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Abstract

The method of identifying human blood is studied from the standpoint of the fibrinolytic enzyme system by means of the "fibrin plate method" and the following results are obtained: 1) "Fibrin plate method" is, in the point of its sensitivity and species-specificity, one of the most excellent methods for the identification of human blood. 2) A small amount of the blood stains left standing as long as for 5 to 30 years can serve in the determination of the human blood. 3) Putrefied fluid blood does not demonstrate fibrinolysis. 4) Blood stains absorbed in various objects can also be identified whether they are of the human origin or not. 5) The pieces of cloth stained with human blood give positive fibrinolysis even after four washings with soap or after heating at 100°C for one hour. On the other hand, positive results are obtained with the pieces of cloths after three washings by benzidine test and with the physiological saline-extracted solution obtained after two washings of pieces of cloth, by precipitation test. 6) Proactivator has been found to contain globulin fraction in human serum protein. 7) The blood type can be determined with the same materials previously examined by the "fibrin plate method".

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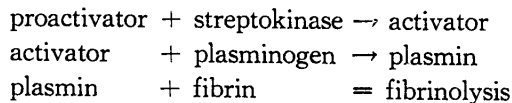
IDENTIFICATION OF HUMAN BLOOD ON THE BASIS OF THE FIBRIN PLATE METHOD

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Recently the studies on the fibrinolytic enzyme system in blood have been developed extensively. *In vitro*, fibrinolytic enzyme system is explained as the following scheme by CHRISTENSEN & MCLEOD (1945)¹:



Proactivator is brought by streptokinase into an activator, which transforms plasminogen into its activated form, plasmin. Utilizing the "fibrin plate method"², the author has studied the fibrinolysis which was induced by plasmin. On the aspect of practical forensic medicine, the blood stains placed under various conditions (polluted, putrefied, heated, washed and left standing for long time) have been examined by the "fibrin plate method" and with the same material agglutinin absorption test has been conducted in order to distinguish the type of blood. By these methods it has become clear that human blood can be readily identified by the "fibrin plate method" and also the types of blood with the same materials can likewise be determined. The findings of these studies are described in this paper.

MATERIALS AND METHODS

Fibrinogen stock solution prepared with the method of ASTRUP & MÜLLERTZ (1952)²: Bovine blood was collected in 0.1 vol. of 2.5 per cent potassium oxalate monohydrate solution and immediately centrifuged for 15 minutes at 3,000 r. p. m. Three hundred ml. of oxalated plasma was stirred for 20 minutes with 18 g. of tricalcium phosphate. After centrifugation for 10 minutes at 3,000 r. p. m. the absorbed plasma was diluted with cold distilled water to 600 ml. and precipitated by slow addition of cold saturated ammonium sulfate of 240 ml. through a funnel and a glass tube with its tip below the surface of the solution with mechanical stirring. After centrifugation at 2,500 r. p. m. for 5 minutes

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the precipitation was dissolved in 150 ml. of 0.9 per cent NaCl solution and diluted with 300 ml. of cold distilled water. After reprecipitation with 180 ml. of cold saturated ammonium sulfate and centrifugation at 2,500 r. p. m. for 5 minutes the centrifuge test tube and the sediment were washed gently with cold distilled water to remove excess ammonium sulfate solution. And then the fibrinogen was dissolved in 120 ml. of diethyl barbiturate buffer. Ten mg. per cent of marzonin were added to the fibrinogen to prevent bacterial contamination. This fibrinogen stock solution contained about 0.6 per cent in fibrinogen, which was kept in ice box (0 to 5°C), to be used up to 3 weeks.

Thrombin solution: thrombin (Mochida Pharm. Co., Japan) was dissolved in the concentration of 100 units per one ml. of physiological saline solution.

Buffer solution: diethyl barbiturate buffer, pH. 7.8, ionic strength 0.05, 662 ml. of 0.1 M sodium diethyl barbiturate were added to 338 ml. of 0.1 M HCl and then diluted with 320 ml. of distilled water.

Streptokinase solution: a stock solution containing 100 mg. per cent of Varidase (Lederle Pharm. Co., U. S. A.) in physiological saline solution was used.

