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A Precipitin-Inhibition Test on Denatured Bloodstains for the Determination of Human Origin

Since the initial discovery of precipitating antibodies by Kraus in 1897 [*1*] and the demonstration of the precipitin reaction with human blood in vitro by Uhlenhuth in 1901 [*2*], this reaction has become one of the essential tools of the forensic immunologist. Oudin's tube technique (single diffusion in one dimension) [*3*], Petrie's plate method (single radial diffusion) [*4*], Oakley's method of double diffusion in one dimension [*5*], Ouchterlony's immunodiffusion (double diffusion in two dimensions) [*6*], or Culliford's crossed-over electrophoresis [*7*] have been the generally accepted methods for the determination of the species origin of bloodstains. Recently, sensitized latex particles have been used with precipitin sera for the identification of the species of origin of bloodstains [*8*]. The basic principle of these methods is to bring the bloodstain extract into close-reacting contact with a suitable antiserum. The success of the reaction usually depends on the quality of the antiserum and the presence of soluble precipitin antigens in the bloodstain extract.

Sensabaugh has made a detailed study of the protein changes which take place during the aging of dried blood [*9–11*]. No difficulty was experienced in obtaining suitable precipitin reactions on an eight-year-old, dried sample. Several authors have studied the effect of heat and boiling water on the precipitin reaction for bloodstains [*12–17*]. Results have been somewhat conflicting, but it may be concluded that, in general, the reaction will be much weaker after such treatment. Therefore, with stains which have been exposed to high temperature, detergent, and extensive washing, the conventional precipitin methods usually no longer work. Such conditions may occur in certain laundering operations or with harsh environmental exposure.

Di Jeso and Faiella reported that heated human and bovine sera lost the ability to be precipitated by their corresponding antisera [*18*]. This loss was assumed to be due to the formation of heteroaggregates during heat treatment [*19*]. Some residual antigenicity in sera has been observed after exposure to urea and heat [*20,21*]. When dried bloodstains are heated, this residual antigenicity is fixed on the stain and difficult to extract. Schech [*22*] also observed that solubility of dry-heat treated bloodstains was very poor and the precipitin reaction was much weaker, if observed at all.

The present study was designed to determine the extent to which extractable species-specific protein antigens remain after a dried bloodstain is subjected to a variety of washing and heat treatments. Furthermore, a study was made of the antigenic activity of

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insoluble species-specific human proteins which remain in the stain following these treatments. This latter point, the main focus of this research, was studied by determining the power of the stain-bound proteins to specifically absorb the precipitins in anti-human serum. The antisera which were incubated with the stains were then diffused against known human serum proteins in Ouchterlony plates. A positive reaction for the presence of human proteins in a stain would be indicated by attenuation or inhibition of the antiserum's precipitating ability against these known human sera. This process is diagrammed in Fig. 1.

