Radioimmunoprecipitation Assay for Australia Antigen, Antibody, and Antigen–Antibody Complexes[1] (35872)

JOHN A. COLLER,[2] IRVING MILLMAN, THERESA C. HALBHERR, AND BARUCH S. BLUMBERG

The Institute for Cancer Research, Fox Chase, Philadelphia, Pennsylvania 19111

The association of Australia antigen, Au(1), with viral hepatitis is well established (1–5). The immunodiffusion (ID) (Ouchterlony) method is widely used (6) and permits specificity control by means of lines of identity with known specimens; however, relatively large amounts of antigen and antibody are required to form a visible precipitin band. "Cross over" or "reaction" electrophoresis described by Lang (7) and used by Krassnitsky *et al.* (8) and Pesendorfer *et al.* (9) for the detection of Au(1) is also contingent upon a visible precipitin reaction. Although more rapid, it is only slightly more sensitive than immunodiffusion, and the advantage gained by the rapidity is compromised by the inability to maintain a specificity comparison. Complement fixation (CF) for the detection of Au(1) first described by Purcell *et al.* (10) is a sensitive test but it also lacks the built in specificity comparison of immunodiffusion since any antigen–antibody combination will permit the fixation of complement.

Although 60–90% of patients developing posttransfusion hepatitis have detectable Au(1) at some time during the course of their illness, only 20–30% of them have received blood from Au(1) positive donors as determined by immunodiffusion or complement fixation. The majority of the patients have therefore contracted hepatitis either from a source other than blood transfusion or from Au(1) negative donors. If transmission has in fact been from Au(1) negative donors, then the inability to detect Australia antigen in these blood samples may be a result of the insufficient sensitivity of the current methods.

Our current investigation, an extension of an earlier report (11)[3] was directed toward the development of a highly sensitive detection method for Au(1) and human antibody to Au(1) without loss of immunologic specificity. Specificity is preserved through the use of purified Au(1) as a constant reactant. Whereas many radioimmunossay systems precipitate immune complexes either as a double antibody complex or by gammaglobulin precipitation with 50% saturated ammonium sulfate the procedure described below combines both methods to accomplish specific immunoprecipitation of double antibody–antigen complexes with a less concentrated ammonium sulfate solution.

Materials and Methods. Purification of Au(1). Susceptible normal protein components of an Au(1)-containing plasma specimen were degraded by enzyme treatment (13). Au(1) was then purified by sequential Sephadex G-200 column gel filtration, sucrose and cesium chloride gradient centrifugation. That fraction which displayed immunoreactivity with human and heterologous anti-Au(1) antisera, but not with antinormal human sera antisera, was used as the antigen

[1] This work was supported by U.S. Public Health Service Grant 5TO1-GM01540-05; by U.S. Public Health Service Grants CA-06551, CA-08069, CA-06927, and RR-05539 from the National Institutes of Health; by an appropriation from the Commonwealth of Pennsylvania and by contract No. NIH 70-2334.

[2] Department of Surgery, Hospital University of Pennsylvania.

[3] At the time of our first disclosure (11), Walsh *et al.* (12) described a radioimmunoassay for the detection of Australia antigen. Their method differed from the one reported here in the manner of determining bound and unbound radioactive precipitate.

source in these experiments. The details of the purification procedure have been described elsewhere (13).

Purified Au(1) conjugation with ^{125}I. Conjugation of purified Au(1) with ^{125}I-iodide was accomplished with chloramine-T using a modification of the method of Greenwood *et al.* (14). The reactants, in small volumes, consisted of 100 μg of purified Au(1), 1 mCi of ^{125}I, 100 μg of Chloramine-T, 244 μg of sodium metabisulfite. Following removal of unconjugated ^{125}I-iodide by both gel filtration and dialysis, 90–95% of the remaining radioactivity was precipitated in 5% trichloroacetic acid. Seventy to 92% of the protein-associated radioactivity was precipitable by human anti-Au(1) in the method described below.

