

[54] PROCESS OF VIRAL DIAGNOSIS AND REAGENT

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[63] Continuation of Ser. No. 33,327, April 30, 1970, abandoned.

[52] U.S. Cl. **424/1, 23/230 B, 250/303, 250/304**[51] Int. Cl. **A61k 27/04**[58] Field of Search. **250/83 SA, 303, 304; 424/1; 23/230 B**[56] **References Cited****OTHER PUBLICATIONS**

"Sandwich" Solid Phase Radioimmunoassay for the Quantitative Determination of Human Immunoglobulins, by Salmon et al., from the Journal of Immunology, Vol. 103, No. 1, July 1969, pp. 129-137.

Radioimmunoassays Employing Immunosorbents, by Wide, L., from the Dept. of Clinical Chemistry, University Hospital, Uppsala, Sweden, Sept. 1969, pp. 207-218.

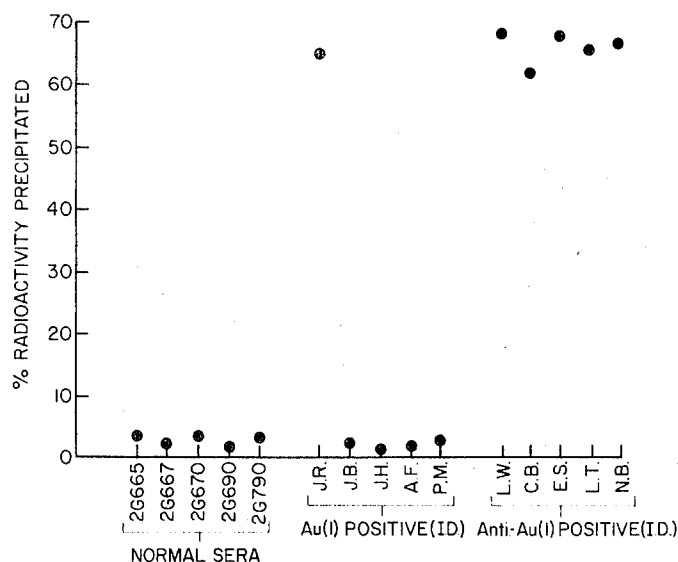
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[57] **ABSTRACT**

The invention makes possible the diagnosis of the

presence of viral moieties, specifically one possible viral moiety, Australia antigen Au(1) and antibody to it (anti-Au(1)) which are associated with hepatitis, in human sera, by radioactively labeling the Au(1) moiety, as an indicator, with a radioactive element, preferably radioactive iodine, and most desirably ^{125}I . By incubating anti-Au(1) antibody with the radioactively labeled Au(1), a portion of the reactants may form a complex. The degree to which a complex is formed as a function of the reactants can be determined by separation between complexed and non-complexed radioactivity. Separation, when by precipitation, can be enhanced and therefore preferably accomplished in the presence of anti-human gamma-globulin of another species and ammonium sulphate (approximately 25 percent final saturation), followed by centrifugation. If antibody is present in the sample of serum to be tested, it will react and form a complex with the ^{125}I -labeled Au(1) added. The complex of ^{125}I -labeled Au(1) with antibody under the appropriate conditions can be separated from any ^{125}I -labeled Au(1) which is not reacted and complexed with antibody. After separation the radioactivity of either or each portion so separated can be measured. In testing for Au(1) antigen it is best to preincubate a standardized anti-Au(1) antibody and an appropriate amount of unknown. To this is added a standard amount of ^{125}I -labeled Au(1) and further incubated. After separation, when accomplished by precipitation, the amount of radioactivity in the precipitate will depend upon the composition of the unknown sample.

The radioactive labeling of the viral moiety Au(1) with radioactive iodine is preferably accomplished by first oxidizing the Au(1) with chloramine-T in the presence of radioactive iodine and then terminating the oxidation reaction by adding sodium meta-bisulphite.