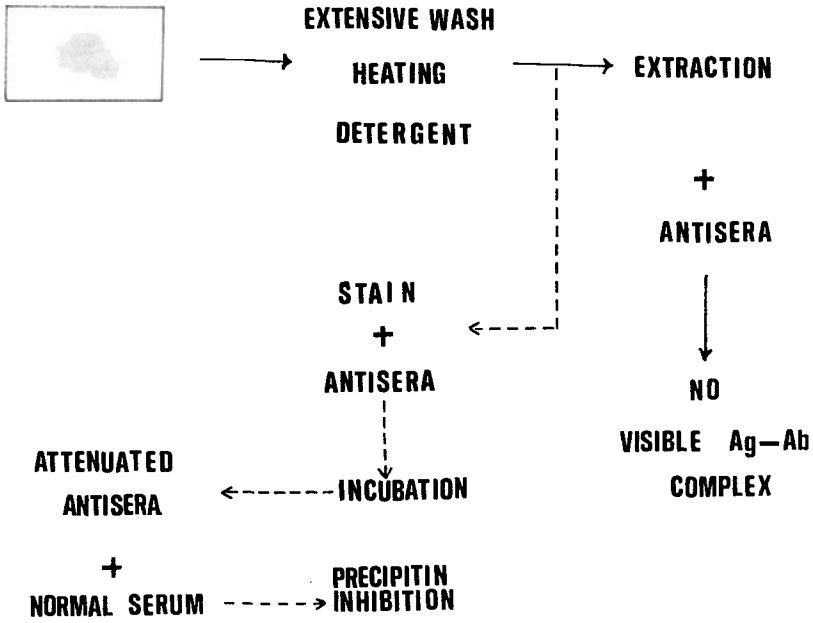

FIG. 1—*Flow chart for the precipitin-inhibition procedure.*

Materials and Methods

Human bloodstains were prepared from capillary blood deposited on clean cotton sheeting. Other animal bloods were obtained from Dr. John Lee, Yale University. Antisera were purchased from Miles Laboratories, Elkhart, Ind.; normal sera and additional antisera were obtained from Cappel Laboratories, Downingtown, Pa.

The titer and specificity of antisera and normal sera were determined by the simple dilution method [*23*]. The protein concentration was determined by a modification of Lowery's method [*24*]. The procedure for the extraction of the bloodstains was essentially the same as that described by Whitehead et al [*8*]. The dried bloodstains were heated in a water bath for 10 min with and without detergent (Triton X-100, Ajax Laundry Detergent) at 25, 50, 75, 90, or 100°C. After being heated selected samples were washed and rinsed in running water for several additional hours. Each washed bloodstain was equally divided and placed into two test tubes. One half was extracted with saline for 24 h at 4°C; the other half was incubated with rabbit anti-human sera (1:16) at 4°C for 24 h. The samples were then centrifuged, and the clear supernatants were transferred by pipet to an Ouchterlony plate (0.9% agarose in borate-saline buffer, pH 8.5 with 0.01% merthiolate as preservative) for the immunodiffusion reactions. The precipitin bands were developed 24 h at room temperature.

Results

As is shown in Table 1, the antibody precipitating ability of bloodstain extract is inhibited by the heating and extensive washing of the stain prior to assay. The combination of heat, detergent and extensive washing either removed or denatured most of the antigenic protein, resulting in a decrease in the amount of saline-extractable human precipitin antigens.

TABLE 1—*Effect of temperature and washing on the direct precipitin reaction.*

Washing Procedure	Heating, °C				
	25	50	75	90	100
Stain in water	positive	positive	weak	very weak	very weak
Stain in water (subsequent rinse in cold water)	positive	positive	very weak	very weak	negative
Stain in water and detergent (subsequent rinse in cold water)	positive	positive	very weak	negative	negative

Figure 2 shows that after extensive washing at different temperatures the amount of protein extractable from a relatively large bloodstain (equivalent to 0.5 ml of whole

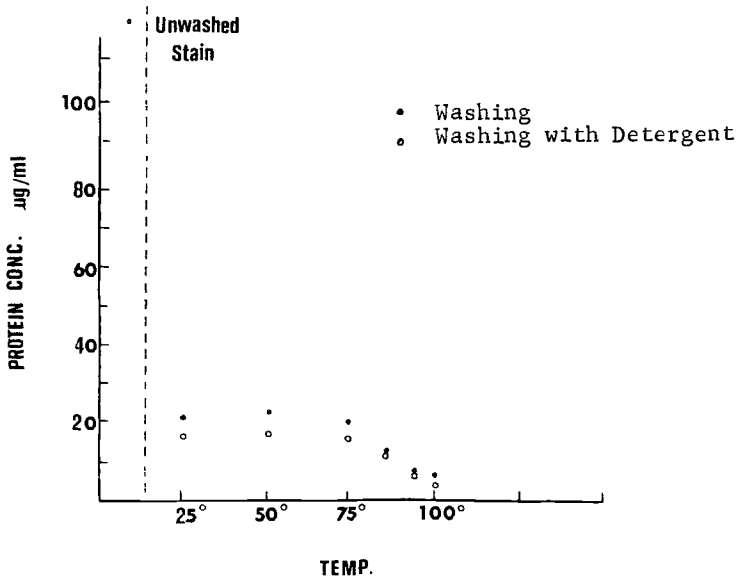

FIG. 2—*The relationship between temperature and the amount of saline-extractable protein remaining after washing.*

blood) decreased drastically. Ninety-five percent of the extractable protein of a bloodstain was lost because of the extensive washing. As the temperature of the washing solution was increased to 50°C a slight increase in the level of saline-extractable protein was observed. This appears to be due to a partial temperature fixation of some blood proteins which become water insoluble but are extractable in saline. But at higher temperatures the fraction of the remaining saline-extractable protein decreases.

