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Quantitative Estimation of Heat-stable Alkaline Phosphatase Activity in Dried Blood Stains and its Application to the Forensic Diagnosis of Pregnancy

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Summary. The placental isoenzyme of alkaline phosphatase is characterized by heat stability (56° C for 30 min). This paper describes a new technique for the forensic diagnosis of pregnancy from blood stains, based on the fact that in serum of pregnant women in the latter half of pregnancy the heat-stable alkaline phosphatase activity rises markedly. In this study the total and heat-stable alkaline phosphatase activities were quantitatively assayed in blood stains of various origins by the method of Bessey-Lowry-Brock with the use of disodium p-nitrophenyl phosphate as substrate. Our results indicate that the principle herein reported may be helpful in the diagnosis of pregnancy from blood stains.

Zusammenfassung. Das placentare Isoenzym der alkalischen Phosphatase ist durch Hitzebeständigkeit charakterisiert (56° C für 30 min). Es wird ein neuartiges Verfahren zur forensischen Schwangerschaftsbestimmung aus Blutspuren beschrieben, das auf der Tatsache beruht, daß im Serum von Schwangeren in der letzteren Hälfte der Schwangerschaft eine deutliche Erhöhung der Aktivität der hitzebeständigen alkalischen Phosphatase eintritt. In dieser Arbeit wurden die Aktivitäten der gesamten sowie der hitzebeständigen alkalischen Phosphatase in Blutspuren verschiedener Herkunft nach der Methode von Bessey-Lowrey-Brock bei Verwendung von Dinatrium-p-nitrophenylphosphat als Substrat bestimmt. Unsere Ergebnisse zeigen, daß das hier angegebene Prinzip zur Schwangerschaftsdiagnostik aus Blutspuren verwendbar ist.

Key words: Examination of blood stains — Forensic diagnosis of pregnancy — Serum alkaline phosphatase.

A test to determine whether or not an unknown blood stain on clothes, weapons or some other articles is derived from a pregnant woman is of practical importance in legal medicine.

It is well known that the activity of a number of enzymes such as histaminase, cystine aminopeptidase, leucine aminopeptidase and alkaline phosphatase increases in the maternal circulation during pregnancy [1]. Berg [2] proposed an assay of histaminase (diamine oxidase) from blood stains as a forensic diagnostic test for pregnancy. We have recently described a method for the diagnosis of pregnancy from blood stains by the electrophoretic separation of leucine aminopeptidase isozymes on cellulose acetate strips [3].

Serum alkaline phosphatase activity rises progressively with advanced pregnancy, reaching a peak at term, and declines gradually during the subsequent

few weeks. In 1964, Neale *et al.* [4] discovered that alkaline phosphatase of placental origin is heat-stable at 56°C, whereas phosphatases from other sources are heat-labile and inactivated within 30 min. McMaster *et al.* [5] showed that the rise of serum alkaline phosphatase level in late pregnancy is predominantly due to the heat-stable enzyme derived from the placenta.

The present study was undertaken to determine the total and heat-stable alkaline phosphatase activities in blood stains of various origins for its possible application to the medicolegal diagnosis of pregnancy.

Material and Methods

Blood samples consisted of:

1. Venous blood of normal healthy men and non-pregnant women.
2. Venous blood of pregnant women in different stages of gestation.
3. Venous blood of women during the puerperium.
4. Retroplacental blood at parturition.
5. Genital blood at abortion.

The blood stains were made on filter paper (Toyo No.1), allowed to dry at room temperature and examined after 1 day, 1 week and 1 month. The total and heat-stable alkaline phosphatase activities were estimated by Bessey-Lowry-Brock's [6] method with disodium p-nitrophenyl phosphate as substrate.

The blood stains were divided into two groups. Each of these, 2 cm^2 in size, was cut into smaller pieces and placed in a test tube. Aqueous extract was prepared by adding 0.5 ml of glycine-NaOH-$MgCl_2$ buffer (pH 10.4). Without removing the filter paper, one group was heated in a water bath at 56°C for 30 min, while the other was kept at room temperature. To each tube 0.5 ml of substrate solution (0.4 g/100 ml of disodium p-nitrophenyl phosphate) was added. The tubes were subsequently incubated at 37°C for 1 hr. The enzymatic reaction was stopped by adding 1 ml of 10% trichlor acetic acid and the tubes were centrifuged at 3000 r.p.m. for 5 min. To 1 ml of the supernatant 4 ml of 0.2 N NaOH were added to develop the yellow color of free p-nitrophenol. The liberated p-nitrophenol was measured spectrophotometrically at 415 nm in alkali. The readings were converted to micrograms (γ) of p-nitrophenol liberated in 1 hr per 1 cm^2 of stained filter paper by means of a calibration curve previously prepared.

