Osamu Suzuki,[1] *M.D.; Takatoshi Matsumoto,*[2] *M.D.; Masakazu Oya,*[3] *M.D.; Yoshinao Katsumata,*[3] *M.D.; and Minoru Asano,*[1] *M.D.*

A New Enzymatic Method for the Demonstration of Choline in Human Seminal Stains

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ABSTRACT: A new, simple method for the identification of seminal stains based on the reaction of choline oxidase with choline is presented. The key reaction is the production of hydrogen peroxide by the oxidase action on choline, whereby hydrogen peroxide reacts with *N*-ethyl-*N*-(3-methylphenyl)-*N'*-acetylethylenediamine and 4-aminoantipyrine (ampyrone) in the presence of peroxidase to produce a purple color so that seminal fluid can be identified. Some data are presented on the sensitivity, stability, and specificity of the present method.

KEYWORDS: pathology and biology, semen, choline, chemical analysis

Various chemical tests have been used for the identification of seminal fluid and stains. Florence [*1*] developed a microscopic method by crystallizing seminal choline. Barberio [*2*] and Puranen [*3*] also succeeded in crystallizing spermine in human semen. These classical methods, however, are not widely used at the present time. More recently, some chromatographic tests for seminal choline were reported [*4-6*]. In the present paper, we report a new enzymatic method for the demonstration of seminal choline using choline oxidase, which is much simpler than the previous methods [*1,4-6*] and thus meets modern medicolegal needs.

Materials and Methods

Chemicals

Choline oxidase (11 U/mg), purified from *Alcaligenes* species [*7*], was purchased from Toyobo Co., Ltd., Osaka, Japan. *N*-Ethyl-*N*-(3-methylphenyl)-*N'*-acetylethylenediamine

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[1] Senior lecturer and professor (director), respectively, Department of Legal Medicine, Hamamatsu University School of Medicine, Hamamatsu 431-31, Japan.

[2] Research associate, Division of Oncology, First Department of Surgery, Nagoya University School of Medicine, Nagoya 466, Japan.

[3] Associate professor and senior lecturer, respectively, Department of Legal Medicine, Nagoya University School of Medicine, Nagoya 466, Japan.

(EMAE)[4] was donated by Kyowa-Hakko-Kogyo Co., Tokyo, Japan. Horseradish peroxidase (Type II) and choline chloride were products of Sigma Chemical Co., St. Louis, Mo., and 4-aminoantipyrine (ampyrone) was a product of Wako Pure Chemical Industries, Ltd., Osaka, Japan. Other common chemicals used were of the highest purity commercially available.

Stains

Various human body fluids, including semen, and juices of vegetables and fruits were dropped or smeared on filter paper (Toyo Roshi No. 2, Tokyo). They were allowed to dry at room temperature for a few hours and cut into small pieces (2 by 5 mm). Seminal stains kept at room temperature for periods of one week to 23 years in our laboratory were also used.

Preparation of Reagents

Buffer solution: 1.4 g (final concentration, 0.1*M*) of monobasic potassium phosphate was dissolved in 100 mL of distilled water and the pH was adjusted to 7.4 by adding 5*N* potassium hydroxide solution.

Reagent I: 2 mg of horseradish peroxidase and 2 mg of choline oxidase were dissolved in 5 mL of the buffer solution.

Reagent II: 2 mg of EMAE and 2 mg of 4-aminoantipyrine were dissolved in 5 mL of the buffer solution.

Recommended Procedure

Various conditions of the assay were tested and the following procedure is recommended as a standard assay. A small piece of the stained material was placed on a hollowed glass plate. One drop of Reagent I and one drop of Reagent II were added to the stain. After 5 to 10 min a strong purple color developed, which was observed by the naked eye. The blank test was unnecessary, because no autoxidation occurred in the present system.

Results

In order to confirm that the color reaction is not an artifact, but is due to choline present in seminal stains, we subtracted choline oxidase or peroxidase from the complete assay mixture. This resulted in no reaction.