Figure 3 illustrates the temperature dependency of precipitin band formation with bloodstain extracts in an agar-gel medium. Wells 5 and 6 illustrate the loss of species protein precipitability as the temperature increased. Figure 4 shows that insoluble human bloodstains incubated with rabbit anti-human serum bind substantial amounts of the anti-human precipitins. This absorption of the antibodies by the stain results in a loss of precipitability with normal human serum. The need to dilute the antiserum prior to incubation with the stain results in precipitin bands that are rather diffuse when compared to those produced by the normal method with full-strength antisera.

Figures 5 and 6 further demonstrate that the precipitin-inhibition reaction is species-specific. Anti-human serum was incubated with various species bloodstains (human, cow, pig, horse, sheep, duck, chicken, rabbit, dog, or cat) prior to diffusion against human serum. Of all the species tested only human bloodstains produce a detectable attenuation of the precipitability of the anti-human serum.

Discussion

The technique we described here illustrates that it is possible to determine the species of origin on both denatured and extensively washed bloodstains. Even where most of the blood proteins are denatured or washed away, residual antigenicity remains trapped on substrate. If the bloodstain has the same origin as the antiserum, a substantial amount of antibody will be absorbed by the stain, resulting in the loss of its ability to precipitate known human serum antigens. In specific instances in case work with denatured and washed bloodstains, this technique may be of great importance. Such situations might be expected where a stained garment has been laundered or where a stain has been treated with harsh chemicals in attempts to remove it. Also, stains on darker-colored surfaces left exposed to the sun could reach temperatures sufficient to denature the antigenic proteins. The results reported here indicate that the species of such stains might be determined by using this precipitin-inhibition method. However, success with this technique depends on antiserum specificity and careful selection of a suitable antiserum dilution. As with any precipitin procedure, adequate controls are essential.

One further application for this technique might arise in a case where a stain or substrate contains a substance which interferes with the normal precipitin reaction. This substance could possibly be removed from an aliquot of the stain without eluting the antigenic proteins by first rendering the stain insoluble with heat. After a period of prolonged washing the denatured stain could be tested for species by the method described here.

The exact limits of this method have not been determined. Further work is necessary to explore the applicability of this technique to the following circumstances:

(1) stains of varying ages, particularly very old ones;
(2) stains which have been immersed in sea water for periods of time;
(3) small stains (determine the smallest usable sample size for different situations);
(4) stains on fabric substrates other than cotton; and
(5) stains exposed to temperatures appreciably above 100°C (determine the maximum temperature to which a stain can be exposed before its ability to specifically absorb homologous antisera is destroyed).

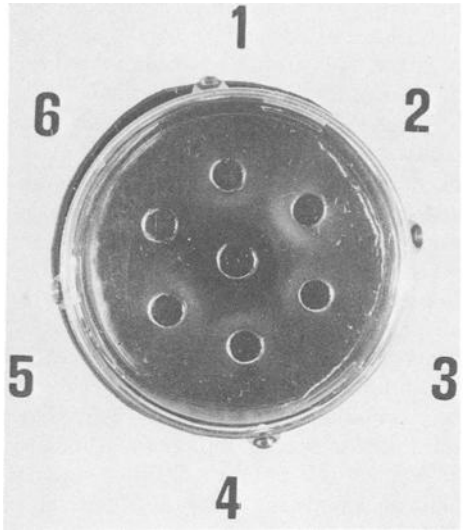

FIG. 3—*Precipitin band in agar medium. The central well contained rabbit anti-human serum. The peripheral basins, clockwise from top, contained (1) control normal human serum (1:1000); (2) serum heated to 25°C; (3) serum heated to 50°C; (4) serum heated to 75°C; and (5) serum heated to 90°C.*