Immunoprecipitation of ^{125}I-labeled Au(1). Human antibody to Au(1) was obtained from a blook-bank employee who had never had recognized clinical or chemical hepatitis but had persistent anti-Au(1) antibody activity as determined by use of immunodiffusion. The fraction of this serum that was precipitated in 50% saturated ammonium sulfate was equilibrated against a 0.1 *M* borate–0.85% NaCl, pH 8.4 buffer without concentration and designated the standard antibody preparation. This preparation has been used extensively for the routine clinical immunodiffusion detection of Australia antigen in our laboratory and is immunoreactive in titers up to 1:8. Immunoprecipitation of ^{125}I-labeled Au(1) with this preparation was performed in the following manner:

Twofold serial dilutions of the standard antibody from 1:40 to 1:1,280,000 were made using 0.1 *M* borate–0.85% NaCl buffer with 0.04% bovine serum albumin (BSA), pH 8.4. Five hundred microliters of each dilution was incubated with 500 μl of ^{125}I-labeled Au(1) (approx 15,000 cpm on the day of labeling), at 37° for 2 hr and 4° for 22 hr. Thereafter, 50 μl of rabbit antihuman IgG (H-chain specific) was added, followed by incubation at 37° for 2 hr and 4° for 18 hr. Centrifugation of immune complexes was performed at either 8000 rpm for 1 hr or at 5000 rpm for 30 min after the addition of sufficient 60.9% saturated ammonium sulfate to provide a final saturation of 25%.

The qualitative detection of anti-Au(1) in an unknown human serum was accomplished by substituting for the standard antibody preparation a 10-μl volume of undiluted serum. Titers of positive specimens were determined using 500-μl dilutions in the same manner as described for the standard antibody preparation.

The detection of Au(1) in human sera by coprecipitation-inhibition is based on the capacity of added unlabeled Au(1) to compete with labeled Au(1) for a predetermined and constant amount of the standard anti-Au(1) preparation and thereby decrease the amount of precipitable radioactivity. In this procedure, 10 μl of a 1:10 or greater dilution of the test specimen is incubated with 500 μl of the standard antibody preparation (generally a 1:20,000 dilution) at 37° for 2 hr and 4° for 22 hr prior to incubation with the ^{125}I-labeled Au(1) and rabbit antihuman IgG and separation with 25% saturated ammonium sulfate. All dilutions are made in 0.1 *M* borate–0.85% NaCl buffer with 0.04% BSA, pH 8.4.

Comparisons were made of sera which were Au(1) positive by immunodiffusion; Au(1) negative but containing antibody to Au(1) by immunodiffusion; and sera found by use of immunodiffusion to contain neither antigen nor antibody. The latter specimens were from first year medical students with no known hepatic dysfunction as determined by historical, clinical, and chemical investigations. In each case, these specimens did not react with a panel of 20–30 Au(1) and anti-Au(1) sera.

Results. Standard anti-Au(1) antibody preparation. The precipitin curve of increasing dilutions of the standard antibody preparation with constant ^{125}I-labeled Au(1) and rabbit antihuman IgG is shown in Fig. 1. There is an area of equivalence between 1:10,000 and 1:20,000 dilution of standard antibody. A nearly identical curve is obtained when whole serum dilutions of standard antibody are used in place of the 50% saturated ammonium sulfate precipitable fraction of standard antibody.

