

1952

The acid phosphomonoesterase of the human prostate.

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Dissertation

THE ACID PHOSPHOMONOESTERASE OF THE HUMAN PROSTATE

by

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Submitted in partial fulfilment of the
requirements for the degree of
Doctor of Philosophy
1952

Ph D
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Acknowledgements

The advice and guidance of the readers, Dr. Burnham S. Walker and Dr. Isaac Asimov, are much appreciated.

Thanks are due Dr. Philip M. LeCompte, Dr. Olive Gates, Dr. G. Kenneth Mallory and the many pathologists and urologists who have cooperated in supplying surgical and autopsy material.

The author is especially grateful to Dr. Matthew A. Derow. Without the benefit of his theoretical knowledge and actual skill in operating the Tiselius apparatus the electrophoretic studies would not have been attempted. He has also made innumerable valuable suggestions concerning other phases of the investigation.

Topical Outline

	<u>Page</u>
Introduction	1
History	2
Experimental Procedures - I.	
A. Purification by $(\text{NH}_4)_2\text{SO}_4$ Fractionation	11
B. Purification by EtOH Precipitation	17
Results	19
Experimental Procedures - II.	
Electrophoretic Studies	28
Discussion of Results	33
Summary and Conclusions	34
Bibliography	35
Abstract	i
Autobiography	

PHOSPHOMONOESTERASE OF THE HUMAN PROSTATEIntroduction

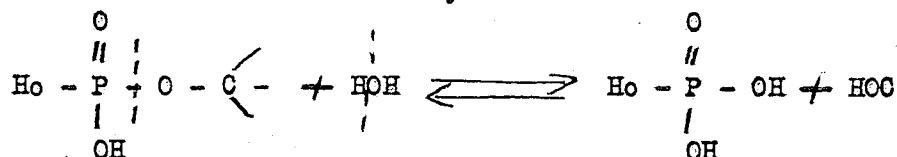
The purpose of the current investigation is to devise a simple and practical method for the purification of the acid phosphatase present in both benign and malignant prostatic tissue and to compare or contrast some of their physical and chemical characteristics.

PHOSPHOMONOESTERASE OF THE HUMAN PROSTATE

History

Occurrence

The human prostate gland produces a phosphomonoesterase exhibiting optimum activity in the vicinity of pH 5.0. The enzyme is therefore a Class A II phosphatase according to Folley and Kay (26). Baldwin (7) considers the action of such an enzyme as:



The prostate enzyme was first reported in 1935 by Kutscher (42) who found that human urine contains a phosphatase that hydrolyzes phenylphosphate and alpha- and beta-glycerophosphate at a pH optimum of 4.0 - 5.0. Kutscher and Wolbergs (44) found the enzyme present in high concentration in the prostate, which they regarded as the source of ejaculate and male urinary phosphatase.

Moore and Hanzel (52) unwittingly confirmed Kutscher and Wolbergs by noting that the secondary softening of the corpora amylacea of the prostate is brought about by the nuclease enzymes in an actively secreting prostate. Class A II phosphatases are also present in many animal tissues - for example, in spleen (20, 28), liver (20, 9, 12, 57), kidney (9, 12) and the axis cylinders of both the central and peripheral nervous systems (60). In some cases (20, 9, 12, 57) tissue extracts were used to demonstrate the presence of the enzyme; in others (28, 60) the histochemical method was employed. Since there is considerable doubt regarding

the validity of the histo-chemical method (47, 29) results obtained by this technique are difficult to evaluate. Lemon and Wisseman (48) used frozen tissue sections for the simultaneous quantitative estimation and cytological localization of the enzyme and found the acid phosphomono-esterase present in the gastro-intestinal tract, uterine myometrium, breast, bronchus, skin and bladder. Since none of these A II phospho-tases has been adequately purified, it is impossible to say whether or not the same enzyme is present in each of the above-mentioned tissues.

Relation to Neoplasia

In 1936 Gutman, Sproul and Gutman (34) noted that osseous metas-tases secondary to carcinoma of the prostate showed increased acid phos-phatase activity. Two years later the Gutmans (32) and Barringer and Woodward (10) independently discovered that in many cases the serum of patients with disseminated carcinoma contained significant amounts of the prostatic phosphatase. With this fact established serum acid phos-phatase determinations became an aid to the diagnosis of prostatic cancer.

It cannot be assumed that the serum enzyme level is raised because the malignant prostate produces increased amounts of phosphatase, since Woodward (61) has demonstrated that the activity of this enzyme in pros-tatic carcinoma averages somewhat less than in the normal or hypertro-phied gland. Gutman (30) assumes that when carcinomatous prostate tissue metastasizes, invasion of lymph or blood channels is accompanied by es-cape of the prostatic secretion into the circulation. Many workers, in-cluding Woodward (62) and Nesbit and Baum (54) concur. It has also been shown that operation (59) as well as digital manipulation (36) of the prostate can cause a transient rise in the serum acid phosphatase. Dean

and Woodward (21) have proved that it is possible to show whether a tumor does or does not originate in the prostate by measuring the acid phosphatase activity of extracts of tissue removed through the cystoscope or proctoscope. This differentiation is possible because the phosphatase activity of prostatic tissue is 100 to 1000 times as great as that of bladder or rectal carcinomatous tissue. In 1941 Gomori (28) showed by histo-chemical means that the acid phosphatase activity is localized in the glandular epithelium of the human prostate. Some years previously Moore, Price and Gallagher (51) established that the maintenance of the secretory cells of rat prostate depends on androgen production. This is in accord with the Gutmans (31) who found that the prostate gland of children contains a negligible amount of phosphatase. It also substantiates their report (33) that the precocious puberty produced in the rhesus monkey by the injection of testosterone propionate causes a maturation of the prostatic epithelium accompanied by a rapid several 100-fold increase in acid phosphatase content to adult levels. These facts are the basis of the work of Huggins and Hodges (39) in demonstrating that castration or injection of large amounts of estrogen causes a sharp reduction of the serum acid phosphatase level in the blood of patients with prostatic cancer. In such cases serum acid phosphatase determinations are now widely used as an indicator of the effectiveness of the control of the disease.

Purification Methods

There has been some progress in the purification of prostatic phosphatase:

A. Method of Kutscher, Wörner and Pany (45, 43)

Prostatic brei was autolyzed at room temperature for several days with 3 - 5 times its weight of water. The autolysate was then centrifuged and the supernate submitted to electro dialysis. This was followed by adsorption on $Al(OH)_3$ and subsequent elution with M/2 citrate buffer at pH 6.0. The enzyme was then precipitated with 0.50 saturated ammonium sulphate. The resulting precipitate was dissolved in one third of its original volume and extraneous protein was removed by 0.40 saturated ammonium sulphate. The enzyme was finally precipitated from solution as a silver salt.