29 Claims, 10 Drawing Figures

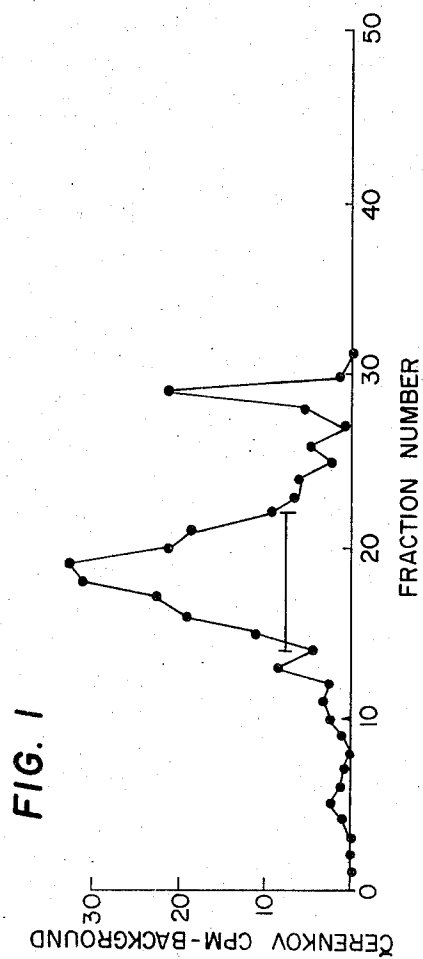


FIG. 2

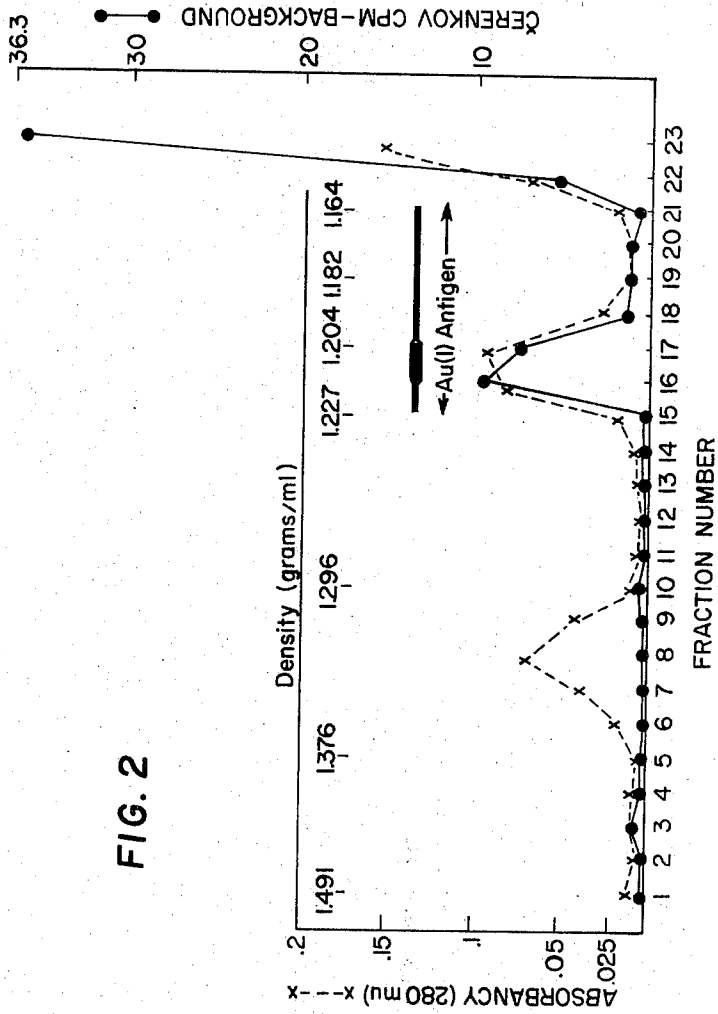