In preliminary investigations, the human

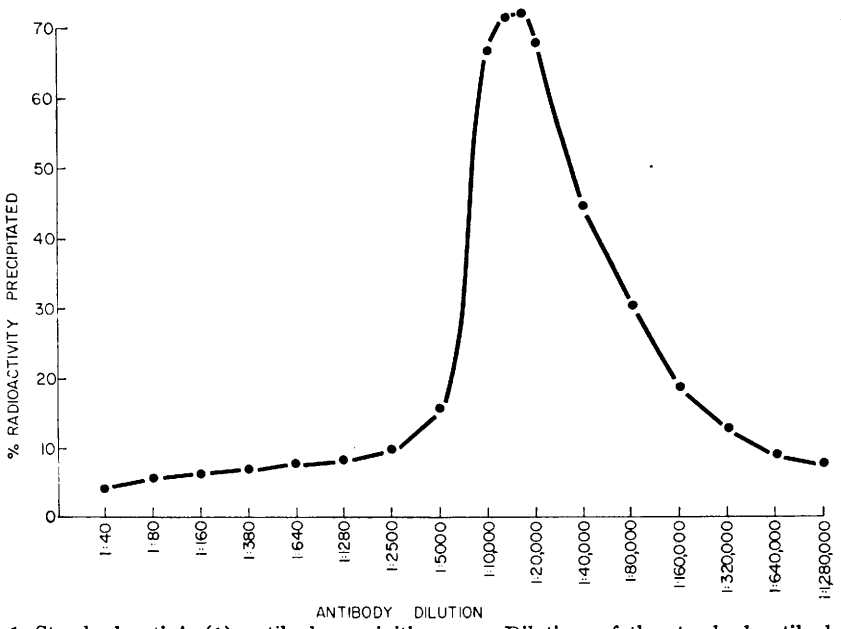

FIG. 1. Standard anti-Au(1) antibody precipitin curve: Dilutions of the standard antibody were incubated with ^{125}I-labeled Au(1), rabbit antihuman IgG and centrifuged at 8000 rpm for 1 hr. The percentage of precipitable radioactivity is plotted.

anti-Au(1) antibody by itself did not form an immune complex with labeled Au(1) that could be separated from free unbound labeled Au(1) by centrifugation. Concentrations of ammonium sulfate that efficiently precipitate gamma globulin and thus any bound labeled antigens were also unsatisfactory in delineating immune complexes. We found that the bulk of free Au(1) is also precipitated at such concentrations. Therefore, a method was developed to detect the immune complexes in the area of antibody excess by salt precipitation of double antibody complexes. A concentration of ammonium sulfate was used that was inadequate to precipitate the individual components but that could quantitatively precipitate the intact double antibody complex. A final concentration of 25% saturated ammonium sulfate followed by centrifugation resulted in the selective precipitation of the double antibody-Au(1) complex.

Following the addition of ammonium sulfate to provide a final saturation of 25%, maximal precipitable radioactivity equal to, but not exceeding, that observed at equivalence is obtained at the dilutions corresponding to anti-Au(1) excess (Fig. 2).

Similar dilutions of normal human sera used in place of the standard antibody preparation failed to precipitate more than 3.5% of the total radioactivity at any dilution. At a standard antibody dilution of 1:640,000 the precipitable radioactivity of the standard antibody dilution exceeds that of the normal sera by 3 SD. There was no significant precipitable radioactivity in the absence of rabbit antihuman IgG.

Detection of anti-Au(1) antibody activity in human sera. Serial dilutions of whole serum with known anti-Au(1) and Au(1) activity as determined by immunodiffusion were made. After sequential incubation with labeled Au(1) and rabbit antihuman IgG, centrifugation was performed at 5000 rpm following the addition of ammonium sulfate. A comparison of titers as determined with immunodiffusion, complement fixation, and radioimmunoprecipitation (RIP) is given in Table I. Antibody was detected by radioimmunoprecipitation in three of five specimens which contained only Au(1) by immunodiffusion or complement fixation; in one there was a moderately high titer (1:30,000).

