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An Improved Staining Technique for Precipitin Bands in Agar or Agarose Gels[1]

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Summary

A method for the staining of proteins in agar and agarose gels using three stains simultaneously and a mordant is described. When compared with conventional Coomassie brilliant blue R-250 staining procedures, it requires a comparable time expenditure but has the following advantages: 1) it is threefold to fourfold more sensitive; 2) there is increased photographic resolution on conventional photographic material; and 3) the stain has a long shelf-life and does not fade under normal lighting conditions. Conditions for the washing and drying of gels are discussed.

Introduction

Precipitation reactions in semi-solid agar or agarose gels are generally stained in order to make them more visible, easier to photograph and intensify the precipitin bands. The most commonly used staining procedures employ CBB[2] (1,2) or ABB (1,2). The combination of metachromasia and the lower sensitivity seen with ABB (3), have led to CBB being the most frequently used stain. However, CBB stained gels are difficult to restain if the destaining process has proceeded too far and occasionally precipitin bands will not pick up the stain. Metachromasia can appear with some proteins and the stain fades when exposed to sunlight, incandescent, fluorescent, or longwave ultraviolet lamps (4). Photographic analysis is complicated with blue stains and requires both special film and filters (3).

Presented herein is a staining procedure for immunological precipitin bands which largely overcomes these problems by utilizing three different stains simultaneously with mercuric chloride as a mordant. The stain will henceforth be called the triple stain.

Materials

Oxoid ionagar #2 (lot 991) was obtained from Consolidated Laboratories and HGT(P) agarose (lot 10439) and Amido black 10B (C.I. 20470 lot 10439)

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[2]Abbreviations used: CBB, Coomassie brilliant blue R-250; ABB, Amido black 10B.

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were purchased from Miles Laboratories. Thiazine red R (C.I. 14780) was from Fluka, Light green SF (C.I. 42095) from MCB and Coomassie brilliant blue R-250 (C.I. 42660) was purchased from Sigma. Slide cover glasses (50x50mm) were obtained from Eastman. Whole human serum antiserum (lot 2913G) was purchased from Calbiochem-Behring Corp. All other reagents were reagent grade.

Methods

Gel Diffusion. Ionagar was used throughout for the double diffusion gels. A 100ml 2% solution of ionagar was prepared with distilled water. This solution was heated in a boiling water bath until clear. Subsequently, 10ml were poured into a series of 10 18x150mm test tubes, cooled, capped and kept at 4 C until future use. Just prior to making a double diffusion plate, a 10ml sample of the 2% ionagar was again heated until completely in solution at which point 10ml of 0.07M sodium phosphate (pH 7.0), 0.85% NaCl (w/v), and sodium azide 1:5000 (w/v) were added. After mixing, 2.5ml of the resulting solution were poured onto a 50x50mm glass slide and allowed to cool. This provides a uniform gel thickness of 1mm and sufficient area to examine 36 samples.

Gel Electrophoresis. Crossed antigen-antibody electrophoresis was performed according to the method of Laurell (5) with the following modifications: after the first electrophoretic separation, the unused portion of the gel was removed. A separate agarose solution containing 0.1 volume of antiserum was poured onto the cleaned portion of the plate to the same level of the original agarose gel. The 50x50mm glass plates used were found to require less antibody than those more commonly in use (1,5). All electrophoretic analyses were performed using HGT(P) agarose.

Staining and Destaining. The stain, a modification of the triple stain of Crowle (3) was made by mixing 0.5g CBB, 0.5g Thiazine red R, 0.5g Light green and 5.0g of $HgCl_2$ in 100ml of 2% acetic acid (final volume).

When the development of precipitation lines was optimal, the gels were washed in 0.85% (w/v) NaCl (pH 7.5-8.0) for 24hr followed by a 24hr wash in distilled water. Several changes of each normally resulted in removal of non-precipitated proteins. This was followed by covering the gel with a piece of Whatman 42 filter paper cut to the size of the gel. After an additional 24hr period at room temperature, the dried gel was pretreated with the destaining solution for 10 minutes and then immediately stained for 10 minutes. This was followed by destaining with a solution of 10% acetic acid, 45% absolute ethanol, 45% distilled water. Once differentiation was complete, the gel was air dried with an Oster hair dryer. The resulting gel was fixed to the glass plate, and transparent.

The Coomassie brilliant blue stain of Weeke (1) was used for a comparison of the sensitivity of the two stains.