This procedure resulted in 100 - 120 fold purification. One mg by weight of the best preparation hydrolyzed 320 mg phosphorus in one hour from .153 M beta-glycerophosphate at pH 5.4 - 5.6 in M/10 citrate buffer. The nitrogen content was 16.4 per cent. Before electro dialysis all preparations contained varying amounts of carbohydrate, as demonstrated by the Molisch test. After electro dialysis the Molisch reaction was negative.

These workers used the same method to purify ejaculate phosphatase. Values for two of their preparations were as follows:

	<u>mg P hydrolyzed</u> <u>mg dry weight</u>	<u>%</u> <u>nitrogen</u>
#1	467	11.7
#2	252	15.1

By the Theorell method of cataphoresis they estimated the isoelectric point at pH 4.4.

B. Method of Dmochowski and Pracowity (22)

Partially purified preparations of spermatic fluid and prostatic

phosphatase were obtained by ammonium sulphate fractionation. The enzyme was precipitated at 0.60 saturation.

The ejaculate preparations gave a negative Molisch reaction and 1 mg of a representative sample in one hour hydrolyzed 300 mg phosphorus from 4.5 per cent glycerophosphate at pH 5.5. When the method was applied to aqueous salt-containing extracts of human prostates the enzyme loss reached 70 per cent and the preparations were consistently contaminated with inactive protein.

C. Method of Anagnostopoulos (4)

A prostatic autolysate was fractionated with ammonium sulphate at 0 C. 0.80 saturation gave the most active precipitate.

5 ml (.005 g dry residue) liberated 7.9 mg phosphorus in one hour at 37 C from 20 ml of 0.25 M beta-glycerophosphate at pH 5.2 in acetate buffer.

D. Method of Hudson and Butler (38)

Purified phosphatase was obtained from human ejaculate by 35 per cent ethanol precipitation at pH 3.0 and 0 C. The resulting precipitate was extracted with water, the extract adsorbed on alumina and eluted with acetate buffer at pH 3.0.

The product contained about 40 per cent of the original activity and had a purity index of 1000 times that of the starting material. The authors state that from the values for tyrosine protein concentration is estimated to be quite low, but no values are given.

Inhibitors and Activators

The literature contains few helpful facts and some conflicting information, presumably because usually only crude enzyme extracts are

available. In such cases it seems unwise to assume that a given enzyme always behaves the same way in the presence of a given inhibitor - in other words, the effect of extraneous material present should be considered.

The action of the following has been studied:

A. Fluoride

Various workers (45, 12, 56, 49, 19) have reported the inhibition of acid phosphatase by fluoride ion. Kutscher and Wüst (46) believe that the action is competitive in nature as evidenced by the fact that a given concentration of fluoride inhibits the hydrolysis of phenylphosphate to a greater extent than it does the hydrolysis of beta-glycerophosphate. Courtois and Anagnostopoulos (19) contend that fluorides do not inactivate acid phosphatase by precipitating Mg, Ca or Zn since saturated solutions of CaF_2 , MgF_2 and ZnF_2 inhibit various phosphatases to the same extent as NaF solutions of like F content. Ohlmeyer⁽⁵⁶⁾ claims that inhibition by F is increased in the presence of Mg salts and considers that this is dependent upon the reversible formation of a complex of phosphatase and MgF_2 . Salts of Ca, Zn, Cd, Mn, Fe, Co, Ni and Cu have a similar action. Lundquist (49) has shown that F inhibition, as well as that by oxalate, maleate and Zn is diminished or prevented by citrate when the substrate is beta-glycerophosphate or phosphorylcholine.

B. Oxalate

Belfanti and co-workers (13) showed that the acid phosphatases of extracts of liver and kidney reversibly inactivated by sodium oxalate can be reactivated without loss. This is done by precipitating the oxalate ions with Ca or by removing them by dialysis. Abul-Fadl and King consider oxalate inhibition non-specific.

C. Tartrate

Abul-Fadl and King (1) found that L- or D-L tartrate has no effect on the acid phosphatase of red cells or normal plasma but that it is a very strong inhibitor of prostate, liver, and spleen phosphatase. They proved by dialysis that this inhibition is reversible.

D. Ethanol

Abul-Fadl and King (2) have shown that the acid phosphatases of adrenal, intestine, liver, pancreas, spleen and thyroid are unaffected by ethanol whereas those of bile, kidney, red cells and prostate are seriously inhibited. Kutscher and Wörner (28) also found that the addition of 2 molar ethanol to the substrate caused a 50 per cent inhibition of prostatic phosphatase. Certain other monohydroxy low molecular weight alcohols and urethane showed varying degrees of inhibition. Herbert (35) has shown that the alcohol sensitivity of prostatic phosphatase depends upon the diluting medium - for example:

1. In water little or no inactivation occurs.
2. In citrate or acetate buffer solutions ranging from pH 4.8 - 7.4 the enzyme is inactivated.
3. In 0.9 per cent NaCl there is partial inactivation.

A serum known to contain prostatic phosphatase or an enzyme preparation from the prostate was mixed with 4/5 volume of ethanol and left at room temperature for 30 minutes. Acid phosphatase activity was then determined.

E. Specific Group Reagents

Kutscher and Wörner (45) found that M/50 cysteine had no effect on their purified prostatic enzyme.

Anagnostopoulos (4) showed that prostatic phosphatase is only slowly inhibited by ketene and nitrous acid and partially inactivated by an excess of formaldehyde or by phenylisocyanate. Abul-Fadi and King (2) claim that prostatic phosphatase in the blood serum is unaffected by formaldehyde and advocate its use in differentiating the prostatic enzyme from the red cell phosphatase, since the latter is completely destroyed by the inclusion of 0.1 ml of 20 per cent neutral formaldehyde in the buffer - phenylphosphate serum mixture used for the determination.

F. Magnesium

According to Kutscher and Wörner (45) Mg has no effect on prostatic phosphatase. Ohlmeyer (56) believes that Mg activates the enzyme. Roche (58) states that in general Mg salts are antagonistic to the inhibiting effect of the phosphate liberated during the reaction.

Possible Functions of Acid Phosphatase

Nothing definite is known about the physiological role of prostatic phosphatase. Lundquist (50) surmises that the splitting of phosphoryl choline, known to be present in human semen, is the sole function of prostatic phosphatase. Hudson and Butler (38) found that their purified preparations of human ejaculate phosphatase failed to hydrolyze this substrate and because of this they suggest that more than one phosphatase exists in ejaculate.

Appleyard (5) basing her work on the findings of Axelrod (6) demonstrated that a partially purified extract of human prostate catalyzed the transfer of phosphate between pentasodiumphenolphthaleindiphosphate and certain aliphatic hydroxy alcohols. The presence of these alcohols increased the liberation of free phenolphthalein without appreciably affect-

ting the formation of free phosphate. Appleyard's assumption is that two enzymes are involved - a phosphatase and a transphosphorylase and that the latter competes for the phenolphthalein more effectively than does the phosphatase.

