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MEASUREMENT OF ACID PHOSPHATASE ACTIVITY TO IDENTIFY SEMINAL STAINS

CLAUDE B. HAZEN

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PART I

Medical legal precedent has required the finding of spermatozoa to positively identify a stain as being seminal. Since sterility in man may be a factor to be considered as a reason for not finding spermatozoa, the forensic investigator is in need of recognized procedures and methods whereby suspect seminal stains may be identified although no spermatozoa are found present.

A considerable amount of research has been done and recorded in England and America with regard to acid phosphatases (1, 3). Acid phosphatases have been found in the sera and in greater amounts in the red cells of the blood, and in other tissues and fluids of the body. Nowhere is acid phosphatase found, according to the literature, in such abundance as in the human and monkey prostatic fluids. It has been found that 40% neutral formaldehyde destroys acid phosphatases of the blood but exerts little or no effect on those of the prostate (2). It has also been found that acid phosphatase is destroyed by exposure and aging. The amount found depends greatly on the conditions to which the specimen is subjected. The writer has found a specimen to contain activity such that one cc. of extract of the stained cloth liberated 39.45 micrograms of inorganic phosphorus after three days of uncontrolled exposure. However, after six days of exposure at room temperature only 13.26 micrograms of inorganic phosphorus was liberated per cc. of extract indicating a 66.5% decrease in enzyme activity.

However, on April 15, 1953, a handkerchief was submitted to the writer which had been examined and spermatozoa reported found in December 1948. The handkerchief had been stored and preserved in a folded condition inside an unsealed brown manila type envelope. After almost five years spermatozoa were found present in an extract of the stained area, and by the method hereinafter set forth acid phosphatase activity resulted in the liberation of 82.98 micrograms of inorganic phosphorus per cc. of the extract.

The writer consulted with Dr. E. M. K. Geiling, Chairman of the Department of Pharmacology, University of Chicago, and Dr. Kenneth P. DuBois, Associate Professor Pharmacology, University of Chicago, with regard to developing a reliable technique for measuring the acid phosphatase activity in extracts of suspect seminal

stains. As a result of these consultations Dr. DuBois suggested the solutions listed below and outlined the chemistry of the test in Part II of this paper. After subsequent experimentations on known and unknown extracts, the method was developed.

Solutions

1. Buffer solution is made by adding the following reagents in the order listed:
   0.025M—Sodium Barbital (2.6 grams)
   0.004M—Magnesium chloride (0.4 grams of $MgCl_2 \cdot 6H_2O$)
   0.015M—Sodium b-glycerophosphate (2.3 grams)
   The above reagents are dissolved in 400 cc. of distilled water, and the pH is adjusted to 6 by adding 10% hydrochloric acid using a pH meter or Alka-Acid test paper as the indicator. Then sufficient water is added to make 500 cc. of solution. To each 100 cc. of the buffer 0.5 cc. of formaldehyde (neutral) are added.
   The solution is kept under refrigeration, and when stored under these conditions it may be used for 6 weeks. It should not be used after it becomes cloudy.
2. 50% Trichloracetic acid.
3. 10 N. Sulfuric acid prepared by diluting 266 cc. of concentrated sulfuric acid to 1000 cc. with distilled water.
4. 2.5% Ammonium molybdate solution prepared by dissolving 2.5 grams of ammonium molybdate in 100 cc. of distilled water. Filter through filter paper if the solution is turbid.
5. Phosphorus reducing reagent is made as follows: Add 125 milligrams of 1-amino-2 naphthol-4 sulfonic acid. 7.5 grams of sodium bisulfite and 250 milligrams of sodium sulfite to 50 cc. of distilled water. Warm gently, if necessary, until the materials are in solution. This reagent is stable for several weeks if kept well stoppered and protected from strong light.
6. Standard phosphorus solution is prepared using potassium dihydrogen phosphate (KH_2PO_4). Add 61 milligrams of KH_2PO_4 to 500 cc. of distilled water. The resulting solution contains 30 micrograms of phosphorus per cc.

Method

The method used in sex cases in which an area on cloth is suspected to contain a seminal stain is as follows:

1. Under ultraviolet light, using the 3650 filter or its equivalent, the stained area is marked off using an appropriate colored wax pencil.
2. A portion of the stained area 2.0 centimeters square, is cut out and placed in a clean test tube and covered with 2.5 cc. of distilled water. After 30 minutes the portion of cloth is removed from the test tube and as much of the liquid as possible is gently squeezed out.
3. 1.0 cc. of the extracted liquid from the test tube is transferred to each of two clean tubes. In a third test tube 1 cc. of distilled water is placed and serves as the "Blank."
4. To each of the three test tubes noted in step 3 is added 1 cc. of buffer solution. To one of the test tubes containing extracted liquid and buffer solution added,

0.5 cc. of 50% trichloracetic acid is immediately added and is henceforth referred to as the "stopped" specimen. The "stopped" specimen and the buffered solutions are then incubated at 38° C for 30 minutes after which 0.5 cc. of the 50% trichloracetic acid solution is added to each of the two specimens not previously so treated. If solid matter is present the material is centrifuged.