Results and Discussion

Table 1 summarizes the minimum and maximum values of the total and heat-stable alkaline phosphatase activities in blood stains of various sources. In blood stains of women in the early stages of pregnancy no remarkable difference was seen in either total or heat-stable alkaline phosphatase. In the latter half of pregnancy the total alkaline phosphatase activity rose progressively up to term. This rise was due to a marked increase of its heat-stable portion. During the puerperium alkaline phosphatase contents in blood stains decreased to some extent although they were still considerably higher than non-pregnant values. These results obtained in blood stains approximate to those of other workers in serum samples [7—11]. Blood stains at parturition showed the highest values, whereas blood stains at abortion were not significantly different from non-pregnant blood stains. The above results suggest that a blood stain containing a large amount of heat-stable alkaline phosphatase originates either from venous blood of a woman in the latter half of pregnancy or a woman during the puerperium or from a hemorrhage at parturition.

Table 1. The minimum and maximum values of the total and heat-stable alkaline phosphatase activities in blood stains of various sources (micrograms of p-nitrophenol liberated in 1 hr per 1 cm^2 of stained filter paper)

Source of blood stains	No. of cases	Age of blood stains					
		1 day storage		1 week storage		1 mouth storage	
		total	heat-stable	total	heat-stable	total	heat-stable
Venous blood of men and non-pregnant women	6	3.2— 9.0	0 — 0.8	3.0— 9.9	0 — 0.3	2.5— 8.3	0 — 0.3
Venous blood of pregnant women in 9—12 weeks	4	2.8— 7.8	0 — 0.6	2.4— 6.0	0 — 0.7	2.9— 4.9	0 — 0.5
in 13—16 weeks	3	4.6— 6.6	0.3— 0.6	4.3— 6.2	0 — 0.4	3.2— 5.6	0 — 0.2
in 17—20 weeks	5	3.6—16.2	0.8— 1.3	4.0—14.7	0.4— 1.5	2.0—12.4	0.5— 1.0
in 21—24 weeks	6	4.1—10.8	0.7— 2.2	2.9—12.8	0.6— 3.8	3.7—10.9	0.6— 2.6
in 25—28 weeks	3	5.6—13.1	1.4— 5.0	4.8— 9.8	1.5— 4.6	5.0—13.2	1.2— 4.3
in 29—32 weeks	5	5.2—14.3	2.4— 6.6	6.5—13.0	2.8— 6.5	5.4—14.0	2.5— 5.8
in 33—36 weeks	5	9.2—18.5	3.3— 8.9	8.8—16.5	3,4—10.2	7.6—15.2	3.0— 7.6
in 37—40 weeks	10	8.0—17.2	3.6— 9.2	7.1—18.3	3.8— 7.0	8.8—17.5	4.1— 8.3
Venous blood of women during the puerperium	6	3.8—19.0	1.2— 8.8	3.4—15.7	1.0— 9.6	2.8—15.3	1.4— 7.5
Retroplacental blood at parturition	8	10.5—28.4	5.4—20.5	8.9—32.4	4.6—26.3	9.2—28.1	6.2—23.8
Genital blood at abortion	4	3.4—12.7	0.5— 3.0	3.2—11.0	0.4— 2.2	3.5—10.7	0.8— 3.0

The increased fraction of alkaline phosphatase in pregnancy serum and the placental alkaline phosphatase share various characteristics such as heat stability, L-phenylalanine inhibition, electrophoretic pattern before and after neuraminidase treatment and antigenicity to antihuman placental alkaline phosphatase antibody [12]. On the basis of these biochemical and immunological properties, the placental isozyme of alkaline phosphatase is distinguished from other phosphatases found in normal serum, liver, intestine and bone. However, a single exceptional case of heat-stable alkaline phosphatase was recently discovered in serum and cancer cells of a patient with a disseminated carcinoma of the lung [13]. This isozyme was biochemically and immunologically indistinguishable from human placental alkaline phosphatase and was designated as Regan isoenzyme.

In experiments involving the effect of time on the heat-stable alkaline phosphatase activity in dried blood stains, we found no remarkable reduction of the enzyme activity as long as 1 month storage. Consequently, this technique permits the estimation of the enzyme activity unaffected by temperature and ageing of blood stains. The simplicity and briefness of this method make it also recommendable for the routine practice of any forensic science laboratory.

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