Preparation of the fibrin plate: in each petri dish which is 4.5 cm. in diameter, 3 ml. of diluted fibrinogen solution (buffer solution, 2 ml. to fibrinogen stock solution, 1 ml.) was prepared. Each petri dish was placed on a horizontal plate, it was mixed sufficiently by shaking for about five seconds and clotted white gelatinous fibrin by addition of 0.02 ml. of thrombin solution. This dish was left in an incubator at 37°C for 30 minutes horizontally to dry the surface of the fibrin layer before using it. After the material was dropped on the surface of this fibrin plate, petri dish was set on cover glass and in an incubator at 37°C again.

Preparation of the Blood

Liquid materials, human and animals blood (monkey, cow, horse, pig, kit, dog, cat, rabbit, guinea pig, hen and fish) and putrefied human blood which had been left standing for room temperature in summer were prepared. These materials were diluted to 1:1, 1:5, 1:10, 1:50, 1:100, 1:500, 1:1,000, 1:2,000, 1:4,000, 1:8,000, 1:10,000, 1:20,000, 1:40,000, 1:80,000, 1:160,000, 1:320,000, 1:640,000 and 1:1,280,000 with physiological saline solution. Three quarter ml. of each diluted blood was taken in each test tube and mixed with 0.25 ml. of 100 mg. per cent of streptokinase (SK) solution, and then a drop of it was placed on the surface of the fibrin plate. Materials which had not mixed with SK-solution were used for the controls.

One ml. of human blood was taken in centrifuge test tube, immediately centrifuged at 3,000 r. p. m. for 10 minutes, and then blood cytes were separated from blood plasma. Blood cytes were washed ten times with physiological saline solution and then was hemolyzed by addition of one ml. of distilled water.

Blood plasma was separated to euglobulin, pseudoglobulin and albumin by means of saturated ammonium sulfate, the method of HEKTOEN and COLE (1927)³. In each fraction of serum protein the fibrinolytic activity was studied by the "fibrin plate method"². Human blood was fractionated by "paper electrophoretic method"⁴. Filter paper which had been developed by "electrophoretic method" was divided from two pieces. The one was stained by the bromphenol blue. The other unstained piece was cut into small pieces (0.5 cm²) to be compared with each stained fraction of albumin, α -globulin, β -globulin and γ -globulin, and each piece was placed on the fibrin layer and a drop of SK-solution was added on it.

Solid materials, the blood stains that were absorbed in the pieces of cloth, stone, sand, wood, leaves, glass and iron plate were made into small pieces (each about 0.5 cm²). Dried pulverized bloods, left standing for 5 to 30 years, were prepared. These were placed directly on the surface of the fibrin plate, and a drop of SK-solution was added on by capillary pipette. The pieces of the cloth stained with human blood had been left for a week in room temperature and washed carefully with soap and dried repeatedly five times, and then placed on the fibrin plate and a drop of SK-solution was added on it. The pieces of cloth stained with human blood were put in test tube and heated at 100°C for one hour over water bath at 200°C and 300°C for one hour over oil bath. These materials were studied by means of the above-mentioned method.

Human blood stain was soaked in the cloth and filter paper was cut into small pieces (0.5 cm²) and placed on the fibrin layer. Dried pulverized blood, which had been left for 5 to 30 years, was placed on the surface of the fibrin layer about 0.05 g. A drop of SK-solution was added on these materials. When fibrinolysis was observed in each material, it was collected in clean petri dish and dried at 37°C for 24 hours in an incubator, and then agglutinin absorption test⁶ was tried with the same material for the purpose of determining the type of blood (O, A, B, AB types).

Fibrinolytic activity was estimated as follows: when fibrinolysis occurred due to digestion by plasmin on the surface of the fibrin layer, a well-defined transparent zone was observed around the material. Fibrinolysis observed within 2 hours is considered as strongly positive (≡), that within 4 hours as moderately positive (≡), within 8 hours as positive (+) and that showing no fibrinolysis over 24 hours as negative (—).