5. Three clean test tubes are numbered for identification purposes and in each of them is placed 0.5 cc. of the respective supernatant incubated solutions. To a fourth test tube 0.5 cc. of the standard phosphorus solution is added.
6. To each of the four test tubes prepared in step 5 the following solutions are added *in the order here listed:*
   4.3 cc. of the distilled water
   0.4 cc. of 10 N sulfuric acid
   0.6 cc. of 2.5% ammonium molybdate
   0.2 cc. of the phosphorus reducing reagent
7. The solutions are permitted to stand for 20 minutes at room temperature and read in a spectrophotometer at 660 wave length. A photoelectric colorimeter with a 660 filter may be used if a spectrophotometer is unavailable. A blue color in the "Blank" is indicative of contamination with phosphorus, and the results are considered unreliable. If there appears to be phosphorus present in the "stopped" specimen, the readings must be corrected accordingly. This may be done by using the "stopped" specimen to adjust the spectrophotometer to 100% transmission. The quantitative result is computed by letting S = optical density of standard, U = optical density unknown stain, X = unknown quantity, 30 = phosphorus concentration in micrograms per cc. of the standard specimen. Then the ratio S:U = 30:X can be applied for a quantitative determination. A value of 18 or more is indicative of the presence of acid phosphatase in the stain in quantity sufficient to indicate the stain to be seminal and of human or monkey origin.

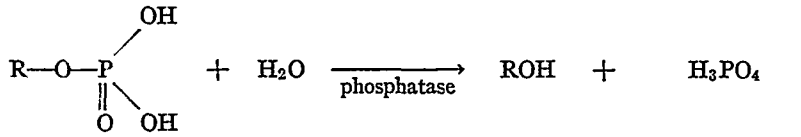
   A value of less than 18 micrograms per cc. of extract is indicative of the presence of acid phosphatase, but in insufficient amount to warrant a conclusion that the stain is seminal. In the event of such a finding, semen identification depends on the presence of spermatozoa on a prepared slide of a portion of the original extract. The finding of spermatozoa and acid phosphatase present in such extract would leave no question as to the origin of the stain extracted.
8. A photograph of the relative blue color density of the respective specimens taken on color film records the qualitative comparison for the file and for court purposes.

PART II

CHEMISTRY OF THE ACID PHOSPHATASE TEST FOR SEMINAL FLUID

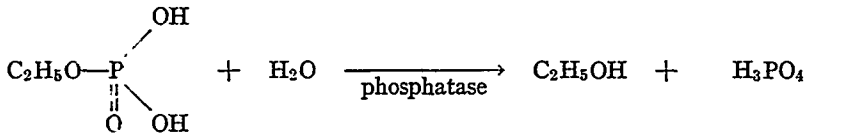
Animal tissues have long been known to contain a group of catalysts which have the ability to hydrolyze phosphorus-containing compounds. Biological catalysts of this type are called enzymes, and the particular enzymes with which we are concerned are called phosphatases. These catalysts are proteins which have specific properties and requirements for activity. All phosphatases have certain properties in

common outstanding among which is their ability to liberate inorganic phosphorus from organic phosphorus containing compounds according to the following type reaction:



organic phosphate — alcohol — phosphoric acid

A specific example of this reaction is the following:

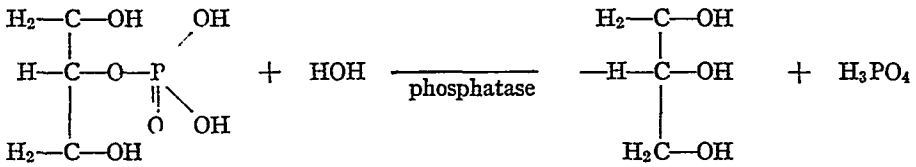


ethyl phosphate — alcohol — phosphoric acid

It may be noted from the above reactions that the phosphatase does not actually enter into the reaction but only facilitates or speeds up a reaction between water and the organic phosphate. One phosphatase molecule can therefore catalyze the hydrolysis of many molecules of an organic phosphate.

In the above discussion statements applicable to phosphatases in general have been made. However, there are actually a number of different phosphatases which vary from one another in the compounds upon which they act and in the conditions under which they function most efficiently.