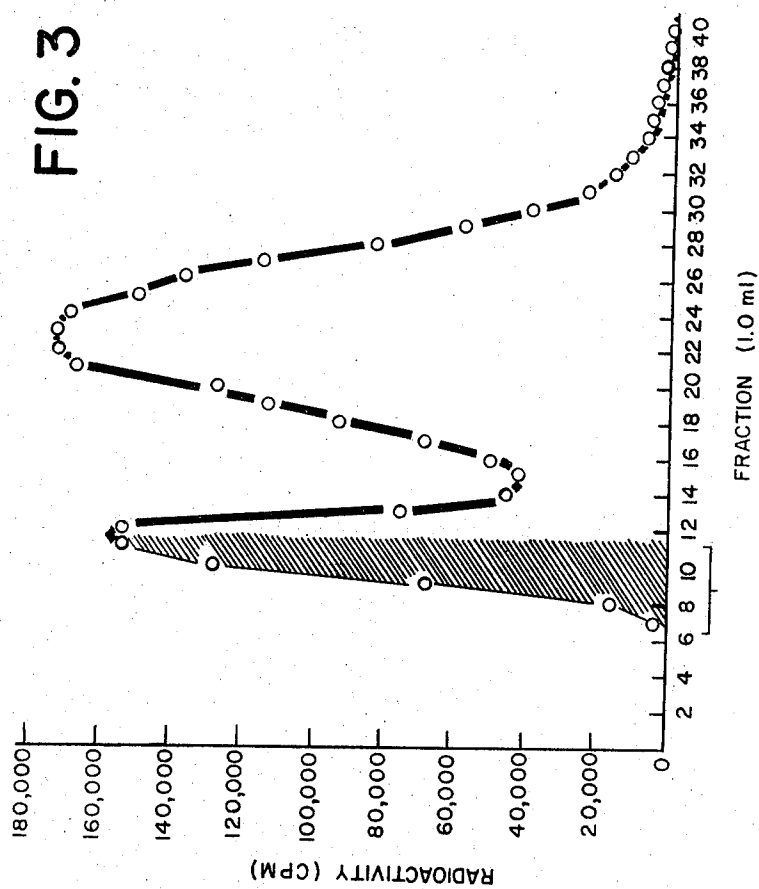
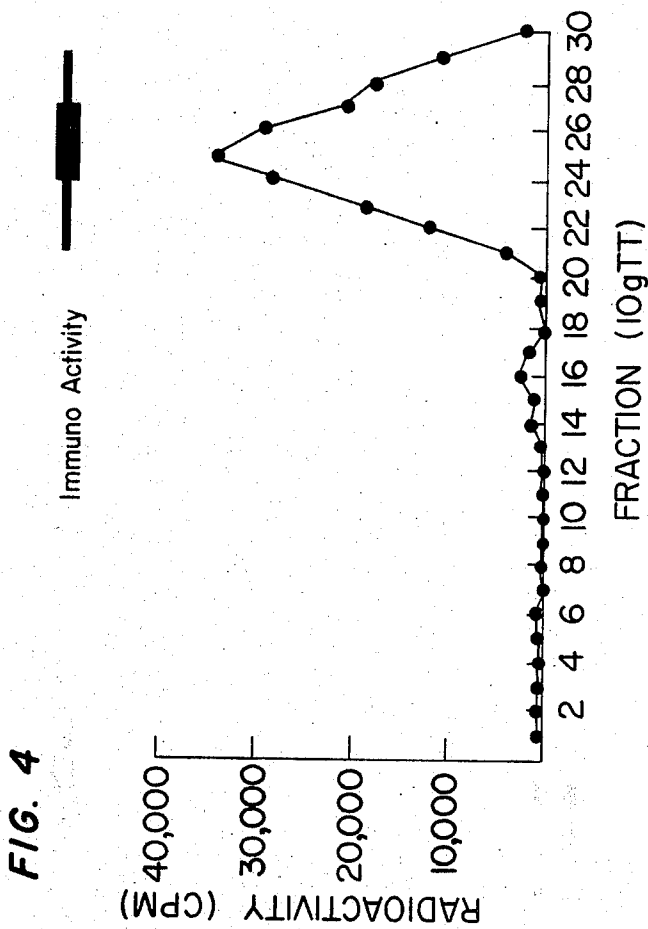