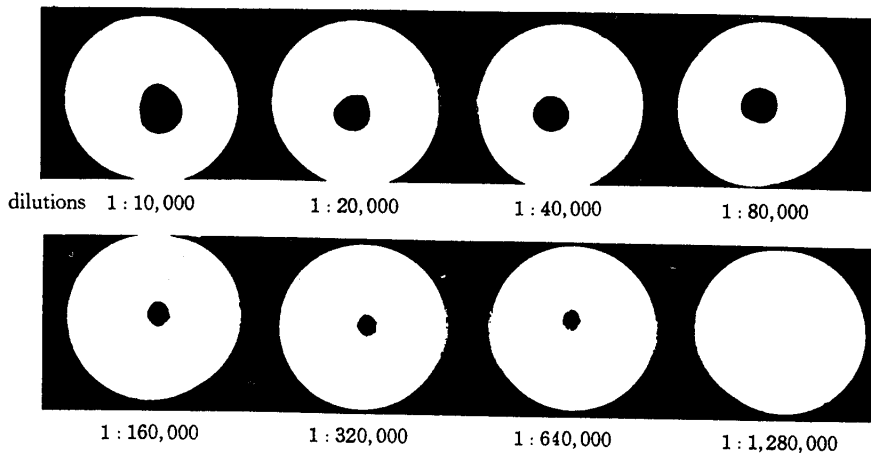
EXPERIMENTAL RESULTS

Positive fibrinolytic phenomenon in normal human blood is observed in dilution of from 1 : 320,000 to 1 : 640,000 (Table 1, Fig. 1) but it is not observable in animal blood except for monkey, dog and cat. Fibrinolytic phenomena

Table 1. Fibrinolytic activity of the blood with 100 mg % SK

Kind of animals	Dilutions of blood																
	Original	50	100	500	1,000	2,000	4,000	8,000	1 × 10 ⁴	2 × 10 ⁴	4 × 10 ⁴	8 × 10 ⁴	1.6 × 10 ⁵	3.2 × 10 ⁵	6.4 × 10 ⁵	1.28 × 10 ⁶	
human	##	##	##	##	##	##	##	##	##	##	##	##	##	##	##	##	##
monkey	##	##	##	##	##	##	##	##	##	##	##	##	##	##	##	##	##
cow	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
horse	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pig	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
kit	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
dog	##	##	##	##	##	##	##	##	##	##	##	##	##	##	##	##	##
cat	##	##	##	##	##	##	##	##	##	##	##	##	##	##	##	##	##
rabbit	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
guinea pig	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
rat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
hen	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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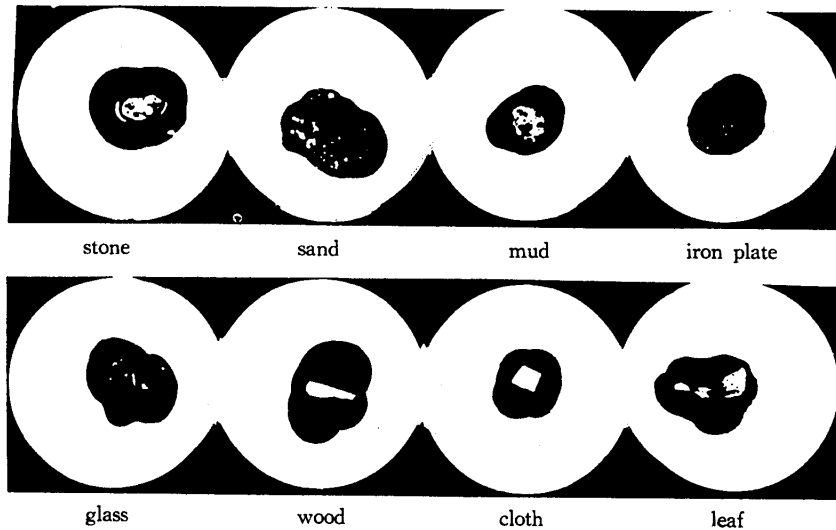
Fig. 1. Fibrinolytic activity of normal human blood



Dissolution of fibrin plate can be seen with 100 mg % SK up to the dilutions of 1 : 640,000 in the blood (8 hrs. after the test)

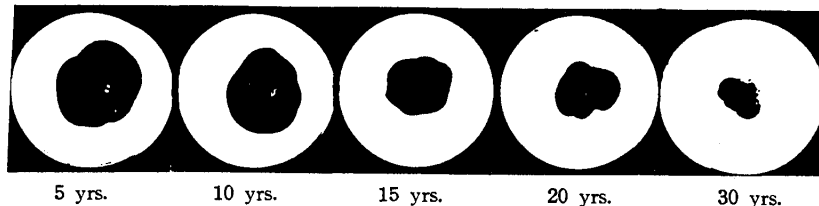
are observed in monkey blood in the dilution up to 1 : 10,000, in dog blood in the dilution up to 1 : 8,000 and in cat blood up to 1 : 4,000. Putrefied liquid human blood shows negative result after ten days or longer at room temperature in summer. The small pieces (0.5 cm²) of cloth, stone, sand, leaves, glass and iron plate absorbed with human blood stains have been directly placed on the surface of the fibrin layer and on each of them a drop of SK-solution is added.