The role of acid phosphatase in other organs is equally obscure. Drabkin and Marsh (23) found that in rats with well-established alloxan diabetes the activity of acid phosphatase was increased 23 per cent and that subsequent treatment with insulin restored the activity to its normal value. These findings suggest that possibly acid phosphatase plays a significant role in the diabetic state. Noberg (55) showed that liver regeneration in the rat is accompanied by a large increase in the several acid phosphatases present. This indicates a possible relation between these enzymes and protein synthesis. Lemon and Wisseman (48) have found that human cancers (prostate not included in the series) exhibit a uniform increase in nuclear acid phosphatase when compared with homologous tissue of origin. They suggest that the increased acid phosphomonoesterase activity of human cancer cells is related to the rapid uptake and retention of labeled phosphorus by malignant tissue, which is known to occur.

The widespread occurrence of acid phosphatase seems to warrant the assumption that the enzyme must have some physiological importance, even though at the present time no definite function can be ascribed.

I. Experimental Procedures

A. Purification by ammonium sulphate fractionation.

Assay

Enzyme - The substrate used was 0.5 per cent sodium beta-glycero-phosphate (Eastman Kodak Co.) in acetate buffer at pH 5.4 to 5.6 and $\mu = 0.2$. 0.5 ml of diluted unknown was added to 5 ml of substrate and the mixture incubated at 37 C for 30 minutes. Enzyme action was stopped by the addition of 4.5 ml of 10 per cent trichloroacetic acid. A blank tube containing 5 ml of substrate and .5 ml of distilled water was treated in a similar manner. Color development was according to the method of Fiske and Subbarow, slightly modified by Hoffman (46). The mg of P hydrolyzed by 1 ml of the enzyme solution was calculated.

Nitrogen - Nitrogen was determined by the micro-Kjeldahl method, using Nesslerization.

The index of purification was expressed as mg P hydrolyzed under the conditions specified, either per mg N or per mg dry weight.

Extraction of the enzyme from non-malignant prostatic tissue.

The source of enzyme was human prostates removed at operation or autopsy and reported by the pathologist to be non-cancerous. Most of the glands showed benign overgrowth. As soon as possible after removal from the body, the specimen was placed in a deep freeze maintained at -18 C. After 2 to 60 days, depending on when it was needed, the tissue was thawed at room temperature. The connective tissue capsule and all areas showing gross fat or blood were discarded. The remainder of the tissue was cut into small pieces averaging about 10 x 10 x 3 mm and extracted at 7 C with

0.85 per cent NaCl. 3 ml of solution were used per gram wet weight of prostate. After 6 to 8 hours the liquid was decanted and the extraction was repeated overnight. For each 100 ml of saline solution 0.2 ml of a 1 - 1 mixture of toluol and ethyl acetate was added as a preservative. The combined extracts were centrifuged at 40,000 rpm for 15 minutes at 7 C. The sediment was discarded and the supernate saved for subsequent manipulation, provided assay showed a suitable amount of enzyme present.

The tissue was frozen prior to extraction to lessen the possibility of contamination by organisms killed or inactivated by low temperature. For the same reason extraction in the cold was preferred to autolysis. In addition to the usual undesirable aspects of bacterial growth there is an added complication here in the fact that most organisms produce a phosphatase (16).

Saline was used as an extractive in preference to water to keep hemolysis at a minimum and so reduce contamination with red cell phosphatases (11, 3).

Two separate extractions were made because it was found that after only one extraction a considerable amount of enzyme remained in the tissue.

In order to estimate the efficiency of saline extraction in the cold as compared with autolysis by a water solution at room temperature, as used by Appleyard (5), comparable portions of the same prostate were submitted to the two methods. The activity per mg dry weight ratio for the autolysis method was 88 per cent that of the extraction method.

The use of the Waring Blendor, as advocated by Fischmann and co-workers (24) was also tried. The activity per mg N ratio for the latter was 90 per cent of that for the saline extraction procedure. It was thought that perhaps inert protein could be removed from the Fischmann homogenate

by filtration through celite. This, however, caused a further reduction in the purity index and was therefore considered undesirable.

In the course of this work two miscellaneous facts were established:

- (1) The enzyme is soluble in 35 per cent dioxane. However, because of the toxicity of dioxane its routine use was considered impractical.
- (2) In order to bring a given enzyme solution to a concentration suitable for assay the use of water, 0.85 per cent NaCl or pH 5.6 acetate buffer $\mu = 0.2$ as the diluent gives identical values for phosphatase activity.

Dialysis and Lyophilization of Saline Extract.

The supernate of the original saline extract was dialyzed overnight against running tap water at 7 to 12 C. The insoluble precipitate which appeared with the removal of salts and possibly other dialyzable material was spun down by centrifugation at 40,000 rpm and discarded. The supernate was freeze-dried and the powder obtained saved for subsequent ammonium sulphate fractionation.

The dialysate showed no inorganic phosphate present; a qualitative test for Cl was negative.

This treatment is desirable for two reasons:

- (1) It provides a means for concentrating the amount of enzyme present.
- (2) The removal of material insoluble in water solution causes an increase in the activity/nitrogen ratio of at least 37 per cent.

Ammonium Sulphate Fractionation.

A 1 to 1.5 per cent aqueous solution of the lyophilized powder was treated for 8-10 hours at 0 C with $(\text{NH}_4)_2\text{SO}_4$ at half saturation. The resulting precipitate was spun down by centrifugation at 7 C for 20 minutes. The $(\text{NH}_4)_2\text{SO}_4$ concentration of the decanted supernate was increased to 0.66 saturation and the solution left overnight at 0 C. It was then spun for at least 30 minutes at 7 C and 50,000 rpm. The sediment was dissolved in water, the volume used being about two thirds that of the 1 to 1.5 per cent lyophilized protein solution. The dissolved sediment was dialyzed against running tap water overnight. The criterion of adequate $(\text{NH}_4)_2\text{SO}_4$ removal was a negative color reaction on the addition of Nessler's reagent to an aliquot of the dialyzed material.

The sediment obtained at 0.50 saturation was dissolved in water and dialyzed overnight against tap water. By repeated passage through the same filter paper a clear solution was obtained which contained approximately 18 per cent of the original enzyme activity. This could be added to the supernate and reprecipitated at 0.66 saturation. However, the large volume resulting from dialyzing out the $(\text{NH}_4)_2\text{SO}_4$ makes the procedure impractical. It is of interest to note that some of the enzyme precipitated, presumably by occlusion, can be recovered. Analysis of the supernate of the 0.66 saturated fraction shows that enzyme is still present. Precipitation at 0.88 saturation instead of at 0.66 was tried with the result that the enzyme/weight ratio showed a 41 per cent decrease and the enzyme/nitrogen ratio a 37 per cent decrease. When fractionation at 0.85 saturation was tried subsequent to the consecutive removal of 0.50 and 0.66 saturated sediments, the enzyme/nitrogen ratio showed a 43 per cent decrease

relative to that of the usual 0.66 saturation. 0.88 saturation directly following 0.50 saturation was then tried. The precipitate so obtained was dialyzed free of $(\text{NH}_4)_2\text{SO}_4$, lyophilized and reprecipitated at 0.66 saturation. Analysis of the supernate showed only a minimal amount of enzyme present, but the enzyme/nitrogen ratio of the sediment was only 34 per cent of that obtained by the usual method of increasing the 0.50 saturated supernate to 0.66 saturation.