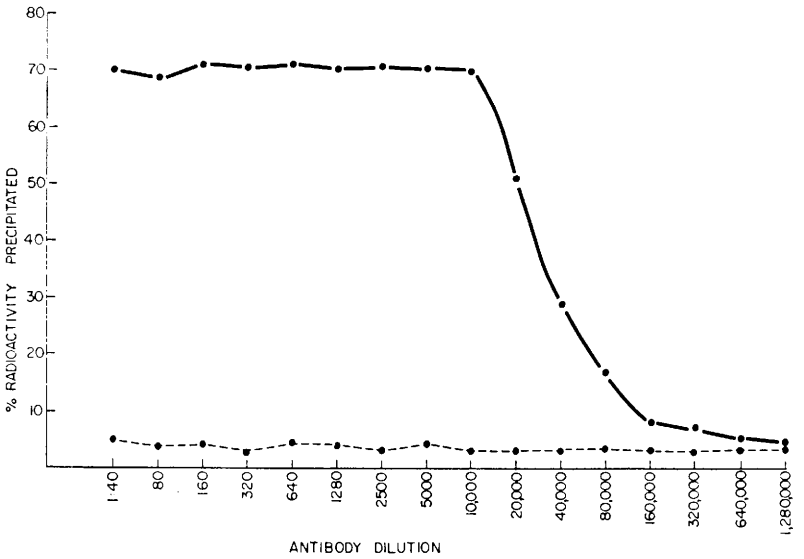

FIG. 2. Ammonium sulfate precipitation of double antibody complexes: Dilutions of the standard antibody were incubated with ^{125}I-labeled Au(1), and rabbit antihuman IgG. Ammonium sulfate to a final saturation of 25% was added before centrifugation at 5000 rpm for 30 min. The percentage of precipitable radioactivity for the standard antibody (●—); and for normal human sera (● - -) is plotted.

Detection and quantitation of purified Au(1) by coprecipitation inhibition. In Fig. 3 is shown the inhibition of precipitable radioactivity as a function of added unlabeled purified Au(1). Ten-microliter dilutions of purified Au(1) are incubated with 1:20,000

TABLE I. Anti-Au(1) Antibody in Human Sera: Comparison of Immunodiffusion, Complement Fixation, and Radioimmunoprecipitation as Assay Methods.[a]

Specimen	Titer		
	ID	CF	RIP
Au(1) negative[b]			
66993	1:8	1:8	1:640,000
41177	1:16	1:32	1:160,000
66821	1:4	1:8	1:160,000
66820	1:8	1:16	1:320,000
67003	1:8	—	1:40,000
Au(1) positive[c]			
66707	—	—	—
45417	—	—	1:50
66751	—	—	1:200
42467	—	—	—
66835	—	AC	1:30,000

[a] ID = immunodiffusion; CF = complement fixation; RIP = radioimmunoprecipitation; AC = anticomplementary.

[b] 66993, blood bank nurse, Hospital University of Pennsylvania; 41177, aplastic anemia, female; 66821, hemophiliac, male; 66820, hemophiliac, male; 67003, Cooley's anemia, female.

[c] 66707, hemodialysis for renal failure, male; 45417, Down's syndrome, male; 66751, infectious viral hepatitis, female; 42467, serum hepatitis, male; 66835, hepatitis of unknown origin, female.

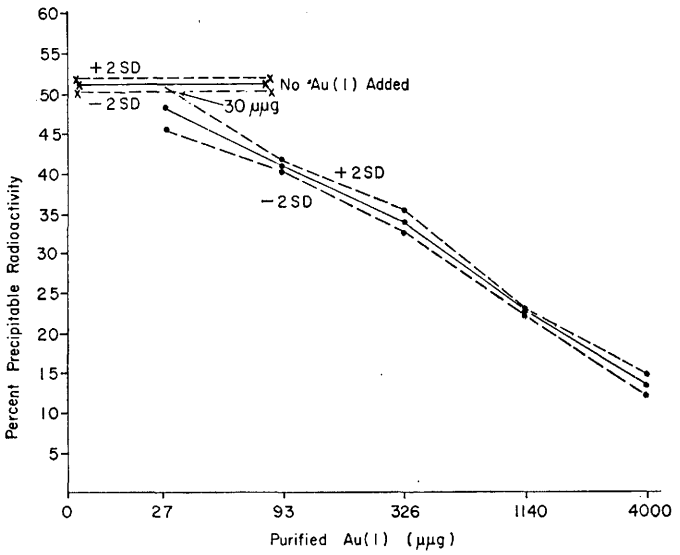

FIG. 3. Inhibition of precipitable radioactivity as a function of added unlabeled purified Au(1): A 1:20,000 dilution of standard antibody was sequentially incubated with unlabeled purified Au(1), ^{125}I-labeled Au(1), and rabbit antihuman IgG, followed by ammonium sulfate separation.

