Rheophoresis: A Sensitive Immunodiffusion Method for Detection of Hepatitis Associated Antigen (36503)

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At the present time, the methods in most common use for detection of hepatitis-associated antigen (HAA) are agar gel diffusion (AGD) (1–3), counterelectrophoresis (CEP) (4), and complement fixation (CF) (5, 6) tests. Whereas the CF test is usually considered to be the most sensitive procedure of the group (5–8), the test is expensive and difficult to perform, and requires 6 to 18 hr to complete. The CEP procedure is rapid (generally requiring 1 to 3 hr/test) and approaches the CF test in sensitivity (7–10). Specificity of a reaction in either the CF or CEP procedures must be determined in AGD. Since the AGD system is less sensitive than the other systems, the sample frequently must be concentrated before testing by the AGD method (3).

Van Oss and Bronson (11) described an immunodiffusion system termed rheophoresis (Rheo) in which a threefold concentration of proteins was demonstrated over their routine immunodiffusion system.

In this report, we describe a rheophoresis system that has been adapted for HAA testing which couples the simplicity and ease of use of the AGD system with the sensitivity of the CEP and CF procedures. This increased sensitivity was obtained by use of a specially prepared agar diffusion cup surrounded by a circular peripheral moat (Fig. 1). For testing, the moat was filled with buffer and the entire plate was covered by a tightly fitting piece of plastic containing a hole placed directly over the antibody well. Dehydration from the central area produced hydrodynamic forces resulting in concentration of proteins toward the central area of the plate.

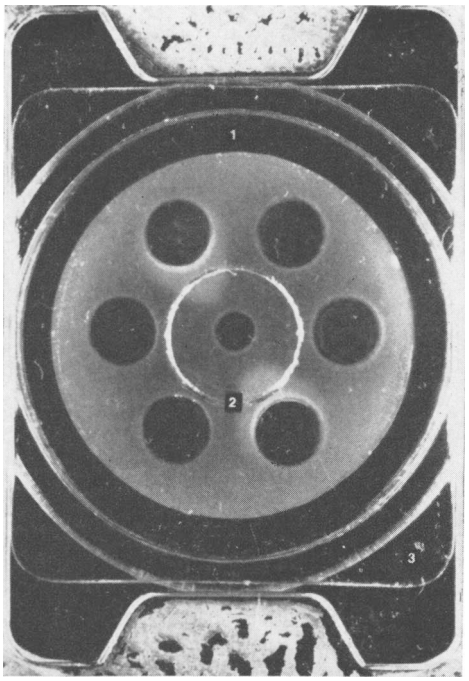
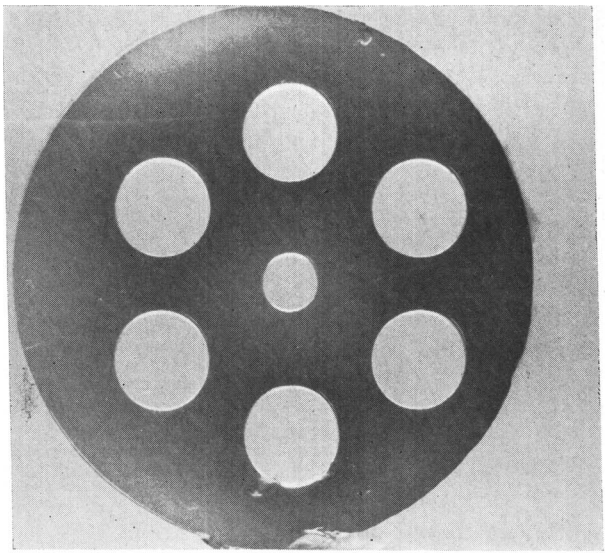
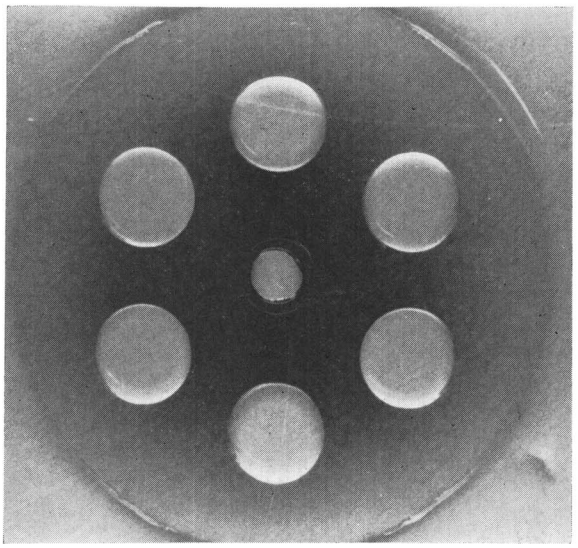


FIG. 1. Rheophoresis plate. Central antibody well is surrounded with six wells for positive controls and sample. Moat (1) is filled with buffer. The central hole (2) of the plastic cover (3) is directly over the antibody well.