Removal of Inert Protein by Adjustment of pH.

A dialyzed solution of the 0.66 saturated sediment was adjusted to pH 5.0 - 4.8 with N/10 acetic acid and left at 0 C overnight. It was then spun for 20 minutes at 7 C and 40,000 rpm. A bulky, inert sediment separated and was discarded. The pH of the supernate was adjusted to 7.0 and the solution again left at 0 C for 4 to 6 hours. A minute amount of inactive sediment was obtained on centrifugation. The supernate was then dialyzed overnight against running tap water, filtered through a #40 Whatman paper and lyophilized. A whitish powder resulted.

As a preliminary procedure in trying to precipitate the enzyme at its isoelectric point the following was done:

A crude saline extract, pH 6.0, was adjusted to pH 5.1 by the addition of pH 5.3 acetate buffer. The solution was set aside for one half hour at 0 C and then spun at 7 C for 15 minutes at 40,000 rpm. After removal of the sediment the supernate was adjusted to pH 4.5 with 1 N acetic acid and left to precipitate as before. The process was also repeated at pH 4.2. Analysis of the supernate showed the following:

<u>pH</u>	<u>Activity/nitrogen ratio</u>
5.1	32% decrease
4.5	10% increase
4.2	11% increase

When the pooled sediments were extracted with water and the filtered extracts added to the supernate the enzyme/nitrogen ratio was doubled. The latter solution was lyophilized in order to decrease its volume. It was then redissolved in a small amount of 0.14 M veronal buffer at pH 6.1 and the procedure repeated in the alkaline range, using 1 N NaOH until pH 10 was reached. This treatment showed the enzyme to be stable to pH 8.4. Up to that point the sediments, reconstituted in pH 5.6 acetate buffer, contained inert material. Beyond that point activity in the supernate diminished and no activity could be recovered from the sediment. When an aliquot of the saline extract was brought directly to pH 3.0, subsequent analysis showed no activity in either supernate or sediment.

On the basis of these results it was felt that isoelectric precipitation of inert material would cause less enzyme occlusion in the sediment if more purified material was used. By carrying out a similar procedure on the dialyzed fraction obtained from 0.66 saturation with $(\text{NH}_4)_2\text{SO}_4$ it was found that the largest amount of inert material precipitated between pH 5.2 - 4.8. Occasionally a small amount of inactive sediment was obtained at pH 7.0.

The procedure described above causes approximately a 4-fold increase in the enzyme/nitrogen ratio. The final product has about 33 per cent of the amount of enzyme present in the original extract. 320 mg of purified powder were obtained from approximately 586 g wet weight of prostate.

B. Purification by Ethanol Precipitation in the Cold.

In regard to this method some background explanation is necessary.

When the present investigation was begun very little human prostatic tissue was obtainable, but a large supply of human liver tissue was at hand. It was erroneously supposed that if a purification method for liver acid phosphatase could be worked out, application of the same method to prostatic tissue, when available, would be successful. It was found that most of the enzyme could be precipitated from a saline extract of liver tissue by ethanol at 0 C and at a final concentration of 70 volumes per cent. Extraction of the alcohol precipitate with water or 26 per cent acetone showed that roughly 60 per cent of the original enzyme activity could be recovered, with an approximate 2.6-fold increase in the original activity/nitrogen ratio. Subsequent ammonium sulphate precipitation similar to that described previously for the prostate, gave a final 9-fold increase in the activity/nitrogen ratio. However, since this final ratio was 0.5 it was decided to abandon work on the liver in favor of a much richer source of enzyme.

When prostatic tissue was procured saline extracts were made and 70 per cent ethanol precipitation at 0 C attempted. In nearly every case no activity in either sediment or supernate could be demonstrated. Lowering the alcohol concentration and varying the pH did not improve the enzyme yield. Ammonium sulphate fractionation was then tried, as described previously.

Cohn, Strong et al. (18) have shown that four variables determine protein solubility: pH, salt concentration, temperature and the protein in the system. Herbert (35) has demonstrated that saline causes partial

inactivation of prostatic phosphatase in the presence of alcohol. With these facts in mind the following experiment was tried some months after the original failure to precipitate active enzyme.

Two 1.4 per cent solutions of lyophilized crude extract previously dialyzed free of salt were made, one in distilled water, the other in 0.85 per cent NaCl. Previous experience had shown the pH of such aqueous solutions to be about 6.2. The preparations were fractionated at 0 C for one hour with ethanol in a final concentration of 25 volumes per cent. After centrifugation at 7 C and 40,000 rpm for 25 minutes the ethanol concentration of the supernates was increased to 40 volumes per cent. The preparations were left at 0 C overnight and then centrifuged as before.

Analysis of both 0.25 fractions showed some enzyme activity in the sediment but most of it in the supernate. Analysis of the 0.40 fraction of the water solution showed no activity in the supernate and good recovery of activity from the sediment. The saline solution showed no activity in the supernate and activity in the sediment was at least 10 times less than that of the water sediment. Dilutions of a solution of the saline sediment were not reduced to a point where it could be said definitely that no activity was present.

Results

Table I shows the values of a representative preparation purified by ammonium sulphate fractionation. The 0.85 saturated fraction is included for comparison with the 0.66 saturated fraction. Carbohydrate was determined by the orcinol reaction, using Friedmann's technique (27). The results reported are referred to dextrose.

Table I

Values for fractions obtained by ammonium sulphate method

Fraction	<u>Enzyme</u> nitrogen	<u>Enzyme</u> weight	Per cent nitrogen	Per cent orcinol- reacting carbohydrate
Saline extract	26	-	-	-
Dialyzed extract	46	5.7	12.3	2.8
.66 fraction after isoelectric precipitation of inert protein	108	12.0	11.1	3.5
.85 fraction after isoelectric precipitation of inert protein	46	4.9	10.8	q.n.s.

Table I indicates the extreme solubility of the enzyme. Since Cohn, McMeekin *et al.* (17) point out the danger in completely characterizing proteins in terms of their solubility without taking account of other properties, no conclusion regarding the chemical nature of the enzyme is warranted.

Table II shows the enzyme/nitrogen ratios of eight saline extracts of non-malignant prostatic tissue. In some cases it was necessary to pool tissue from two or three individuals in order to have a convenient amount. The extracts were all lyophilized after dialysis and saved for subsequent manipulation. All extracts containing activity of less than 16 mg P/ml were discarded and therefore are not included in the table.

Table II

Saline extracts of non-malignant prostatic tissue

Specimen	<u>mg P hydrolyzed</u> mg nitrogen	Ratio
1.	$\frac{94}{.70}$	92
2.	$\frac{40}{.11}$	56
3.	$\frac{68}{.80}$	85
4.	$\frac{24}{.51}$	47
5.	$\frac{22}{.43}$	51
6.	$\frac{26}{.99}$	26
7.	$\frac{64}{.81}$	80
8.	$\frac{23}{.61}$	38

Table II suggests that strict quantitative predictions regarding the amount of enzyme present in non-malignant tissue are not possible.

