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ACID PHOSPHATASE REACTION AS A SPECIFIC TEST FOR THE IDENTIFICATION OF SEMINAL STAINS

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In a variety of sexual offences, the seminal stain is usually encountered in a dried form on clothing worn by the participants of the offence as well as on other extraneous objects such as carpets, floor, grass, linoleum, mat, turf, wool, wood, and on the vaginal and rectal parts of the passive agent, depending upon the nature and circumstances of offence.

The acid phosphatase reaction has now become an indispensable chemical test in the hands of a forensic scientist to identify the presence of semen which is an abundant source of the enzyme acid phosphatase (1). This enzyme acts optimally on monoesters of phosphoric acid at pH values around 5 to 6 (2). This test has been successfully employed to obtain a proof of the presence of seminal stains (3–12).

The acid phosphatase reaction is a typical test in which the suspected seminal stain or an extract of it on a filter paper is reacted with a solution of the substrate, a monophenolic phosphoric acid or its ester, in acetate buffer of pH 5. The enzyme acid phosphatase hydrolyses the substrate to the corresponding phenol and phosphate ion. The phenol formed is simultaneously coupled with a suitable diazonium salt as a chromogen to give a characteristic colored dye stuff, which is a positive test for the presence of a seminal stain. The hydrolysis and coupling are carried out in one and the same reaction by using a reagent prepared by dissolving the required amounts of substrate and chromogen in acetate buffer. A negative reaction means the absolute absence of semen. But where the reaction is positive, a proof that it is due to the presence of semen, must be established by performing control tests.

The seminal stain as visible to the naked eye, usually has an yellowish grey tint, with appearance of an outline of a counter map. It is stiff or starchy in the dried form. In most cases of unlawful sexual indulgence a seminal stain in a garment or other articles may be simulated or obscured by similar other stains of biological or physiological origin, or pertaining to vegetables, fruits, leaves and plants, cereals and beverages, stains due to secretions of animals and birds, and also of miscellaneous substances containing free phenols such as wood, antiseptics, carbolic soap, etc., depending upon the place and circumstances of the offence. Such stains may be either enzymatic or non-enzymatic in character (6) and may produce a typical color with the acid phosphatase reagent or directly with the chromogenic diazonium salt employed and hence cause interference while testing for the presence of semen, by giving a false positive reaction. However, Kind (6), Brackett (7), Eeva Levonen (11), Weyrich (13), Leithoff and Kuzias (14) and Nicholls (15) have claimed that false positive and false negative results could be obtained from various other simulatory stains which may or may not have the nature and appearance of a seminal stain. According to Nicholls (15) good evidence may be lost, by not collecting further articles in the field for examination, if a stain is considered as semen by color or appearance.

Eeva Levonen (11) and Nicholls (15) have described the advantage of acid phosphatase reaction as a reliable chemical test to identify seminal stains particularly in cases of Azoospermia, Oligospermia and Asthernospermia where the microscopical detection of spermatozoa is very difficult. Kind (6) and Nicholls (15) assert that the acid phosphatase reaction is the only sensitive test to detect semen, particularly in the examination of large areas like

turf, mat, wool, leaves, carpets, and linoleum where microscopic detection of spermatozoa often fails. Pinto (16) has stated that the intensity of color reaction in the acid phosphatase test on vaginal secretions decreases in direct proportion to the lapse of time since the last coitus up to 40 hours.

Experimental

100 mg of Alpha naphthyl phosphoric acid and 200 mg of Brentamine fast blue B. salt are dissolved in 50 ml of acetate buffer of pH 5, at room temperature (20°C to 30°C). The solution is shaken for 10 minutes. After half an our it is filtered into a clean glass stoppered amber bottle and preserved at room temperature (20°C to 30°C). This reagent has an amber color, and we have found that it keeps stable and sensitive for 14 days at room temperature. A precipitate forms after 24 hours, but it does not affect the reaction. After 14 days it turns bluish violet and loses its specificity.

Extraction of Seminal Stain from Cloth. We have adopted the mode of extraction described by Jones (17) which is more or less similar to the one used by Kind (6).

A Whatman No. 1 filter paper is dampened with distilled water and is pressed firmly on to the surface of the suspected stain in the cloth and left in position for 5 minutes. The paper is now removed and sprayed with the acid phosphatase reagent prepared above, using a chromatographic micro glass sprayer.

An intense purplish color appears on the paper at the portion where the stain has been extracted, in 30 seconds to 1 minute depending upon the amount of stain and the efficiency of extraction as well as the age of stain.

The purplish color turns purplish violet after one hour and as such remains vivid for over one year and can be preserved for presentation in the court, if necessary.

We have observed that seminal stained clothes kept exposed to air and light at atmospheric pressure and temperature for a period of 2 years respond readily to this test producing the same intensity of color as obtained from fresh stains. Very dilute solutions of semen in the low concentration of 1 drop of a 2% solution of semen in distilled water readily respond to this test even after a period of one year.

It is always very necessary to perform a control test on a piece of clean cloth free from any stain, which tested similarly gives a negative reaction. Likewise, we have also carefully extracted and tested seminal stains from bricks, carpets, floor, grass, leaves, turf, mat, different colored clothes, metals, wood, and wool and found them to give a positive reaction with the same specificity as the stains obtained from clothes.