To check the sensitivity of the present method, five seminal samples were serially diluted, dropped on filter paper, and dried to make small seminal stains, which were then treated by the recommended procedure. As a result, all samples were positive up to an eightfold dilution. By using authentic choline, the color intensity of the undiluted seminal stains was comparable to that of about 100 nmoles of choline; it was also estimated that the seminal stains of the eightfold dilution contained about 10 nmoles of choline.

Seminal stains left at room temperature for various periods were subjected to the present method. As listed in Table 1, all the samples were positive up to 5 years. From 6 to 23 years, some of the samples gave negative results.

Table 2 shows the results on various human body fluids. The color could not be detected except in semen. The stain made of azoospermatic seminal fluid was strongly positive.

The enzymatic test was checked on stains made by various common vegetables and fruits likely to be encountered in forensic science work (Tables 3 and 4). Although a majority of the

[4]EMAE will be kindly provided, on request, by Kyowa-Hakko-Kogyo Co., 1-6-1 Ote-machi, Chiyoda-ku, Tokyo, Japan.

TABLE 1—*Choline test on human seminal stains of various ages.*

Age of Stain	Tested, *n*	Positive, *n*	Negative, *n*
1 day to 1 month	15	15	0
2 to 6 months	13	13	0
7 months to 1 year	10	10	0
2 years	10	10	0
3 to 5 years	12	12	0
6 to 10 years	5	1	4
10 to 23 years	10	6	4

TABLE 2—*Choline test on the stains of various human body fluids.*

Human Body Fluid	Tested, *n*	Positive, *n*	Negative, *n*
Semen	57	57	0
Blood	10	0	10
Serum	24	0	24
Saliva	11	0	11
Nasal discharge	7	0	7
Tears	4	0	4
Breast milk	9	0	9
Vaginal fluid	18	0	18
Urine	25	0	25
Feces	4	0	4

TABLE 3—*Choline test on the stains of vegetable extracts.*

Vegetable	Tested, *n*	Positive, *n*	Negative, *n*
Cabbage	2	0	2
Carrot	2	0	2
Radish	2	0	2
White potato	2	0	2
Barley malt	2	0	2
Green pepper	2	0	2
Spinach	2	0	2
Onion	2	0	2
Kidney bean	2	0	2
Green pea	3	0	3
Broad bean	3	2	1
Cucumber	2	1	1
Lettuce	2	2	0
Cauliflower	3	3	0
Broccoli	2	1	1

samples was negative, some vegetable stains, such as broad bean, cucumber, lettuce, cauliflower and broccoli, gave positive results, but the color intensities were much weaker than that of semen. All fruit stains showed negative results.

Since seminal fluid may coexist with blood, vaginal fluid, or rectal fluid in actual cases, we tested the effects of these materials on the present reaction by adding their stains to the reaction mixtures. No interference of the materials with the choline reaction was observed.

TABLE 4—*Choline test on the stains of fruit extracts.*

Fruit	Tested, *n*	Positive, *n*	Negative, *n*
Grape	2	0	2
Grapefruit	3	0	3
Loquat	3	0	3
Melon	3	0	3
Mandarin orange	3	0	3
Watermelon	3	0	3
Banana	2	0	2
Mango	3	0	3
Pineapple	2	0	2
Papaya	1	0	1
Tomato	2	0	2
Kiwi fruit	1	0	1
Strawberry	2	0	2
Apple	2	0	2
Plum	3	0	3
Peach	2	0	2

Discussion

In the present paper, we have presented a new method for the demonstration of choline in human seminal stains. It is based on the choline oxidase-dependent production of hydrogen peroxide; the hydrogen peroxide-dependent reaction of EMAE with 4-aminoantipyrine in the presence of peroxidase produces a strong purple color. The reaction is shown in Fig. 1.

Choline oxidase used in the present assay is highly specific for choline and betaine aldehyde [*7,8*]; the latter is a reaction intermediate formed from choline by the same enzyme. The tests on the specificity using various human body fluids (Table 2), vegetables (Table 3), and fruits (Table 4) showed that our present method is fairly specific for seminal stains.