Table III shows the final ratios of the purified 0.66 saturated fractions of ten preparations. In some cases, here too, pooling of the dialyzed fractions was necessary. There is therefore no relation between the numbers of the specimens in Table I and of the preparations in Table III.

Table III

Final purification values obtained by ammonium sulphate method

Preparation	$\frac{\text{mg P hydrolyzed}}{\text{mg nitrogen}}$	Ratio	$\frac{\text{mg P hydrolyzed}}{\text{mg dry weight}}$	Ratio	Per cent nitrogen
1	$\frac{8.0}{.074}$	108	$\frac{8.0}{.66}$	12	11.1
2	$\frac{18.0}{.16}$	112	$\frac{18.0}{1.4}$	13	11.5
3	$\frac{21.0}{.12}$	175	$\frac{21.0}{1.0}$	21	11.7
4	$\frac{15.0}{.062}$	242	$\frac{15.0}{.55}$	27	11.1
5	$\frac{40.0}{.25}$	160	$\frac{40.0}{1.8}$	23	14.0
6	$\frac{47.6}{.23}$	207	$\frac{47.6}{1.6}$	30	14.6
7	$\frac{32.0}{.13}$	242	$\frac{32.0}{1.1}$	30	12.7
8	$\frac{48.0}{.22}$	219	$\frac{48.0}{1.4}$	34	15.5
9	$\frac{50.0}{.26}$	194	$\frac{50.0}{1.8}$	28	14.5
10	$\frac{24.0}{.14}$	178	$\frac{24.0}{1.1}$	21	11.9

Table III shows nitrogen values below the expected 16 per cent. These figures may be explained in part by the following facts. The first is that impurities in the tap water against which the final fractions were

Table IV shows the results of chemical analyses. Five separate fractions were used, one for each analysis. Hexosamine was determined by the method of Blix (14). In order to estimate total phosphorus the classical Fiske and Subbarow method (25) was used, subsequent to wet ashing. Cysteine analysis was according to Nakamura and Binkley (53), since Block and Bolling recommend this method.

Table IV

Some chemical constituents of purified ammonium sulphate fractions

Determination of:	Per cent present
Kjeldahl nitrogen	11.1
Orcinol-reacting carbohydrate	3.5
Hexosamine	0.76
Total phosphorus	if present, less than 0.15
Cysteine	if present, less than 0.57

Table IV (and Table I) show the presence of carbohydrate in all fractions analyzed. Since this material does not pass through a dialyzing membrane and since hexosamine is one of the products obtained on hydrolysis of the purified fraction, there is reason to suspect the presence of glycoprotein or mucin. The value for total phosphorus rules out significant amounts of phospholipid and nucleic acid. The cysteine value indicates that -SH groups, if present, are not numerous. Since it is generally accepted that the detection of -SH groups is a complicated analytical problem, this value may well have no significance.

Table V

Tests for presence of specific phosphatases in a purified fraction

Phosphatase tested for:	Concentration of purified enzyme solution added to substrate mg/ml	pH and buffer	Substrate	Activator or inhibitor added	% activity
Prostatic acid	.0017	5.4-acetate	Na β -G-PO ₄	None	100*
Prostatic acid	.01	5.4-acetate	Na β -G-PO ₄	.01 M NaF	4.7
Prostatic acid	.002	5.4-acetate	Na β -G-PO ₄	None	100*
Prostatic alkaline	1.0	9.3-veronal	G-PO ₄ (Merck)	.01 M MgCl ₂	0
Prostatic acid	.00029	5.4-acetate	Na ₂ - ϕ -PO ₄	None	100*
Erythrocyte	.00029	5.4-acetate	Na ₂ - ϕ -PO ₄	0.1 ml 20% neutral formaldehyde	75

* Arbitrary value used for comparison

Table V lists the results of tests designed to show the presence or absence of three phosphatases which could well be present in the purified extract.

In determining the fluoride sensitivity of the prostatic enzyme the usual assay method was used. In testing for alkaline phosphatase the same procedure was followed using an appropriate buffer and Mg as an activator (8). A relatively low concentration of $MgCl_2$ was chosen because it seemed probable that an extract dialyzed against tap water would not be devoid of Mg. The incubation time was increased to 60 minutes. Formaldehyde was used to differentiate prostatic and red cell phosphatases, following the technique of Kintner (41), except that no alcohol inhibition studies were done. The members of each pair were assayed simultaneously, using the same enzyme solution at a satisfactory dilution, which was taken into account in the final calculation in column 6.

Table V demonstrates the pronounced sensitivity of prostatic acid phosphatase to fluoride. It is of interest that Kutscher and Wörner (45) found 82.6 per cent inhibition by .01 M NaF, using sodium beta-glycerophosphate in citrate buffer. A 25% inhibition by neutral formaldehyde, as shown, indicates that 25 per cent of the activity of the purified extract is due to erythrocyte phosphatase, if Abul-Fadl and King (3) are correct in their contention that neutral formaldehyde completely inhibits the red cell enzymes but has no effect on prostatic phosphatase. There is a slight possibility that the crude saline prostatic extract used by these investigators contained more non-enzymatic amino groups than did the purified extract used in the present study. If this be so, and in the presence of an excess of formaldehyde, it is conceivable that crude extracts might show no inhibition while more highly purified preparations would show varying degrees of inactivation.

Table VI shows the results obtained when the previously described ammonium sulphate purification method was applied to pooled tissue removed at operation from three patients and diagnosed by the pathologist as adenocarcinoma of the prostate.

TABLE VI

Ammonium sulphate purification method - adenocarcinoma of prostate

Fraction	<u>mg P hydrolyzed</u> mg nitrogen	<u>mg P hydrolyzed</u> mg dry weight	Per cent nitrogen	Per cent orcinol- reacting carbohydrate
Original saline extract	$\frac{240}{.61} = 39$	-	-	-
Final purified	$\frac{20.6}{.14} = 147$	$\frac{20.6}{1.2} = 18$	12.7	4.7

Table VI shows that the enzyme is decreased in amount when compared to an average value for non-malignant tissue (See Table II), a finding in accord with Woodard (61). No further characterization was possible due to a limited supply of malignant tissue.

Liver tissue almost completely replaced by metastases from a primary adenocarcinoma of the prostate was extracted with saline in the usual manner. Only areas showing gross, non-necrotic tumor were used. The enzyme/nitrogen ratio of the preparation was 2.0. In view of this low value no further purification was attempted. Tissue containing the primary tumor was unavailable. It is of interest that blood removed antemortem from the same patient showed a serum acid phosphatase value of 52 Gutman units per cent*.

* Determined by Clinical Chemistry Laboratory, Mass. Memorial Hospitals. 3.25 units per cent represent the upper limit of the normal range.