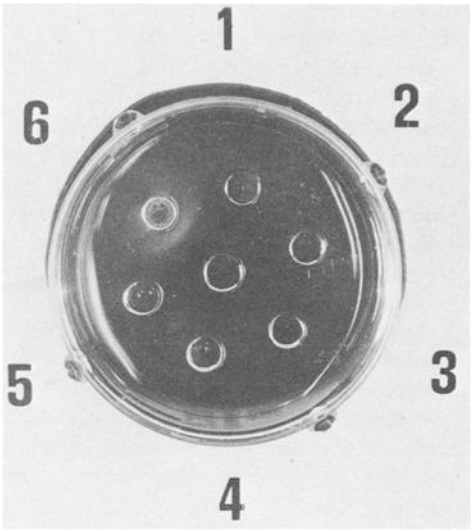

FIG. 4—*An Ouchterlony plate illustrating the loss of precipitability of anti-human serum after incubation of bloodstains. Center well, normal serum; (1) serum heated to 25°C; (2) serum heated to 50°C; (3) serum heated to 75°C; (4) serum heated to 90°C: (5) serum heated to 100°C; and (6) control, blank.*

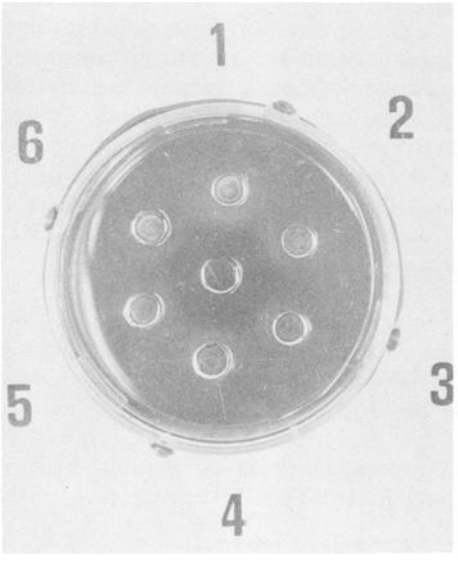

FIG. 5—*Species specificity of the precipitin-inhibition reaction. Center well, normal human serum; (1) anti-human serum control; (2) anti-human serum plus cow bloodstain; (3) pig bloodstain; (4) horse bloodstain; (5) sheep bloodstain; and (6) human bloodstain.*

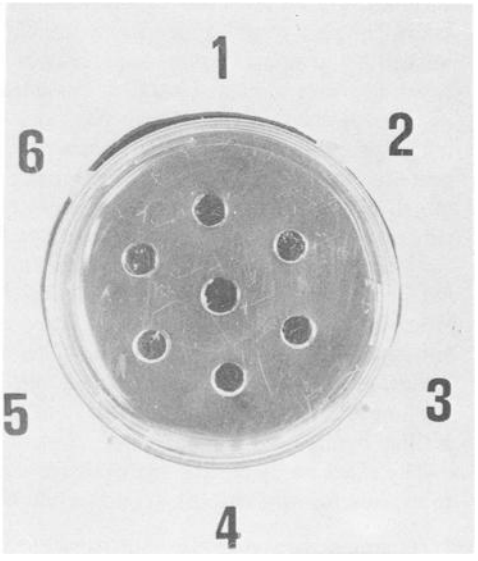

FIG. 6—*Species specificity of the precipitin-inhibition reaction. Center well, normal human serum; (1) anti-human control; (2) duck bloodstain; (3) chicken bloodstain; (4) rabbit bloodstain; (5) dog bloodstain; and (6) cat bloodstain.*

Further work is also necessary to elucidate the nature of the antigenicity remaining in denatured stains. Knowledge of this would help in finding ways of improving the method.