Materials and Methods. Rheophoresis plates. A molded plastic cup (3 cm in diam and approx 6 mm deep) was fitted with a cylindrical Teflon ring (3 cm o.d., 2.5 cm i.d., and 0.55 cm length). Melted agarose (1.25 ml, 0.8%), adjusted to pH 7.6, was poured into the cups. After the agarose had solidified, a pattern of six peripheral wells (5 mm in diam) and a center well (3 mm in diam) was cut with a center to center distance of 7 mm. For performance of the test, the Teflon ring was carefully lifted out of the cup, leav-





FIGS. 2 and 3. AGD plate (2) and Rheo plate (3) in which wells were filled with protein solution and incubated 24 hr at 28°.

ing a circular moat around the periphery of the cup. The moat was filled with 0.01 *M* Tris buffer, pH 7.6. Two opposing peripheral wells were filled with positive control, and the remaining peripheral wells were filled with unknown samples. The center well was filled with HAA antiserum. [Hepatitis-associated antibody—guinea pig anti-Australia antigen (AUS-tect) was used throughout these studies.] One minute after addition of HAA antibody, the central well was covered with a 3 mm plastic cover (to prevent evaporation). A plastic cover (approx 3 cm^2) with a central hole (0.8 cm in diam) was placed over the entire plate with the center of the plastic cover located directly over the antibody well. The plate was incubated at 28–32° and read after 16–24 hr, or at 35–37° and read after 8–16 hr incubation.

Complement fixation. The method of Sever (12) was used with 1.7 exact units of complement, 4 units of antigen, 4 units of anti-

body, and overnight fixation. Concentration of plasma for testing was done by methods recommended by Dr. Peters (USC and John Wesley Clinic, Los Angeles, CA) using Lyphogel (Gelman Instrument Company, Ann Arbor, MI) and achieving a final concentration of approximately fivefold.

Counterelectrophoresis. CEP was done by the methods described by Gocke and Howe (4), and modified as follows: Double columns of wells of two different sizes were cut in agarose. The larger size wells (3.0 mm) were for antigen, and the smaller wells (2.5 mm) were for antisera (near anode). The voltage across the plate was regulated to a level of 5.0 to 6.0 V/cm. Plates were read at 30- and 60-min intervals.

Agar gel diffusion. AGD was performed in plates supplied by Abbott Scientific Products Division which contain an agarose gel cut with a center well (3.0 mm in diam), and six outside wells (5.0 mm in diam). The distance from the middle of the center well to the middle of the peripheral wells is 8 mm. For Australia antigen testing, the center well was filled with guinea pig antibody (10 to 15 μl/well), and the outside wells were filled with antigen (35 to 40 μl/well). Positive and negative antigen controls were included in each test.

Antiserum. Antiserum was prepared in guinea pigs by injection of selected Australia-positive human plasma (AUS-tect guinea pig anti-Australia antigen—Abbott Scientific Products Division). The antigen was concentrated and purified by a process similar to that employed by Gerin *et al.* (13). The guinea pig anti-Australia antiserum gave lines of identity to Australia-1 antiserum supplied by Dr. Baruch Blumberg (The Institute for Cancer Research. Philadelphia, PA), and was found to react with a panel of antigens in tests performed in Dr. Blumberg's laboratory.

Experimental and Results. The peripheral wells of both AGD and rheophoresis plates were filled with plasma preparations, incubated overnight, and stained with Ponceau S. In the AGD system (Fig. 2), the proteins were spread evenly over the plate, as contrasted to the rheophoresis plate (Fig. 3) in which the proteins were concentrated in the central area of the plate.

Several experiments were carried out to compare the sensitivity of the rheophoresis procedure for detection of HAA with that of other methods in current use.

Titrations of HAA positive plasma with rheophoresis and other HAA test methods. Two HAA positive plasma samples were serially diluted and tested by Rheo, CF, CEP, and AGD methods to determine the maximum dilution in which the samples gave a positive reaction (Table I).

Both HAA-positive plasma samples gave higher titration endpoints by the CF procedure than was found with the other methods. CEP and Rheo gave comparable titers, whereas AGD titers were four to eightfold lower than those seen with CEP, Rheo, and CF methods.

Sensitivity of Rheo method for screening blood donors. The sensitivity of the Rheo method for detection of HAA was compared with CEP and AGD in 1000 randomly selected donors from a commercial blood bank. In the original screen, seven positive samples were detected by Rheo, four by CEP, and one by AGD. Careful retesting of the seven positive samples were confirmed by Rheo and, in addition, were positive by CEP. This observation lends support to the recommendations of Schmidt, Gee, and Lennette (8) for inclusion of positive controls, since if a weak positive reaction is present in AGD or Rheo, it will be readily recognized by the turning of the line, whereas in the CEP system, the operator must rely on only the observation of a visible line.