Table VII shows the results when the alcohol fractionation method is combined with that of precipitating inert protein at pH 4.9 and dialyzing the supernate free of acetate formed in the process.

TABLE VII

Values obtained by ethanol fractionation - non-malignant prostate

	Y	
	<u>mg P hydrolyzed</u> mg nitrogen	Per cent nitrogen
Dialyzed powder	247	not determined
Final purified	600	12.5

The figures shown in Table VII are of interest in explaining the alcohol inhibition of prostatic phosphatase, noted by various investigators.

II. Experimental Procedures
Electrophoretic Studies
(With Dr. Matthew Derow)

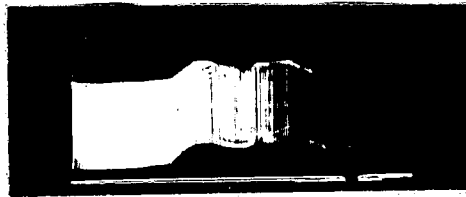
Methods Used

Extracts prepared as previously described were submitted to electrophoretic analysis in a Perkin-Elmer Tiselius instrument, using a 2 ml cell. Acetate buffers were made according to Boyd (15) and $(\text{CH}_2\text{OH})_3 - \text{C} - \text{NH}_2$ buffers according to a nomogram constructed by Derow (unpublished). The pH was determined at room temperature by a glass electrode. When the best possible resolution of components was obtained the levels of the desired cuts were estimated from the final Schlieren patterns. Each fraction was carefully removed by means of a special long BD needle and a syringe. The activity/nitrogen ratio was determined by the methods described in Section I. To reduce error in the measurement of the distance travelled by a component, electrophoretic patterns were projected at a 3-fold magnification.

FIGURE I

Non-malignant prostatic tissue-saline extract

$(\text{CH}_2\text{OH})_2\text{-C-NH}_2$ buffer
 pH 7.3 $\mu = 0.2$
 conc. 1.21 mg N/ml



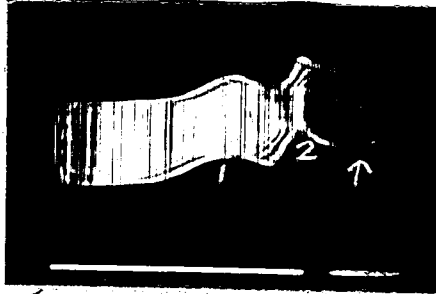
125 minutes

FIGURE II

Non-malignant prostatic tissue-purification by two methods

A.

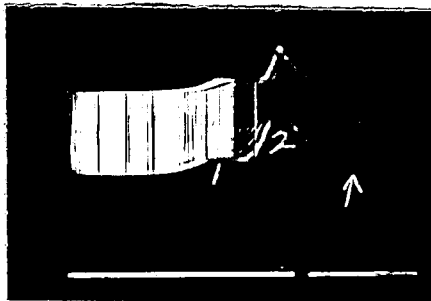
$(\text{NH}_4)_2\text{SO}_4$ method
 acetate buffer
 pH 4.6 $\mu = .05$
 conc. 0.996 mg N/ml



120 minutes

B.

EtOH method
 acetate buffer
 pH 4.6 $\mu = .05$
 conc. 0.447 mg N/ml



103 minutes

Results

Figure I shows that the crude saline extract contains two major components in an apparently polydisperse system.

Figure II suggests that despite two different methods of precipitation the resolved components of (A) and (B) are similar, if not identical. Outlets made at 165 minutes (A) and at 202 minutes (B) showed increased activity/nitrogen ratios at the points indicated by the arrows. Table I lists the mobilities.

TABLE I

Electrophoretic mobilities of components shown in Figure II

	Peak 1	Peak 2
A. $(\text{NH}_4)_2\text{SO}_4$ method	(a) 2.87×10^{-5} (b) 1.91×10^{-5} *	Mobility too low for accurate calculation
B. EtOH method	2.44×10^{-5}	"

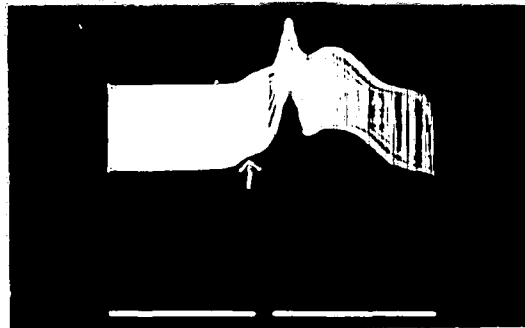
* Values represent $\text{cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$

FIGURE III

Non-malignant prostatic tissue- $(\text{NH}_4)_2\text{SO}_4$ fractions at two concentrations

A.

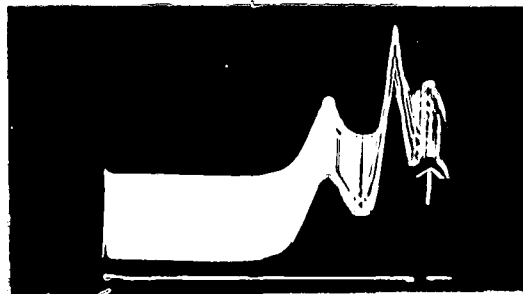
Low protein
acetate buffer
pH 4.6 $\mu = .05$
conc. 0.786 mg N/ml



81 minutes

B.

High protein
acetate buffer
pH 4.6 $\mu = 0.1$
conc. 2.50 mg N/ml

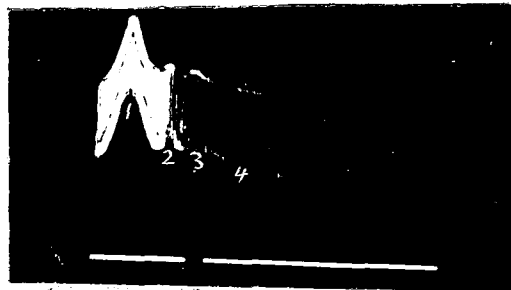


113 minutes

FIGURE IV

Adenocarcinoma of prostate - $(\text{NH}_4)_2\text{SO}_4$ method

Acetate buffer
pH 5.2 $\mu = 0.1$
conc. 0.70 mg N/ml



95 minutes

Figure III shows that in both (A) and (B) the component with the highest activity/nitrogen ratio (indicated by the arrows) migrates toward the anode. This suggests that its isoelectric point is acid to pH 4.6. The electrophoretic mobility of this component is $5.43 \times 10^{-6} \text{ cm}^2 \text{ volt}^{-1} \text{ sec.}^{-1}$ in (A) and $5.47 \times 10^{-6} \text{ cm}^2 \text{ volt}^{-1} \text{ sec.}^{-1}$ in (B).

A scan of (A) at 133 minutes was enlarged and the relative areas of the components calculated. By this method the active component is approximately 11 per cent of the total area. The activity/nitrogen ratio was 573 in contrast to the starting ratio of 254. A similar analysis of (B) was not possible because at 113 minutes some of the active component had passed into the upper section of the cell and is therefore not represented in the Schlieren pattern.