standard antibody prior to incubation with labeled Au(1), rabbit antihuman IgG and separation with ammonium sulfate. In the absence of added unlabeled Au(1), the 1:20,000 antibody dilution precipitates approximately 50% of the immunologically reactive radioactivity. Thirty picograms (micromicrograms) of purified Au(1) caused a significant inhibition of precipitable radioactivity.

Detection and quantitation of Au(1) in human sera by coprecipitation inhibition. The detection of Au(1) in human sera, unlike the inhibition obtained with purified unlabeled Au(1) may be complicated by the presence of other serum components. Principally, this includes the possible coexistent presence of anti-Au(1) antibody and the competition for rabbit antihuman IgG by varying amounts of nonspecific gammaglobulin.

For the most part, nonspecific interference due to components of the test specimen other than Au(1) or anti-Au(1) can be eliminated by the selection of an appropriate serum dilution. In the absence of added normal human serum a 1:20,000 dilution of standard antibody preparation precipitates approximately 50% of the ^{125}I-labeled Au(1). If this standard antibody dilution is preincubated with 10 μl of a 1:10 dilution of normal human sera prior to the addition of ^{125}I-labeled Au(1), the mean precipitable activity is 44.1% ± 7.8 (1 SD). Greater dilutions result in a much smaller deviation among normal sera, *e.g.*, 1:20 dilution permits recovery of 54.1% ± 1.9 (1 SD) precipitable activity.

In Fig. 4, the inhibiting capacity of 5 sera which contain Au(1) according to immunodiffusion is compared with that of 5 normal sera. The mean precipitable activity ± 2 standard deviations for each 10-μl dilution of the normal specimens is indicated. The titer of Au(1) in each specimen is the greatest serum dilution that results in precipitable activity of more than 2 SD below the corresponding dilution of normal serum.

The quantity of serum Au(1) (μg of protein/ml of serum) is determined by comparing, under identical conditions, the degree of inhibition of an appropriate dilution of the test specimen with that of purified Au(1).

Immunodiffusion and complement fixation testing is compared in Table II with the antigen titer and Au(1) protein concentration as determined by radioimmunoprecipitation for the same Au(1) positive specimens that were tested for antibody (Table I).

