Univ. Coll. Agr. Bull., 7, 503.

Waygood, E.R. (1948), Can. J. Research, 26C, 1948.

White, F.D. (1950), Personal communication.

Woodward, H.Q., (1950), J. Hematology, 5, 660.