Figure IV suggests that at least four components are present in the preparation from malignant prostatic tissue. At pH 5.2 resolution of the three minor components is poor. Table II lists the mobilities. The pattern resembles that of Figure V B.

TABLE II

Electrophoretic mobilities of components shown in Figure IV

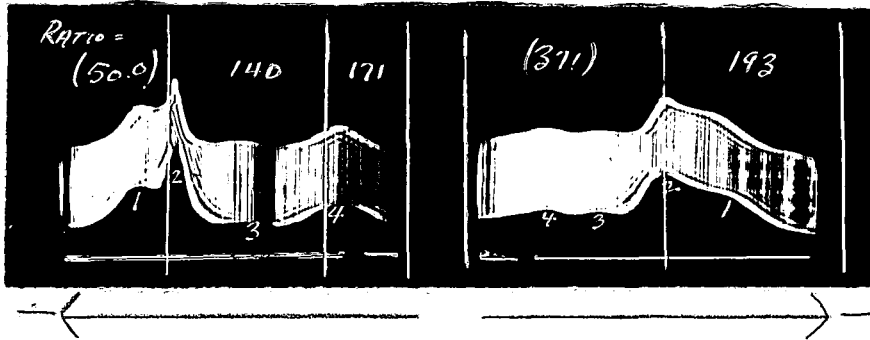
Peak 1	Peak 2	Peak 3	Peak 4
$2.30 \times 10^{-5} *$	1.05×10^{-5}	0	1.05×10^{-5}

* Values represent $\text{cm}^2 \text{ volt}^{-1} \text{ sec.}^{-1}$

FIGURE V

Non-malignant prostatic tissue - $(\text{NH}_4)_2\text{SO}_4$ fraction - effect of pH on resolution

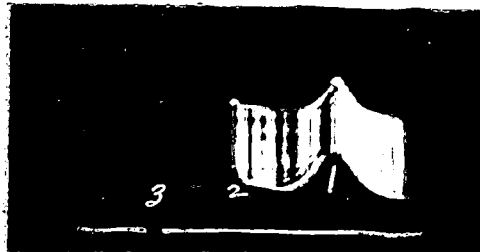
A.



Acetate buffer
 pH 4.05 $\mu = .05$
 conc. 1.02 mg N/ml

118 minutes

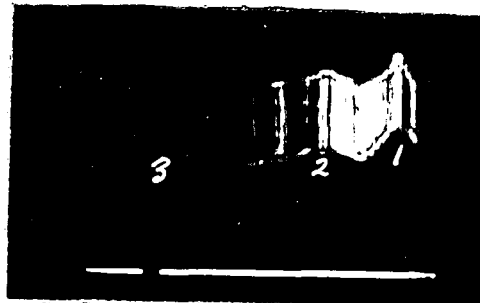
B



180 minutes

Acetate buffer
 pH 5.55 $\mu = .05$
 conc. 0.975 mg N/ml

C



120 minutes

$(\text{CH}_2\text{OH})_3\text{-C-NH}_2$ buffer
 pH 7.60 $\mu = .05$
 conc. 1.14 mg N/ml

Figure V shows that under the conditions stated at least four components are detectable. Table III lists the mobilities.

TABLE III

Electrophoretic mobilities of components shown in Figure V

	pH	Peak 1	Peak 2	Peak 3	Peak 4
A.	4.05	6.14×10^{-5} *	5.14×10^{-5}	3.26×10^{-5}	(4.85×10^{-6})
B.	5.55	3.24×10^{-5}	1.39×10^{-5}	0	
C.	7.60	8.31×10^{-5}	5.94×10^{-5}	(1.41×10^{-6})	

* Values represent $\text{cm}^2 \text{ volt}^{-1} \text{ sec.}^{-1}$

The peaks 1 of Figure V and also of Figure IV appear to represent the same components. The isoelectric point is definitely alkaline of pH 4.05, and Figure III A suggests that it is just alkaline of pH 4.6. The peaks 2 of Figure V seem to correspond. The component represented appears similar to peak 2 of Figure IV. If this is so the isoelectric point is slightly acid to pH 5.2. Analyses of cuts of peaks 1 and 2 consistently showed a low activity/nitrogen ratio.

It is difficult to correlate the peaks 3 and it seems logical to assume that they do not correspond. With the cuts shown it is impossible to know whether in (A) peak 3 or peak 4 represents the enzyme. On comparing the patterns of Figures V A and III A it seems reasonable to assume that peak 3 represents the enzyme. In (B) peak 3 is at its isoelectric point. In (C) peak 3 exhibits such slight mobility that it seems safe to assume either that the component is at its isoelectric point or that the peak is

due to a salt boundary. These two alternatives also apply to peak 4 of (A).

Figure V A shows that at pH 4.05 the enzyme is acid to its isoelectric point. From the mobility of the active component in III A and the mobility of peaks 3 - V A, which is assumed to represent the enzyme, the isoelectric point is determined by interpolation to be at pH 4.5.

Discussion of Results

On the basis of solubility the enzyme could be considered an albumin or a pseudoglobulin. Its relatively low electrophoretic mobility favors the latter classification. However, most protein classifications pertain to the plasma proteins and it seems unwise to assume analogous behavior for all tissue proteins.

It would be of interest to know something about the inactive components present in the prostatic extracts. Here, too, deficient knowledge concerning the body proteins makes the problem difficult.

Due to the difficulty of procuring sufficient neoplastic tissue only a very limited comparison between extracts from non-malignant and malignant prostate was possible. Save for a decreased amount of enzyme activity and an increased amount of orcinol-reacting carbohydrate in the carcinomatous tissue, the present investigation showed no significant differences. The first finding is a familiar one; the second is difficult to explain because the source of the presumed polysaccharide is not known. A study of a large series of preparations from both types of tissue would show whether or not this finding is incidental.

A true comparison of the acid phosphomonoesterases from the two sources would require the use of crystalline preparations. However, considering the fact that the enzyme in question is a product of a functional cell of the prostate, it seems logical to assume that the malignant process might alter the amount of enzyme produced as the degree of anaplasia increases. Whether or not the chemical nature of the enzyme might be altered is a debatable question and reveals our current lack of knowledge concerning neoplasia.

Summary and Conclusions

- A. The phosphomonoesterase of the human prostate gland exhibits a high degree of solubility, shown by its reaction to precipitation by $(\text{NH}_4)_2\text{SO}_4$ and by EtOH. Even at 0.80 per cent saturation with $(\text{NH}_4)_2\text{SO}_4$ some enzyme remains in solution. A concentration of 25 vols. per cent EtOH at 0 C fails to precipitate an appreciable quantity of enzyme.
- B. The enzyme exhibits a low degree of electrophoretic mobility.
- C. Electrophoretic studies place the isoelectric point of the enzyme between pH 4.05 and pH 4.60. However, the marked solubility is again evidenced by the fact that after twelve hours at 0 C acetate buffer at pH 4.6, 4.3 and 4.0 - $\mu = 0.2$ - fails to precipitate the enzyme.
- D. A comparison of extracts of non-malignant and malignant prestatic tissue purified under identical conditions showed:
1. Decreased enzyme activity in the malignant tissue.
 2. Similar nitrogen contents.
 3. Orcinol-reacting carbohydrate content of the malignant tissue slightly above that of the benign.
 4. Electrophoretic mobilities apparently similar.

