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THE USE OF RECORDING SPECTROPHOTOMETER FOR THE DETECTION OF ACID PHOSPHATASES IN SEMINAL STAINS

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The acid phosphatase test has been widely recognized today in forensic chemistry as a reliable tool for the detection of semen in stains and it has been useful in limiting the area of search for spermatozoa. Many methods and their adaptations have been described in the biochemical literature for the measurement of acid phosphatase activity, and some of these have been adapted for the detection of seminal stains. These methods depend on the formation of colored products resulting from the reaction of either the liberated phenol (1, 2) or inorganic phosphorus (3) with a specific reagent. There has been a number of modifications of these methods.

Phenolic esters have been commonly used as substrates in the acid phosphatase detection. It has been shown (4) that many substances interfere or may cause serious errors in this type of test unless rigid control measures are exercised. The colored substances produced from a hydrolytic product of an added substrate are nonspecific in nature and could possibly be formed from interfering substances during the course of the reaction.

The use of a recording spectrophotometer such as a model known as DK2 manufactured by Beckman Instruments, Inc. obviates the possible interferences and errors attending the production of colored substances with liberated phenol. Such an instrument is equipped with an automatic scanning and recording device which plots on a graph sheet the absorption spectra of the phenol liberated during the course of the reaction and of the reagent blank or "zero-time" reaction.

The liberated phenol is examined for its maximum absorption peak and its characteristic shape. While many interfering substances will form non-specific colors in the colorimetric methods, these substances will be manifested by their characteristic absorption curves in the instrumental method.

EXPERIMENTAL

The standard condition used by this laboratory for the detection of acid phosphatase is as follows: One square centimeter of the stain area is extracted with one ml. of distilled water. One drop (approximately 0.05 ml.) of the extract is mixed in a small glass tube with 1.5 ml. of substrate-buffer solution (0.01% disodium monophenylphosphate in 0.2 N acetate buffer, pH 6.0). The "zero-time" reaction is measured immediately on the recording spectrophotometer in the ultraviolet ray range of 240–290 millimicrons wave length. The tube is then placed in a constant temperature bath or an incubator at 37 degree C., and readings are made at a 30-minute and/or two-hour interval in the same ultraviolet range.

The two curves, test and control, are compared and any appreciable increase in the maximum absorption peak in the test reaction curve is interpreted as phosphatase activity. The maximum absorption peak of the curve produced by liberated phenol is at 270 millimicrons and its characteristic shape is shown in figure 1.

Figure 1 also shows the higher absorption characteristic of free phenol as compared with the substrate, sodium phenylphosphate, from

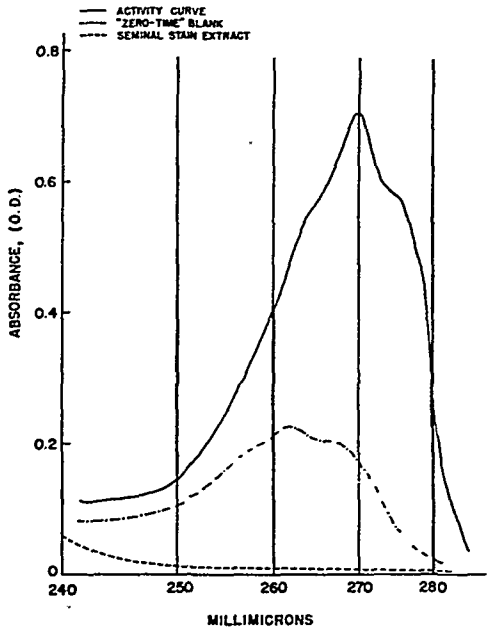

Figure 1

A typical test reaction showing the absorption curves of a reaction mixture under the standard condition at "zero-time" and 30-minute intervals.

which it was liberated under the standard condition. This wide difference facilitates the measurement of liberated phenol by instrumentation. Also it is noted that under this condition, the extract of seminal stain per se exhibits little or no absorption.

Since the test reaction usually contains unhydrolysed substrate, various proportions of equimolar solutions of free phenol and sodium phenylphosphate were mixed, and the absorption characteristics of these mixtures were determined (figure 2). The molar extinction coefficient for the phenol used was 1660 at 270 millimicrons wave length while sodium phenylphosphate was 580 at 262 millimicrons.