FIG. 3





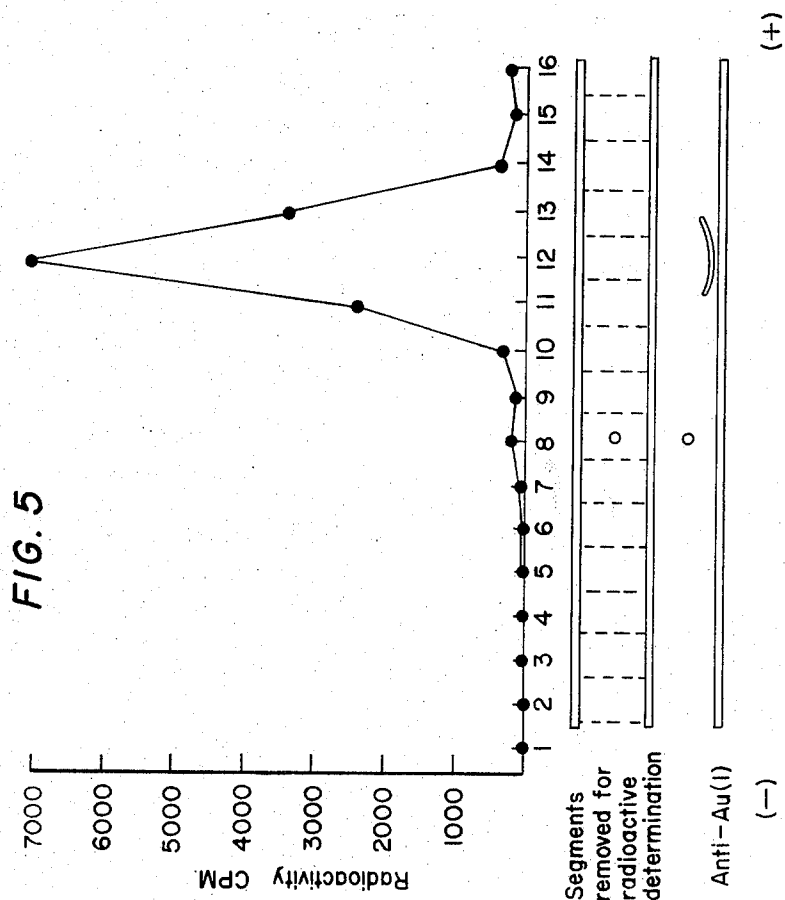


FIG. 6