Detection of anti-Au(1) antibody by co-

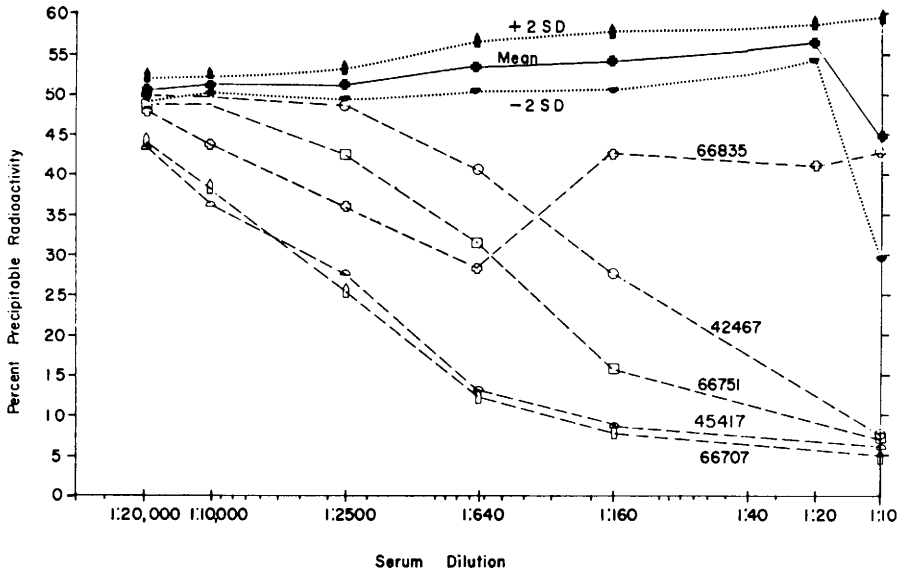

FIG. 4. Detection of Au(1) in human sera by coprecipitation-inhibition: Dilutions of 10-μl volumes of either normal human sera (—); or immunodiffusion Au(1) positive specimens (- - -); were incubated sequentially with the standard antibody (1:20,000), ^{125}I-labeled Au(1), rabbit antihuman IgG and separated with ammonium sulfate.

precipitation enhancement. The method of antigen detection employs a concentration of standard antibody that is sufficient to precipitate about one half of the ^{125}I-labeled Au(1). If the test specimen contains antibody rather than antigen, the total amount of antibody in the system will be increased and the final percentage of precipitable ^{125}I-labeled Au(1) will exceed the 50% control level. With increasing dilutions of the test specimen, less antibody is added and the precipitable activity will decrease to the 50% level.

We refer to this method of detecting predominantly antibody activity in a specimen being tested for antigen as "coprecipitation enhancement."

Delineation of antigen–antibody complex in human serum. 1. Antigen excess. Antigen testing of specimen 66835 at the initial 10-μl dilution of 1:10 (Fig. 4) fails to demonstrate Au(1) by inhibition. As greater dilutions are examined, however, inhibition is clearly seen, increasing first to a maximum before decreasing to a nondetectable level. That this nonlinear inhibition curve is related to the previously detected antibody activity is shown by comparison of the amount of test

TABLE II. Comparison of Immunodiffusion, Complement Fixation, and Radioimmunoprecipitation in the Detection of Au(1) in Human Sera.

Specimen[a]	Titer		RIP	
	ID	CF	Titer	Protein (μg/ml)
66707	1:16	1:128	1:30,000	174
45417	1:32	1:256	1:26,000	(149)[b]
66751	1:4	1:32	1:5000	(26)[b]
42467	1:8	1:16	1:2000	10
66835	1:8	1:256	1:15,000	(60)[b]

[a] See Table I.

[b] Antigen–antibody complex, see Table I.

specimen added relative to the constant amount of standard antibody used. Ten-microliter dilutions greater than 1:600 of No. 66835 do not contribute appreciable antibody in terms of the 1:20,000 standard antibody and the degree of inhibition is predominantly a function of antigen activity. On the other hand, even though the test specimen contains a large quantity of antigen, dilutions less than 1:600 result in the addition of significant amounts of antibody of test specimen origin thereby increasing the amount of antibody available for subsequent complexing of labeled antigen. Although the actual influence of the additional antibody on inhibition in this region of greater concentration is determined by several factors, *e.g.*, antigen–antibody ratio in the test specimens, antibody affinity, dissociability, etc., the net effect is usually less inhibition than expected for the amount of antigen. Significant inhibition may not be discernible unless the specimen is tested in dilution.

2. *Antibody excess.* In most circumstances, 10 μl (1:10 dilution) of human serum with ID detectable antibody will, when added to the standard antibody (1:20,000), precipitate 95–100% of the immunologically reactive ^{125}I-labeled Au(1) that is subsequently added. A major exception to obtaining full precipitation occurs when coexistent antigen is present in the test specimen. In this situation, a relatively constant level of precipitation, short of full precipitation, may occur for several dilutions before the progressive decrease of the 50% level begins.

While it is evident that an unknown specimen may be evaluated in a single test by observing coprecipitation inhibition when antigen is present or coprecipitation enhancement when antibody is present, this technical simplification should not be relied upon to provide complete characterization of the specimen because both antigen and antibody may frequently be present in the same specimen. If complexes of antigen and antibody are present, only the predominant activity will be manifested at any single dilution; and, if the components of these complexes are at equivalence (equivalent amounts of antigen and antibody), detection of either antigen or antibody may be missed unless tested further.