Comparative sensitivity of various methods for detection with several panels of HAA

TABLE I. Titrations of HAA-Positive Plasma by Rheo, CF, CEP, and AGD Methods.

	Test method			
Test sample	Rheo	CF	CEP	AGD
L23812	128[a]	512	128	32
Y700326	32	128	32	4

[a] Reciprocal of maximum dilution that gave positive reaction.

TABLE II (Panel A). HAA-Positive Blood Donors Comparison of Sensitivity of Various Test Methods.

Test method	No. positive/no. tested (%)
CF	87/90 (97)
CEP	86/90 (96)
AGD	80/90 (89)
Rheophoresis	90/90 (100)

plasma. Comparative sensitivity of the Rheo procedure to that of CF, CEP, and AGD was determined by testing against several panels of HAA-positive plasma.

Panel A was composed of 90 samples from a commercial blood bank operation. Panel B was a group selected from samples submitted to a clinical laboratory for HAA testing, Panel C was a randomly selected panel, and Panel D was obtained from the Center for Disease Control (courtesy of Dr. Robert Kissling). In Panel A (Table II), rheophoresis detected antigen in all samples (90/90), compared to 87/90 by CF, 86/90 by CEP, and only 80/90 by AGD. With Panel B (Table III), composed of plasma samples from cases of suspected hepatitis, the CEP test detected 62/72 HAA-positive specimens, in contrast to 57/72 by AGD, and 61/72 by Rheo. In Panel C (Table IV), composed of samples collected from many sources, rheophoresis detected 67/79 (85%) compared to 68% by CEP, and only 43% by AGD. Interpretation of the sensitivity of complement fixation tests was questionable since 23 samples were anticomplementary (AC). All 23 samples were negative at dilutions above the AC level. Of the 56 samples that were not AC, 47 were positive and 9 were negative. Table V summarizes data on a panel obtained from the Center for Disease Control. The CF test

TABLE III (Panel B). Cases of Clinical Hepatitis Comparison of Sensitivity of Various Test Methods.

Test method	No. positive/no. tested (%)
CEP	62[a]/72 (86)
AGD	57/72 (79)
Rheophoresis	61/72 (85)

[a] One specimen could not be confirmed by AGD or rheophoresis.

TABLE IV (Panel C). Comparative Assays for HAA on a Selected Group of Specimens.

Test method	No. positive/no. tested (%)
CF	47[a]/79 (59)
CEP	54/79 (68)
AGD	34/79 (43)
Rheophoresis	67/79 (85)

[a] Twenty-three samples were anticomplementary at 1/8 while 9 samples were negative.

detected 64/100 samples, compared to 63/100 by CEP, 56/100 by AGD, and 70/100 by the Rheo procedure.

Discussion. The greater sensitivity of the CEP system (greater than that of the AGD method) for detection of HAA is thought to be due to the concentration of antigen and antibody into a narrow zone, resulting in a greater opportunity for antigen and antibody molecules to react.

In agar gel diffusion, proteins diffuse in all directions from the point of origin, while only those molecules that interact can be expected to participate in formation of a precipitate.

In the rheophoresis system, protein solutions can be forced to migrate toward areas of dehydration by flow of water from a moat toward a dehydrated area. In these studies, we achieved these conditions by cutting a circular moat around the periphery of a circular agar plate and filling the moat with buffer. The center area of the agar plate was left uncovered, permitting increased drying from that area of the plate.

Results of studies with this system indicate that the rheophoresis procedure combines the sensitivity of the CEP and CF tests with the simplicity of the AGD system. The question of nonspecific readings is minimized since an identity test can be performed in

TABLE V (Panel D). Comparative HAA Assays on Center for Disease Control Panel.

Test method	No. positive/no. tested (%)
CF	64/100 (64)
CEP	63/100 (63)
AGD	56/100 (56)
Rheophoresis	70/100 (70)

each rheophoresis plate. In addition, results of these preliminary studies in blood donor screening indicate that the rheophoresis procedure may detect a number of weakly positive samples that would be missed in a large scale blood bank testing program using the CEP system.

Summary. An immunodiffusion system for detection of HAA that is termed rheophoresis is described. In this system, samples containing the hepatitis-associated antigen are forced to migrate toward the antibody section of the plate. This concentration effects an increased sensitivity for detection of HAA. Comparative tests with CF, CEP, and AGD indicate that rheophoresis has sensitivity similar to that of CF and CEP, and is more sensitive than the AGD system. In addition, the Rheo test is simple to perform and identity can be readily established.

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Received Nov. 15, 1971. P.S.E.B.M., 1972, Vol, 140.