Antigens. Human serum albumin (Sigma) was purified by passage through a column (2.5x90cm) of S-200 Sephacryl, equilibrated and developed with 50mM imidazole (pH 6.9). Following dialysis and lyophilization, 1mg of albumin was dissolved in 1ml of 0.07M sodium phosphate (pH 6.9), 0.85% NaCl (w/v). The sensitivity of the stains was determined by maintaining a fixed antibody concentration while making serial dilutions of the albumin solution.

Outdated human blood was obtained from a local blood bank. The serum was isolated by standard procedures and used for electrophoretic analysis.

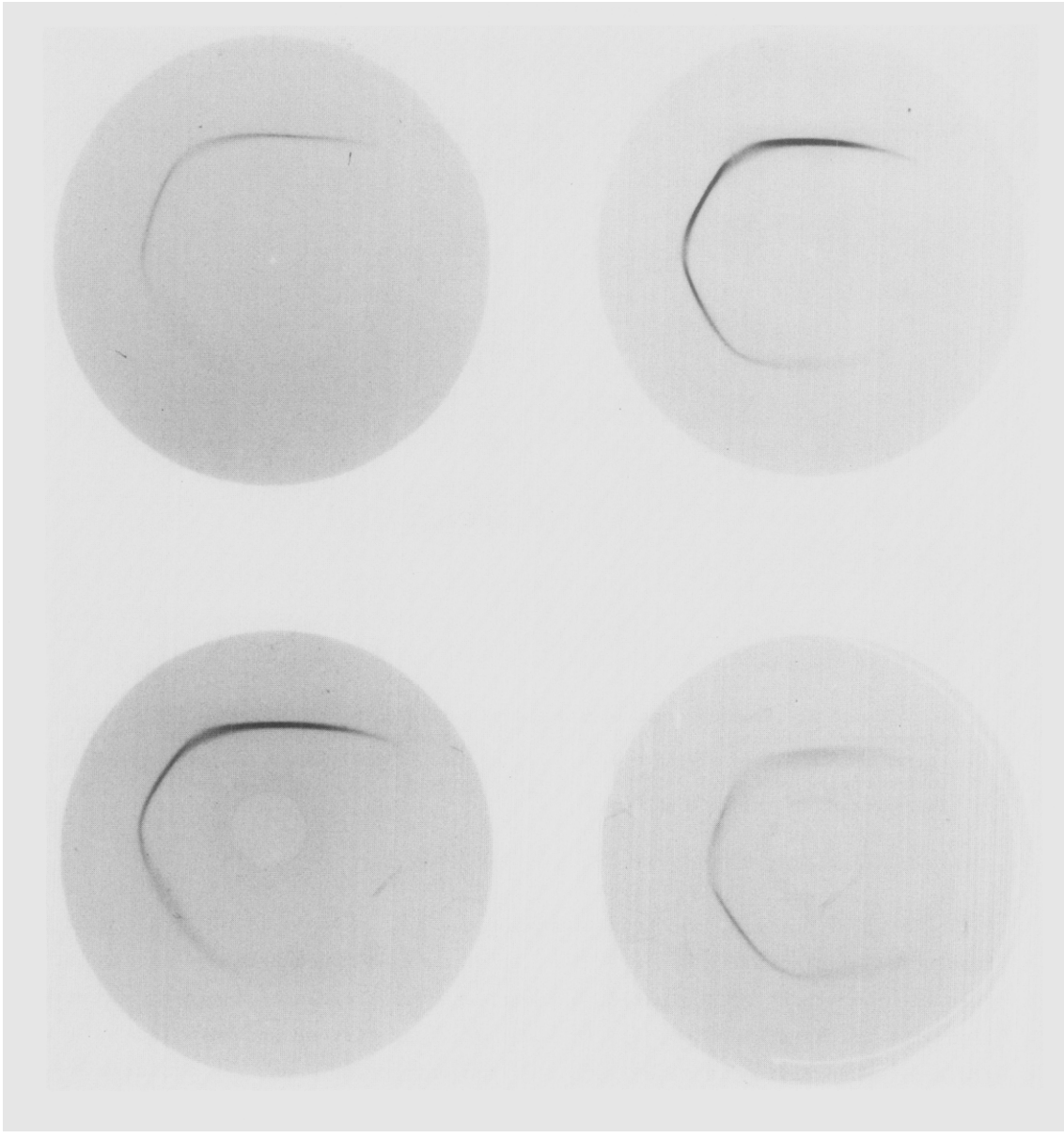

Figure 1. A comparison of differently stained double diffusion plates. Antibody: Rabbit antihuman serum (5ul), placed in the center well. Antigen: Purified human serum albumin was placed in the peripheral wells starting in the 12 o'clock position. Antigen concentration per well, going counter clockwise: 1, 5μg; 2, 2.5 μg; 3, 1.25μg; 4, 0.62μg; 5, 0.31μg; 6, 0.15μg. Gels left to right. Top: CBB stained. Triple stained containing CBB and $HgCl_2$. Bottom: Triple stained containing CBB and $CdCl_2$. Triple stained with ABB in place of CBB.