Inasmuch as the phenylphosphate substrate does possess its own absorption characteristic with a maximum peak near 262 as shown in figure 1, a determination was made for the minimum amount of substrate required to produce a full enzyme activity curve. For the liberation of each mole of phenol, at least one mole of the sodium phenylphosphate must be present in the reaction mixture. On this basis, the minimum substrate concentration of 0.01% disodium monophenylphosphate was found to be convenient to obtain an activity curve in the range of 0.50 to 0.80

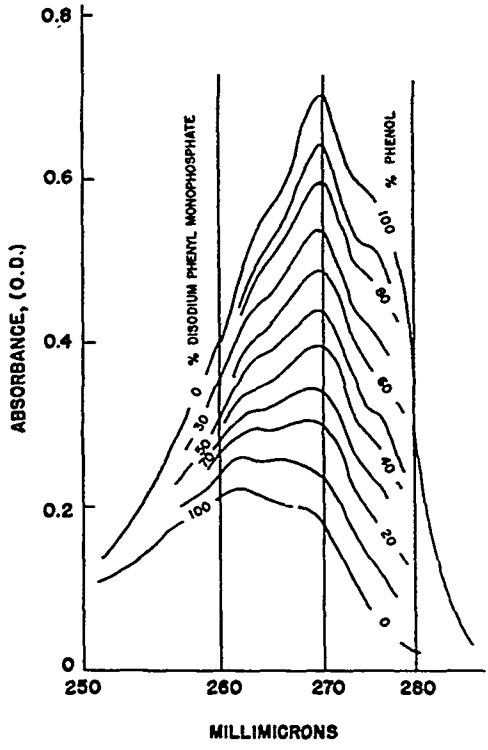

Figure 2

Absorption curves of equi-molar solutions of phenol and sodium phenylphosphate mixed in various proportions. The 100% phenol curve represents total liberation from the amount of phenylphosphate represented in the 100% phenylphosphate curve.

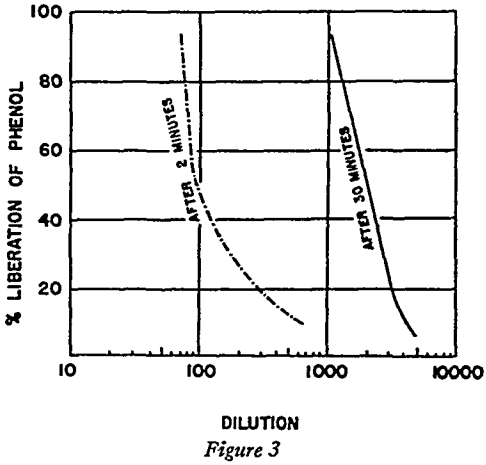

Figure 3

Effect of seminal fluid dilutions on phosphatase activity, as represented by the amount of phenol liberated under the standard condition.

absorbance. This is possible if the phosphatase content of the extract is sufficiently active.

Inasmuch as the amount of seminal fluid in a stain cannot be determined, experiments were