The phosphatase of seminal fluid has certain distinguishing characteristics. Its activity is greatest in an acid medium at a pH around 6. The reaction is therefore carried out in a buffer (barbital) adjusted to this pH. The activity of phosphatases is generally increased by magnesium ions. Although prostatic phosphatase activity is not appreciably increased by magnesium this ion is nevertheless added to the reaction mixture to insure maximum activity. The phosphatase of seminal fluid hydrolyzes b-glycerophosphate readily, and this compound is therefore used for the phosphatase measurements. The chemical reaction which takes place through the action of the phosphatase on b-glycerophosphate is as follows:



b-glycerophosphate — glycerol — phosphoric acid

The amount of phosphoric acid liberated by the hydrolysis of glycerophosphate serves as an index of enzyme activity. Measurements of the inorganic phosphorus in the reaction medium at the end of the incubation period are therefore made. The

reactions which occur in the phosphorus analysis are as follows:

$$(NH_4)_2MoO_4 + H_3PO_4 \rightarrow (NH_4)_3PO_4 \cdot 12MoO_3$$

ammonium molybdate — phosphomolybdic acid

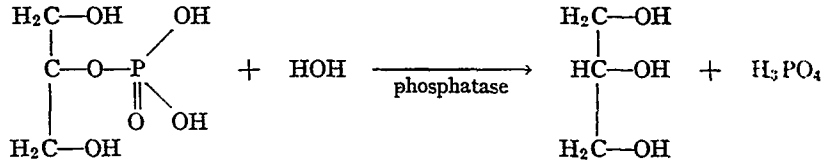
$$(NH_4)_3PO_4 \cdot 12MoO_3 + \text{reducing agent} \rightarrow \text{molybdenum blue}$$

(aminonaphthol sulfonic acid) — (mixture of lower oxides of phosphomolybdic acid of undetermined structure)

The essential phases of this reaction consist of the interaction of phosphoric acid and ammonium molybdate to form ammonium phosphomolybdate which is also known as phosphomolybdic acid. By treating phosphomolybdic acid with a reducing reagent (aminonaphthol sulfonic acid plus sodium sulfite and sodium bisulfite) the phosphomolybdic acid is changed to a blue compound called molybdenum blue. The exact formula for this compound is not yet known, but it is believed to be a mixture of the lower oxides of phosphomolybdic acid. The important fact is that molybdenum blue contains one phosphorus atom per molecule. The quantity of this colored compound can be estimated using a colorimeter or spectrophotometer and compared with the amount of color produced by a known quantity of inorganic phosphorus. B-glycerophosphate will not react with the phosphorus reagents. Thus, only the phosphorus liberated as a result of the action of the phosphatase is capable of reacting with the reagents, and the amount of phosphorus liberated is therefore strictly related to the enzyme activity.

In summary the following reactions take place during the course of the measurement of the phosphatase activity of seminal fluid.

A.



b-glycerophosphate + H_2O → glycerol + phosphoric acid

B. $H_3PO_4 + (NH_4)_2MoO_4 \rightarrow (NH_4)_3PO_4 \cdot 12MoO_3$

Phosphoric acid — ammonium molybdate — phosphomolybdic acid

C. $(NH_4)_3PO_4 \cdot 12MoO_3$ + reducing compound → molybdenum blue

Phosphomolybdic acid

PART III

COMMENTS AND CONCLUSIONS

The method outlined and solutions indicated were used in 102 sex cases submitted for examination, and in 22 experimental cases since June 1952. 4 of the 102 cases submitted for examination resulted in the finding of acid phosphatase in greater amount than 18 micrograms per cc. of extract and in which there were no spermatozoa

found. There were no instances in which the acid phosphatase findings were negative and spermatozoa found.

Although trial courts have accepted and admitted into evidence testimony in which the outlined procedure was presented, the writer has had no personal experience with a case which was reviewed and the procedure ruled on specifically by a court of record. However, Dr. Charles P. Larson, Director of Laboratories, Tacoma General Hospital, Tacoma 3, Washington, wrote that he testified in the case of State of Washington vs. Johanne Pederson to a negative finding for spermatozoa in vaginal smears, and to a positive finding of acid phosphatase in aspirated vaginal fluid in sufficient quantity to indicate the presence of semen. The Washington State Supreme Court reviewed the case and affirmed the verdict of first degree murder. Dr. Larson reported that it was significant in the case that the defendant was well represented by both American attorneys and representatives of the Crown of Norway; that at no time during the trial did the defendant or counsel contest Dr. Larson's testimony or deny that rape had occurred.

The writer is convinced, on the basis of his experiences, that the methods and procedures herein outlined are reliable as a test for identifying seminal stains.

The basic purpose of presenting this paper is to point out a reliable qualitative and quantitative procedure to identify seminal stains for the benefit of forensic investigators. It is presented with the hope that the procedure will be used by many investigators with a view to possible modifications and improvements in the technique, with the ultimate objective to establish a universally accepted qualitative and quantitative procedure for identifying seminal stains.

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