For the identification of semen, the microscopic detection of spermatozoa is most definitive, but their absence is not always indicative of the absence of semen; aspermatic and old seminal stains may give negative results. For this reason and for simplicity, chemical methods are generally employed as preliminary tests. The concentration of choline is higher in human semen than in most other body fluids and tissues [*9*]. The use of seminal choline as an indicator of human semen has an advantage over the use of enzyme proteins such as acid phosphatase [*10*] in that this small molecule is resistant to denaturation.

We have used, in this method, EMAE, a new coloring reagent for the hydrogen peroxide-peroxidase system. In our previous study on the demonstration of seminal spermine [*11*], we used 2′, 7′-dichlorofluorescin (DCF) for the same system. Although the DCF method is more sensitive than the present EMAE method, it suffers from its autoxidation; EMAE is resistant to autoxidation and to interference by bilirubin and proteins. The EMAE system produces a beautiful purple color, which can be easily discriminated from yellow or bloody stains.

The original crystal method of Florence [*1*] for the detection of choline often gives false negative results, especially with old seminal stains. Even with old stains our present method demonstrates, with high certainty, the presence of choline. This is evidenced by the result that all seminal samples were positive after five years of aging at room temperature (Table 1). More recently, methods for the identification of seminal choline by paper and thin-layer chromatography were reported [*4–6*]. Our method requires no separation of choline and thus is much simpler than the chromatographic methods [*4–6*].

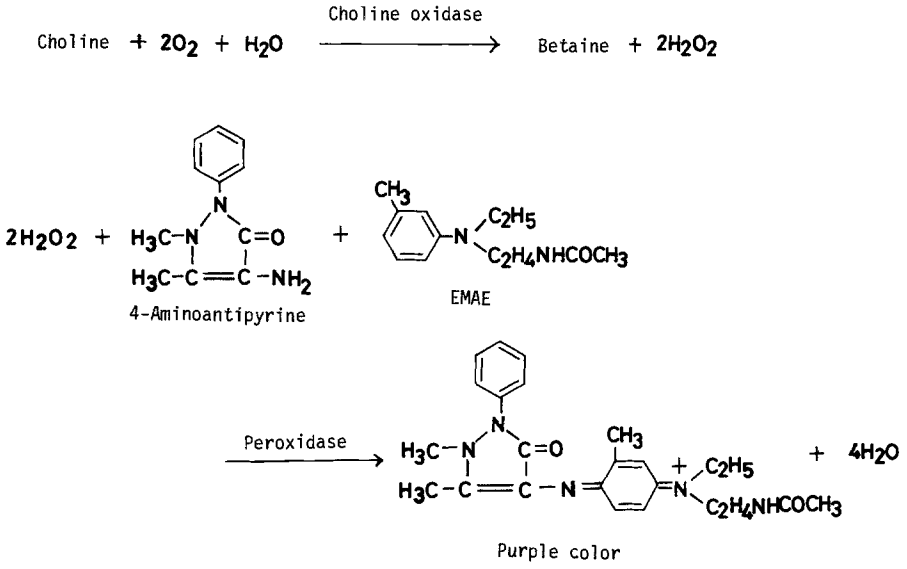

FIG. 1—*Reaction of choline test.*

Free choline is formed in seminal fluid, after ejaculation, from phosphorylcholine and glycerylphosphorylcholine by the action of acid phosphatase [*9*]. The amount of phosphorylcholine secreted into human semen is much larger than that of glycerylphosphorylcholine [*9*]. Phosphorylcholine is known to be secreted from the seminal vesicle [*9,12*]. Therefore, the present method is suitable for the examination of seminal stains excreted by vasectomized individuals.

Our present method is quicker and much simpler than other methods reported so far [*1,4-6*] for the identification of seminal choline. More than 100 samples can be treated within 30 min. Therefore, its simplicity together with its sensitivity, stability (Table 1), and specificity (Tables 2 to 4) makes it a valuable preliminary test for the identification of seminal fluid in medicolegal practice.

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Address requests for reprints or additional information to
Dr. Osamu Suzuki
Department of Legal Medicine
Hamamatsu University School of Medicine
3600 Handa-cho, Hamamatsu 431-31
Japan