We have therefore found it necessary to test each specimen for antibody directly (method in Sect. 2 of Results) as well as for antigen by inhibition. If antibody is present in the direct test and coprecipitation enhancement is not observed when testing for antigen then it is probable that there is coexistent antigen that will become evident only when antigen is tested for in dilution.

Discussion. In view of the established association of Australia antigen with viral hepatitis it is apparent that a highly sensitive and specific method for its detection would have both pragmatic and experimental utility. In addition to the obvious advantage of lowering the threshold level of detection, such an assay might provide the means for characterizing the nature of the Au(1) association with different "types" of viral hepatitis as well as with other disease states in which it has been implicated. (i) Whereas Australia antigen is frequent in parenterally derived viral hepatitis it is usually absent in most point-source epidemics of hepatitis. Recently Del Prete *et al.* (15) reported the occurrence of an unstable antigen in the blood of patients involved in hepatitis epidemics. The antigen is detected with an antiserum which has antibody to Au(1) and the precipitin reaction shows a line of partial identity with Au(1). In view of conflicting data and impressions concerning the similarity of the agents responsible for serum and infectious hepatitis, it is important to determine whether epidemics are associated with Australia antigen. (ii) A qualitative association of Au(1) with Down's syndrome, leukemia, lepromatous leprosy, chronic renal disease, and certain population groups has been established (16) but the direct quantitative relationships of Au(1) with these entities as well as with viral hepatitis have not been investigated. (iii) The current methods are particularly insensitive with respect to the detection of antibody. Previous investgations have failed to demonstrate an antibody incidence or titer in convalescent sera or among large populations that approaches that which might be anticipated in response to a viral infec-

tious agent.

In general, immunodiffusion and complement fixation procedures do not detect antigen–antibody complexes. Such complexes, at equivalence, may remain precipitated and undetected in an immunodiffusion plate well or cause anticomplementary activity by complement fixation test. Shulman and Barker (17) observed a very high incidence of anticomplementary activity among sera from patients with hepatitis during the incubation stage and early course of the disease. Millman *et al.* (18) have shown that the supernatant fraction following ultracentrifugal pelleting of Au(1) derived from plasma, may possess antibody activity capable of reacting with the pellet. Presumably, centrifugation permitted sedimentation of the heavier Au(1) from free antibody after promoting the separation of an antigen–antibody complex.

The degree of sensitivity of the radioimmunoprecipitation assay method for the detection of human antibody to Au(1) and antigen–antibody complexes compared with immunodiffusion and complement fixation has been illustrated (Table I). Although undiluted specimens 66993, 41177, 66820 produce strong precipitin bands for antibody, reactivity is lost above dilutions of 1:8 to 1:32. In contrast radioimmunoprecipitation assay evidence of antibody activity persists up to dilutions of 1:160,000–1:640,000. Antibody titers do not always correlate. The antibody titer of 66993 is four times higher than 41177 when tested by RIP but only ¼ when tested by CF. The reason for this discrepancy may be due to differences in the specificities of antibodies used in each test. Specimen 66993 from a blood bank nurse contained an unusually high RIP antibody titer. Work is currently in progress to determine whether this is a common situation among these nurses. Three of the five Au(1) positive specimens (45417, 66751, 66835) demonstrate concurrent antibody activity only when tested by radioimmunoprecipitation assay. Specimen 66835, though not having detectable antibody titer by immunodiffusion, has a 1:30,000 radioimmunoprecipitation assay titer.

The degree of sensitivity of radioimmunoprecipitation assay for the detection of Australia antigen compared with immunodiffusion and complement fixation has been illustrated (Table II). Among the specimens used as little as a 1:30,000 dilution of 10 μl of serum contains sufficient Australia antigen to cause coprecipitation inhibition. In comparison of equal volumes of Au(1) containing sera, detection by radioimmunoprecipitation assay is possible at dilutions that are 1000 times greater than that which is detectable by immunodiffusion. The concentration of Au(1) (μg/protein/ml) may be determined by relating the degree of inhibition of purified Au(1) to that of an appropriate dilution of serum.