Fig. 2. Fibrinolytic activity of human blood stained in the pieces of various objects



Positive fibrinolysis is observed around the materials which have been directly placed on the fibrin plate (with 100 mg. % SK, 8 hrs. after the test).

Fig. 3. Fibrinolytic activity of the dried pulverized human blood which had been left standing for 5 to 30 years



Positive fibrinolysis can be seen in all the materials (with 100 mg. % SK, 8 hrs. after the test).

As the result positive fibrinolytic phenomena can be observed with them (Fig. 2). Even in the dried pulverized human blood stain which had been left standing for 5 to 30 years positive fibrinolysis is observable (Fig. 3). The pieces of cloth stained with human blood reveal positive fibrinolysis even after four washings (Fig. 4). On the other hand, the benzidine test gives positive result at the third washing of the pieces of these cloths, and the precipitation test conducted up to the dilution of 1:20,000 human blood reacts positively by the physiological saline solution of the extracts only after two washings. The pieces of the cloth with blood stain show positive fibrinolysis after being heated at 100°C for one hour, but with the materials heated at 200°C and 300°C for one hour, they give negative results,

Fig. 4. In the cases of pieces of the cloth stained with human blood and washed with soap are placed on the fibrin plate

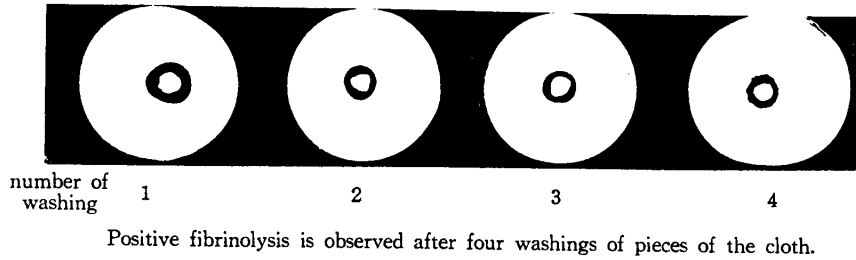
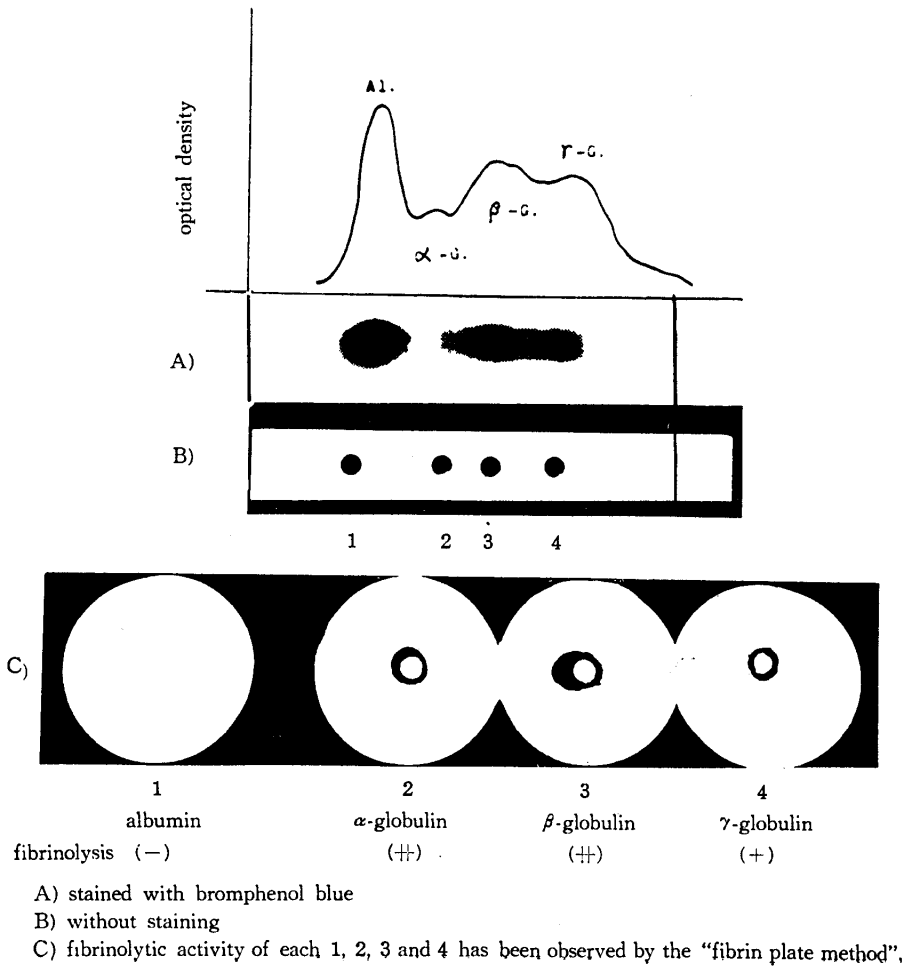