From every batch of acid phosphatase reagent prepared and used in testing these stains, we have performed a test on a seminal stain side by side without any sort of contamination of the latter with the former. This has been necessary in order to check the specificity and sensitivity of the reagent from time to time.

We have also prepared stable test papers for seminal acid phosphatase, as described by Kind (18) using alpha naphthyl phosphoric acid and found them to be very useful for quick identification of seminal stains.

Work on Different Stains. With an object to study the different colors produced by a variety of stains which are likely to simulate or obscure a seminal stain and also to ascertain the specificity of this reagent to detect a seminal stain, we have carried out, in our laboratory the acid phosphatase test on 100 stains from different sources. We have observed that with proper control tests on materials in the unstained portions, the acid phosphatase test is very specific for the detection of a seminal stain in spite of a false positive or a false negative reaction caused by other simulatory stains.

In each of the 100 stains tested a generous quantity of stain is obtained on a piece of cloth and extracted on to a Whatman No. 1 filter paper as in the case of semen. Each one is sprayed with the acid phosphatase reagent. The original color and appearance of the stain in the cloth, the changes in color after spraying with acid phosphatase reagent and the time interval under which such changes are observed have been found to be remarkably different from that of a typical seminal stain.

Chromatography of Seminal Stains. We have also carried out circular filter paper chromatography of seminal stains using aqueous extracts and Whatman No. 1 filter papers with a view to locate the enzyme acid phosphatase and to obtain a possible Rf value. However, it was not successful although we tried as many as 4 different solvents such as:

1. Solvent used by Eeva Levonen (11)
2. Solvent used by Fiori (19)

3. Acetate buffer pH 5, and
4. Citrate buffer pH 4.85

Using the first two solvents, the enzyme could not be located even at the original point of application as apparently it was inactivated by the organic esters. With the two buffer solvents, only trailing occurred, and the resolution was not good, although the enzyme could be located at the point of application.

Electrophoresis of Seminal Stains. We have carried out paper electrophoresis of seminal stains using aqueous extracts with a view to locate the movement of the enzyme acid phosphatase, but we observed that due to the heat involved in the electrophoresis the enzyme was inactivated and as such electrophoresis was not successful.

Discussion. We have used alpha naphthyl phosphoric acid as the substrate and diazoorthodianisidine reagent under the name Brentamine fast blue B. salt (I.C.I.) as the chromogen in our procedure which gives a characteristic purple color with alpha naphthol, resulting from the hydrolysis of the former by the enzyme acid phosphatase. Kind (6), Brackett (7), Eeva Levonen (11), and Nicholls (15) have tried anthraquinone 1 diazoniumchloride as the chromogen which gives a red color with alpha naphthol. The former reagent has a definite advantage over the latter in the sense that the purple color is quite distinguishable from the various colors produced by other stains, and this color is stable for a period of over one year. The enzymatic stains give a gradual color which increases in intensity with time. The colors given by non-enzymatic stains are immediate and constant in their intensity.

Conclusion

The 100 stains from different sources tested by us comprised 37 vegetables, 17 fruits, 16 biological and physiological fluids, 6 leaves and plants, 7 secretions of different animals and birds, 4 cereals and beverages, and 13 other miscellaneous substances.

Of these, only stains due to 12 vegetables and 8 fruits as well as Basil (sacred) and coffee have been found to produce a final purple outer edge. It is also interesting to note that even among these, 6 vegetables and 4 fruits are colorless in their original state. A striking feature to be observed in these cases is that the intensity of the purple color in the outer edges is much less compared to the characteristic vivid purplish color produced by seminal stains (both fresh and aged stains) and also with a considerable variance in time factor. No purple tint was seen in any of the biological or physiological stains tested although 9 of them were colorless in their original state and the different changes in color were quite typical in these cases. A noteworthy point to be observed is the final pink color produced by these stains. Colors produced from stains due to asparagus beans, snake gourd, mango ginger, garlic, turnip, embic myrobalan, and raisin are characteristic in the sense that they offer a remarkable display of 3 to 4 different colors at different timings with a final purple outer edge in each of them, particularly because snake gourd, garlic, turnip, and embic myrobalan are colorless in their original state.

Among the miscellaneous stains tested, those due to substances containing free phenols produced different typical and instantaneous colors although no purple tint could be seen in any of them even after 10 minutes. Out of the 100 stains tested, the dried stains on clothes due to sweetgourd, lady's finger, dates, fig, and secretions of crow, hen, and duck seemed to resemble a seminal stain in appearance and color. Even among these cases, a faint purple outer edge could be observed only in stains due to fig and dates where also the intensity and time of formation of purple tint were remarkably different in comparison with that from a seminal stain. It is observed that the colors produced by these stains from 100 different sources are distinguishable from that due to seminal stains (both fresh and aged stains) and also that there is remarkable difference in the time taken for these color formations between the former and the latter. The only stain which had original purple color was of red spinaches, and this was changed to greenish yellow immediately.

From the foregoing experiments, it can be concluded that the acid phosphatase reaction using Brentamine fast blue B. salt as the coupling agent is quite specific for the identification of seminal stains, both fresh and old, found on materials commonly encountered in cases of sexual offences.

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A mature society would pay its policemen at least twice as much as they make today, give police work the status of a profession, divorce it utterly from politics, and put all candidates through a rigid psychological examination to weed out the psychopaths, misfits, and sadists.

From Sydney J. Harris' column *Strictly Personal*
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