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ABSTRACT

Experimental

A simple and practical method of purifying the acid phosphomonoesterase of the human prostate gland was developed. The procedure was used to obtain preparations of the enzyme present in non-malignant and malignant prostatic tissue. Preparations from the two sources were compared in regard to: (a) activity; (b) nitrogen content; (c) orcinol-reacting carbohydrate content; (d) components demonstrated by electrophoresis.

The following is a brief description of the procedure:

As soon as possible after removal from the body human prostatic tissue was placed in a deep freeze maintained at -18°C . After two to sixty days the tissue was thawed, cut into small pieces and extracted overnight at 7°C with 0.85 per cent NaCl. After centrifugation at 7°C to remove blood cells and other inert material, the supernate was dialyzed overnight against running tap water at about 9°C . After removal of insoluble material by centrifugation the supernate was freeze-dried. A 1.5 per cent solution of the resulting powder was fractionated with $(\text{NH}_4)_2\text{SO}_4$ at 0°C . The 0.50 saturated fraction was discarded and the bulk of the enzyme precipitated at 0.66 saturation. The precipitate was dissolved in water and dialyzed free of $(\text{NH}_4)_2\text{SO}_4$. Inert protein was then removed by isoelectric precipitation at pH 4.9 and the pH was adjusted to 7.0. After a final dialysis against running tap water the solution was freeze-dried.

The purity index was expressed by the ratio

$$\frac{\text{mg P hydrolyzed under specified conditions}}{\text{mg of dry weight or mg nitrogen}}$$

The substrate used was Na- β -glycerophosphate (Eastman Kodak Co.) in acetate buffer at pH 5.5.

The above procedure produces approximately a 4-fold increase in the activity/nitrogen ratio. The final product has about 33 per cent of the amount of enzyme present in the original extract.

EtOH fractionation at 0 C was also investigated. A 1.5 per cent aqueous solution of salt-free dialyzed extract of non-malignant prostate was precipitated with 25 vols. per cent of EtOH. After centrifugation at 0 C the precipitate was discarded and the concentration of the supernate was increased to 40 vols. per cent. This caused complete precipitation of the enzyme. The sediment was dissolved in water and inert protein removed at pH 4.9.

Because of repeated early failures with EtOH fractionation the $(\text{NH}_4)_2\text{SO}_4$ method was used almost exclusively in the present study. Very recently it was discovered that in the absence of salt adequate alcohol precipitated preparations could be obtained. The method seems promising and deserving of additional investigation.

The following is a tabulation of results obtained by the ammonium sulphate purification method.

Non-malignant prostate

Fraction	<u>mg P hydrolyzed</u> mg nitrogen	Per cent nitrogen	Per cent orcinol- reacting carbo- hydrate
Original saline extract	59 (average of 8 preparations)	-	-
Final purified	184 (average of 10 preparations)	12.9 (average of 10 preparations)	3.5 (1 determination - pool of 2 prepara- tions)

Adenocarcinoma of prostate*

Fraction	<u>mg P hydrolyzed</u> mg nitrogen	Per cent nitrogen	Per cent orcinol- reacting carbo- hydrate
Original saline extract	39	-	-
Final purified	147	12.7	4.7

Some additional characterization of $(\text{NH}_4)_2\text{SO}_4$ purified fractions from non-malignant prostatic tissue was possible:

	<u>Determination</u>	<u>Per cent present</u>
Hexosamine	(1 determination - 1 preparation)	0.76
Total P	(1 determination - pool of 2 preparations)	if present, < 0.15
Cysteine	(1 determination - pool of 3 preparations)	if present, < 0.57
Alkaline phosphatase	(1 determination - 1 preparation)	None
Inhibition by .01 M NaF	(1 determination - 1 preparation)	95.3

No comparison with malignant prostatic tissue was possible due to a limited supply of pathological material.

* All determinations were run on a single pool of malignant prostatic tissue removed from three patients.

Various extracts of prostatic tissue prepared as previously described were submitted to electrophoresis. After resolution the desired components were removed from the cell by a needle and syringe. The activity/nitrogen ratio of the fractions so removed was determined. A summary of the results obtained follows:

A. Fractions of non-malignant tissue prepared by the $(\text{NH}_4)_2\text{SO}_4$ purification method show three or four major components, depending on the pH of resolution.

B. A component showing an increased activity/nitrogen ratio and therefore considered to be the enzyme has a mobility of $5.43 \times 10^{-6} \text{ cm}^2 \text{ volt}^{-1} \text{ sec.}^{-1}$ and moved toward the anode at pH 4.60. At pH 4.05 a component presumed by analogy to be identical, has a mobility of $3.26 \times 10^{-5} \text{ cm}^2 \text{ volt}^{-1} \text{ sec.}^{-1}$. At this pH no component moved toward the anode. Thus, the isoelectric point must lie between pH 4.05 and pH 4.60.

C. A comparison of 2 fractions, one purified by the $(\text{NH}_4)_2\text{SO}_4$ method, the other by the ethanol method, show similar patterns at pH 4.6.

D. The pattern of an $(\text{NH}_4)_2\text{SO}_4$ purified extract of non-malignant prostatic tissue resolved at pH 5.55 is similar to the pattern of a like extract of malignant prostatic tissue resolved at pH 5.2.

Summary and Conclusions

- A. The phosphomonoesterase of the human prostate gland exhibits a high degree of solubility, shown by its reaction to precipitation by $(\text{NH}_4)_2\text{SO}_4$ and by EtOH. Even at 0.80 per cent saturation with $(\text{NH}_4)_2\text{SO}_4$ some enzyme remains in solution. A concentration of 25 vols. per cent EtOH at 0 C fails to precipitate an appreciable quantity of enzyme.
- B. The enzyme exhibits a low degree of electrophoretic mobility.
- C. Electrophoretic studies place the isoelectric point of the enzyme between pH 4.05 and pH 4.60. However, the marked solubility is again evidenced by the fact that after twelve hours at 0 C acetate buffer at pH 4.6, 4.3 and 4.0 - $\mu = 0.2$ - fails to precipitate the enzyme.
- D. A comparison of extracts of non-malignant and malignant prostatic tissue purified under identical conditions showed:
1. Decreased enzyme activity in the malignant tissue.
 2. Similar nitrogen contents.
 3. Orcinol-reacting carbohydrate content of the malignant tissue slightly above that of the benign.
 4. Electrophoretic mobilities apparently similar.

Autobiography

Born: 22 June, 19~~57~~¹⁵ in Banger, Northern Ireland.

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