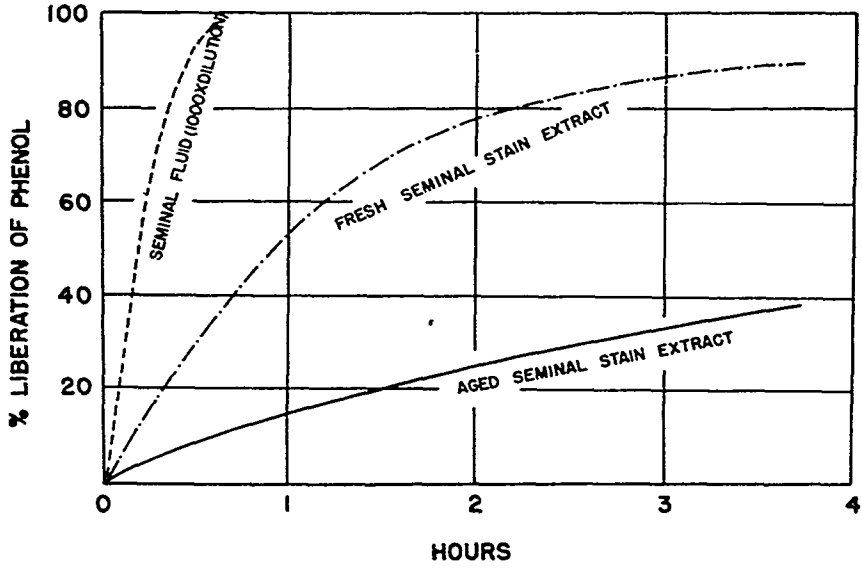

Figure 4

Comparison of phosphatase activity of different semen samples as a function of time under the standard condition. The seminal stains were typical and concentrations not known.

conducted with various dilutions of fresh seminal fluids to determine the maximum dilution to obtain full enzyme activity (figure 3). It was found that a 1000-fold dilution of fresh seminal fluid could produce a full activity curve under the standard condition; however, for average seminal stains, a twenty-fold dilution was considered to be of convenient strength.

Figure 4 shows curves obtained under the standard condition varying the reaction time and dilution of enzyme. A thousand-fold dilution of seminal fluid cleaved nearly 100% of the phenol from the phenylphosphate within 30 minutes. An extract of a comparatively fresh seminal stain cleaved only 30% phenol while an older stain (4 months old) was half as active as the fresh stain. Therefore, it was found advisable to make two readings, one at the end of 30 minutes and another at the end of two hours.

Discussion

The detection of the liberated phenol as a result of the action of acid phosphatases on phenylphosphate by instrumental method has certain advantages over the methods in which liberated phenol is measured by the formation of a colored product with an addition of another reagent. The discrete separation between the curves of the substrate and of the liberated phenol plotted on the graph chart is discernible, more than a gradual change in the intensity of the colored products; this advantage becomes more evident in seminal stains which contain interfering substances.

The production of color entails an additional step and introduces possible errors caused by the contamination or instability of the color producing reagents, changes in pH, and the instability of some colored products. The uncertainty of the nature of color or the lack of color produced due to possible experimental errors requires technicians to repeat tests. The entire step in which these factors are introduced is eliminated.

In the instrumental analysis, the need to prepare an additional tube for the control, or "zero-time", is not required, as the entire procedure is performed in a single tube, or a cuvette in which the reaction mixture is read. The enzyme activity can be measured as fast as the pen of the instrument moves across the paper chart automatically plotting the absorption curve of the reaction mixture.

The expense of a recording spectrophotometer is an obvious limitation if a laboratory is not already equipped with one. If many samples are being determined simultaneously, the timing of the reactions becomes a problem. However in an average forensic laboratory the few samples to be determined per case are hardly comparable to the number assayed by a clinical laboratory. Inasmuch as a forensic laboratory is not concerned

primarily with the assay of phosphatase activity but with its identification, the length of reaction time is not too critical.

The absorption characteristic of phenylphosphate does impose a limitation as to the amount of substrate which can be used, and consequently, a substrate solution cannot be employed which is excessively saturated with respect to the amount of enzyme present as in colorimetric methods. However, in practice the acid phosphatases in seminal stains have proven to be highly active, and the substrate concentration need only to be kept at a minimum to obtain a full activity curve.

While some kinetic study was undertaken in this Laboratory with seminal stain extracts and its action on phenylphosphate, there was no illusion that accurate data could be obtained. The results produced by using seminal stain extracts are of approximate value because for one reason, the stain may have become contaminated with bacteria. The presence of blood, saliva, and fruit juices which also contain phosphatase will possibly interfere. As it is commonly known, the presence of these contaminants do not interfere with the identification of seminal phosphatase because of latter's extremely high activity (more than a hundred times as high as in any of these fluids). An indication of this is that in two minutes under the standard condition, a seventy-fold dilution of seminal fluid can hydrolyse the substrate almost completely in two minutes (figure 3).

Summary

A recording spectrophotometer has been adapted for the determination of acid phosphatases in seminal stains. Free phenols liberated from organic phenols by enzymatic action are determined by ultraviolet light absorption. Some of the advantages have been described as compared to procedures depending on the measurement of colored products formed with liberated phenol.

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