The radioimmunoprecipitation assay has been found to be not only highly sensitive, but also reliable and reproducible. When Au(1) is present *in vivo* as an immune complex the radioimmunoprecipitation assay can be relied upon to detect both components of such a complex. This ability to detect Australia antigen and antibody in extremely small quantities whether or not they exist as complexes may prevent the transmission of hepatitis by the transfusion of sera that are negative by other methods of assay.

Summary. Purified Australia antigen [Au(1)], conjugated with ^{125}I, was reacted with and quantitatively precipitated by human anti-Au(1) in the presence of heterologous antihuman IgG and "dilute" ammonium sulfate. The sensitivity of the devised radioimmunoprecipitation assay procedure proved to be up to 1000 times more sensitive for the detection of antigen and up to 20,000 times more sensitive for the detection of antibody when compared with either immunodiffusion or complement fixation assays. This method is extremely valuable not only in the quantitative detection of antigen and antibody but also in the delineation of naturally occurring antigen–antibody complexes.

The authors thank Dr. Scott Mazzur who performed the complement fixation assays; and acknowledge the generous assistance of Drs. Alton Sutnick and W. Thomas London.

1. Blumberg, B. S., Gerstley, B. J. S., Hungerford, D. A., London, W. T., and Sutnick, A. I., Ann.

Intern. Med. **66**, 924 (1967).

2. Sutnick, A. I., London, W. T., Gerstley, B. J. S., Cronlund, M. M., and Blumberg, B. S., J. Amer. Med. Ass. **205**, 670 (1968).

3. Sutnick, A. I., London, W. T., and Blumberg, B. S., Amer. J. Dig. Dis. **14**, 189 (1969).

4. Blumberg, B. S., Sutnick, A. I., and London, W. T., J. Amer. Med. Ass. **207**, 1895 (1969).

5. London, W. T., Sutnick, A. I., and Blumberg, B. S., Ann. Intern. Med. **70**, 55 (1969).

6. Blumberg, B. S., and Riddell, N. M., J. Clin. Invest. **42**, 867 (1965).

7. Lang, N., Protides Biol. Fluids, Proc. Colloq. **14**, 517 (1966).

8. Krassnitsky, O., Pesendorfer, F., and Wewalka, F., Deut. Med. Wochenschr. **95**, 1 (1970).

9. Pesendorfer, F., Krassnitsky, O., and Wewalka, F., Klin. Wochenschr. **98**, 58 (1970).

10. Purcell, R. H., Holland, P. V., Walsh, J. H., Wong, D. C., Morrow, A. G., and Chanock, R. M., J. Infec. Dis. **120**, 383 (1969).

11. Coller, J. A., and Millman, I., J. Clin. Invest. **49**, 20a (1970).

12. Walsh, J., Yallow, R., and Berson, S., J. Infec. Dis. **121**, 550 (1970).

13. Millman, I., Loeb, L. A., Bayer, M. E., and Blumberg, B. S., J. Exp. Med. **131**, 1190 (1970).

14. Greenwood, F. C., Hunter, W. M., and Glover, J. S., Biochem. J. **89**, 114 (1963).

15. Del Prete, S., Constantino, D., Doglia, M., Graziina, A., Ajdukiewicz, A., Dudley, F. J., Fox, R. A., and Sherlock, A., Lancet **2**, 579 (1970).

16. Blumberg, B. S., Friedlaender, J. S., Woodside, A., Suntick, A. I., and London, W. T., Proc. Nat. Acad. Sci. U.S.A. **62**, 1108 (1969).

17. Shulman, N. R., and Barker, L., Science **165**, 304 (1969).

18. Millman, I., London, W. T., Sutnick, A. I., and Blumberg, B. S., Nature (London) **226**, 83 (1970).

Received April 26, 1971. P.S.E.B.M., 1971, Vol. 138.