The diffuse precipitin bands characteristic of this method are apparently unavoidable because of the need to use titered (dilute) antisera. When adequate controls are utilized, these diffuse bands can be properly interpreted and do not detract from the accuracy of the method. However, the present authors are currently developing and evaluating a mixed-agglutination method using tannic acid-treated red cells which, although not thoroughly investigated at the time of this writing, is expected to yield more easily interpretable results.

Summary

A new method has been developed for determination of the species of origin of denatured bloodstains. Antiserum with known titer is incubated with the denatured bloodstain. If the bloodstain has the same origin as the antiserum, a substantial amount of antibody will be absorbed by the stain, resulting in an attenuation of the antiserum titer. By reacting the supernatant with a known serum by using immunodiffusion it is possible to detect any appreciable attenuation of the antiserum titer. We have been able to determine the species of origin of various denatured bloodstains using this inhibition method.

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References

[1] Kraus, R., *Wiener Klinische Wochenschrift*, Vol. 10, 1897, pp. 736–738.
[2] Uhlenhuth, P., *Deutsche Medizinische Wochenschrift*, Vol. 27, 1901, pp. 499–501.
[3] Oudin, J., *Comptes Rendus*, Vol. 222, 1946, pp. 115–116.
[4] Petrie, G. F., *British Journal of Experimental Pathology*, Vol. 13, 1932, pp. 380–394.
[5] Oakley, C. L. and Fulthorpe, A. J., *Journal of Pathology and Bacteriology*, Vol. 65, 1953, pp. 49–60.
[6] Ouchterlony, Ø., *Arkiv foer Kemi*, Vol. 1, 1949, pp. 43–46.
[7] Culliford, B. J., *Nature* (London), Vol. 201, 1964, pp. 1092–1094.
[8] Whitehead, P. H., Brech, A., and Cayzer, I., *Journal of the Forensic Science Society*, Vol. 14, 1974, pp. 103–107.
[9] Sensabaugh, G. F., Wilson, A. C., and Kirk, P. L., *Journal of Biochemistry*, Vol. 2, 1971, pp. 545–557.
[10] Sensabaugh, G. F., Wilson, A. C., and Kirk, P. L., *Journal of Biochemistry*, Vol. 2, 1971, pp. 558–568.
[11] Sensabaugh, G. F., D.Crim. Dissertation, University of California, Berkeley, 1969.
[12] Carra, M., as quoted by Schleyer, F., *Methods of Forensic Science*, Vol. 1, John Wiley & Sons, New York, 1962, pp. 291–333.
[13] Ferrai, J., *Bollettino Della Academia Medica di Genova*, Vol. 16, 1901, pp. 272–274.
[14] Graham-Smith, A. S. J., *Hygiene* (Algiers), Vol. 3, 1903, pp. 354–363.
[15] Jones, F., *Journal of Experimental Medicine*, Vol. 46, 1927, pp. 303–314.
[16] Schleyer, F., *Zeitschrift fuer Gerichtliche Medizin*, Vol. 39, 1948, pp. 167–179.
[17] Smith, S. and Glaister, J., *Recent Advances in Forensic Medicine*, Churchill, London, 1939.
[18] Di Jeso, F. and Faiella, A., *Giornale di Biochimica*, Vol. 9, 1960, pp. 146–150.
[19] Di Jeso, F. and Faiella, A., *Bollettino Della Societa Italiana di Biologia Sperimentale*, Vol. 36, 1960, pp. 1939–1942.
[20] Callahan, P. and Martin, N. H., *Biochemical Journal*, Vol. 79, 1961, pp. 28–29.
[21] Jadric, S. and Abinum, A., "Binding of Bilirubin to Human Serum Albumin: Determination of the Dissociation Constants," *FEBS Letter*, Vol. 5, 1969, pp. 112–114.
[22] Schech, E., *Zeitschrift fuer Gerichtliche Medizin*, Vol. 15, 1930, pp. 343–345.
[23] Kirk, P. L., *Crime Investigation*, Interscience, New York, 1960.

[24] Lowery, O. H., Rosebrough, N. J., Fan, A. L., and Randall, R. J., *Journal of Biochemistry*, Vol. 193, 1951, pp. 265–275.

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