Fig. 5. Fibrinolytic activity of the fractionated protein in normal human serum



The fractions of human serum protein, obtained by the HEKTOEN & COLE's method³, demonstrate positive fibrinolysis only in euglobulin, but in the part of blood cytes no fibrinolysis can be observed. In the case of the "paper electrophoretic method"⁴, positive fibrinolysis has been observed in the fractions of α -globulin, β -globulin and slightly in γ -globulin (Fig. 5).

By means of agglutinin absorption test⁵, each of O-, A-, B- and AB- blood types can be distinguished with the material previously used to identify human blood by the "fibrin plate method".

DISCUSSION

One of the most important problems in the forensic medicine is to determine whether a blood stain is of human or animal origin. The precipitation test (UHLENHUTH⁶, NUTTALL⁷) for the identification of human blood is generally employed using anti-human serum or anti-human hemoglobin. In the case of polluted, muddy or colored materials, precipitation test is not suitable for the determination because it results in pile reaction at the interface between transparent liquids.

Utilizing the "fibrin plate method" (ASTRUP & MÜELLERTZ, 1952)² the author has studied the identification of human blood from the stand point of the fibrinolytic activity of blood. Fibrinolytic phenomenon may be explained as follows (CHRISTENSEN & MCLEOD)¹ (ASTRUP 1951)⁸: proactivator (the details of it have been reported by ASTRUP⁸ 1954⁹, MÜELLERTZ & LASSEN 1953¹⁰, CLIFFTON 1953¹¹, SHERRY & TROLL 1954¹²) is transformed into an activator by streptokinase and the activator transforms inactive fibrinolytic enzyme, plasminogen, into its active form, plasmin, which induces fibrinolysis. Proactivator contained abundantly in human blood is called "human factor" by SZOLLOSY and RENGEI 1960¹³.

Fibrinolytic activities of human and animal bloods have been studied by addition of 100 mg. per cent of SK-solution, and the fibrinolytic activity in human blood is estimated up to the dilution of 1:640,000. It has also been found that the direct identification of human blood is possible even with a small amount of polluted, muddy or colored materials. The above mentioned results clearly indicate that the "fibrin plate method" is a very excellent one for the identification of human blood in forensic medicine.

The thermostability of proactivator as reported by LASSEN in 1958¹⁴, 1960¹⁵ is also observed at 100°C for one hour in the present experiments. Proactivator in human serum reported by ABE in 1961¹⁶, has likewise been found to contain a part of the globulin fraction by means of the HEKTOEN-COLE's method³ and "paper electrophoretic method"⁴. Using the agglutinin absorption test, human blood types have been determined with the materials previously examined by the "fibrin plate method",

CONCLUSION

The method of identifying human blood is studied from the standpoint of the fibrinolytic enzyme system by means of the "fibrin plate method" and the following results are obtained:

1) "Fibrin plate method" is, in the point of its sensitivity and species-specificity, one of the most excellent methods for the identification of human blood.

2) A small amount of the blood stains left standing as long as for 5 to 30 years can serve in the determination of the human blood.

3) Putrefied fluid blood does not demonstrate fibrinolysis.

4) Blood stains absorbed in various objects can also be identified whether they are of the human origin or not.

5) The pieces of cloth stained with human blood give positive fibrinolysis even after four washings with soap or after heating at 100°C for one hour. On the other hand, positive results are obtained with the pieces of cloths after three washings by benzidine test and with the physiological saline-extracted solution obtained after two washings of pieces of cloth, by precipitation test.

6) Proactivator has been found to contain globulin fraction in human serum protein.

7) The blood type can be determined with the same materials previously examined by the "fibrin plate method".

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