Results and Discussion

For the purpose of comparing the two stains, identical gels were prepared. That the stain reported here is more sensitive than that of CBB alone is shown in Figure 1. While the limits of the CBB stain appears to be at the level of

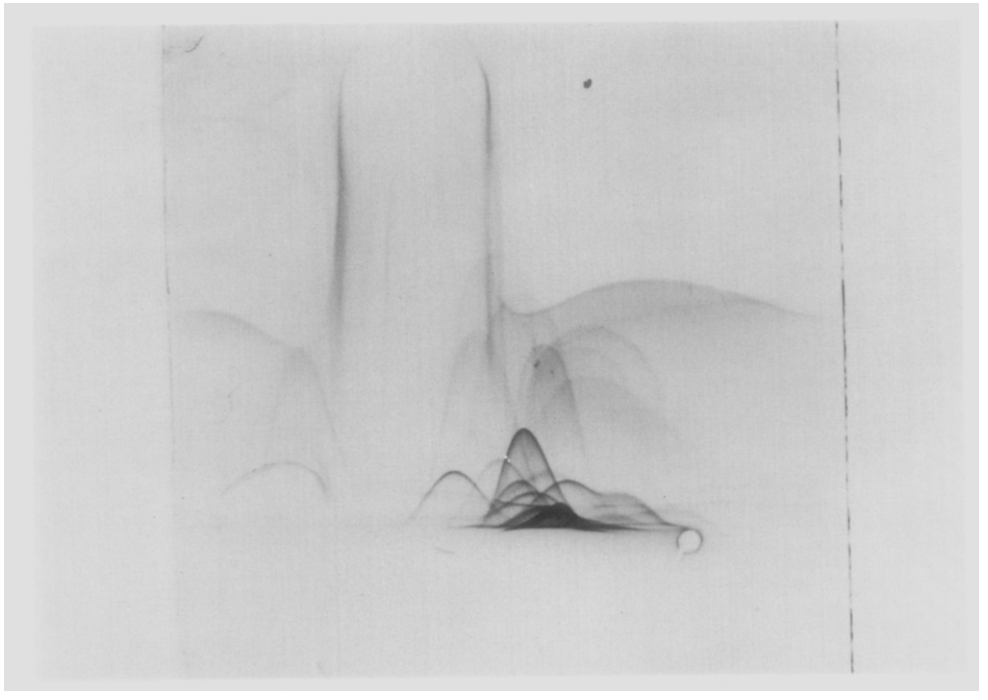

Figure 2. Crossed immunoelectrophoresis of 2ul of human serum. First dimension: 70 minutes, 10V per cm, 4°C in 1% agarose (HGT[P]agarose) in barbital buffer pH 8.6, ionic strength 0.02. Antibodies: Rabbit antihuman serum (120ul) corresponding to 12.5ul per cm^2. Second dimension: Overnight, 2V per cm, 4°C. The anode is at the left and top.

1.25-2.5 µg of antigen, the sensitivity of the triple stain is 0.31 µg - 0.62 µg. Resolution of precipitin bands at the higher antigen concentrations is similar when the two stains are compared, however, at lower levels of antigen concentration, the triple stained precipitin bands are less diffuse and more intensely stained. When cadium is substituted for the mercury mordant, the staining intensity decreases (Figure 1). Clearly, the use of a mercury mordant increases the sensitivity of the stain.

In contrast to previous reports (3,6), staining after an initial drying of the gel leads to an increased sensitivity. It seems reasonable to suppose that a thinner gel matrix will allow the mordant and stain more complete access to proteins precipitated within the gel.

When ABB or CBB containing triple stains are compared (Figure 1), the latter is more sensitive. Figure 2 demonstrates that the triple stain can also be successfully used on crossed-antigen antibody gels. It is also apparent that the washing and drying procedure can be transfered to immunoelectrophoretic gels.

The advantages of this stain are the simplicity and rapidity of the procedure, the long shelf-life of the single staining solution, and its increased sensitivity. Incorporation of both red and green stains increases photographic resolution of precipitin bands on conventional photographic material.

Finally, extreme care should be exercised whenever this stain is used to prevent mercury poisoning and to prevent mercury from entering the ecosystem.

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