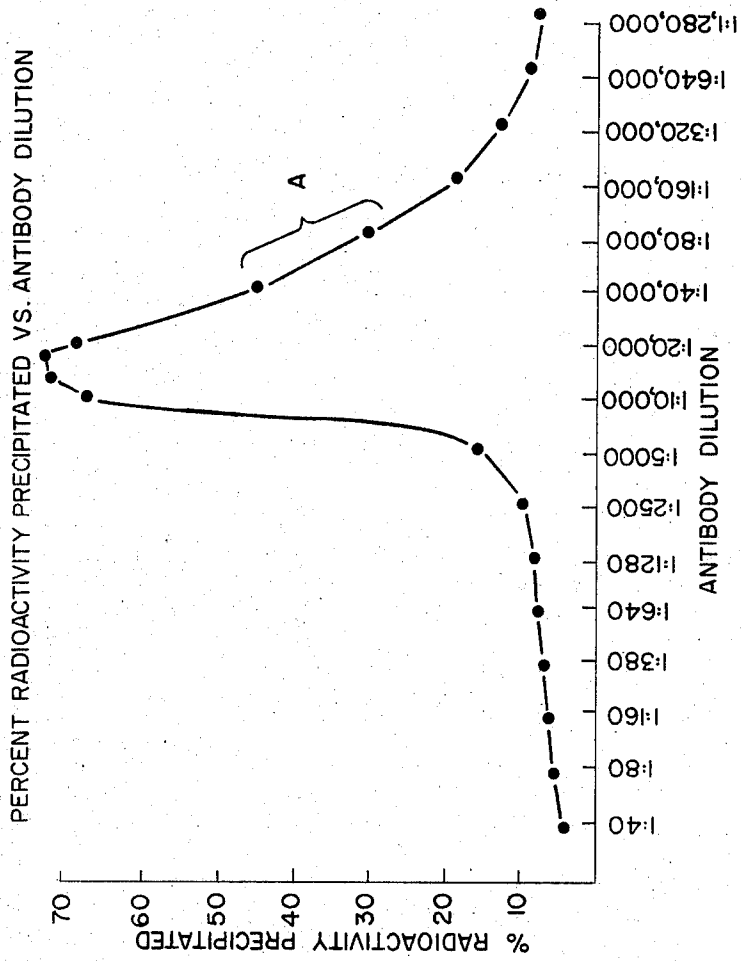


FIG. 7

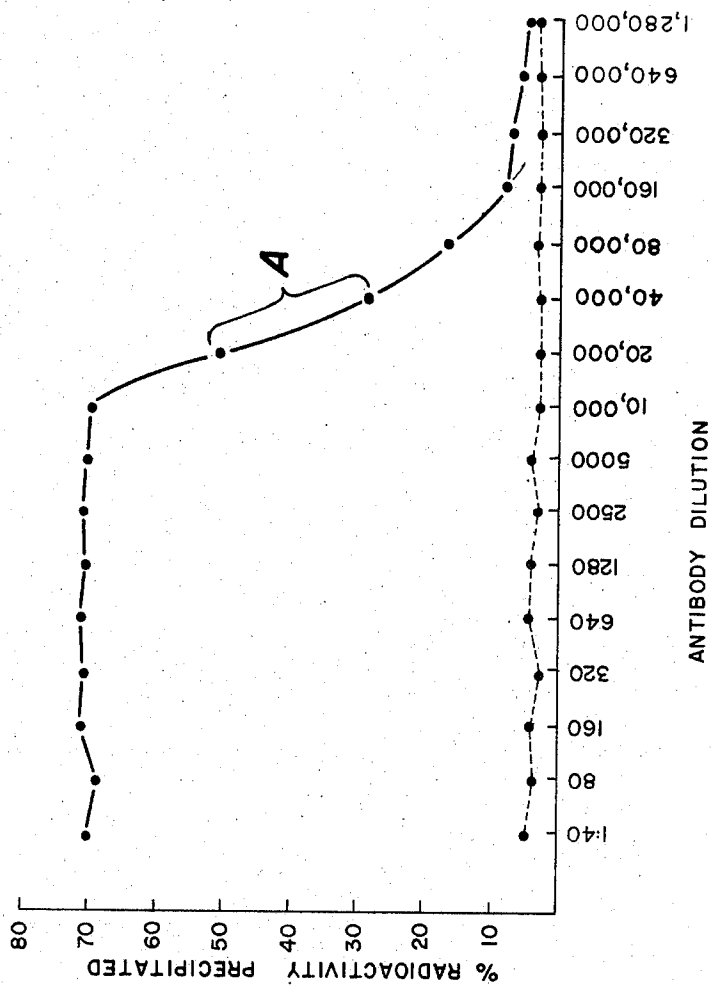


FIG. 8.

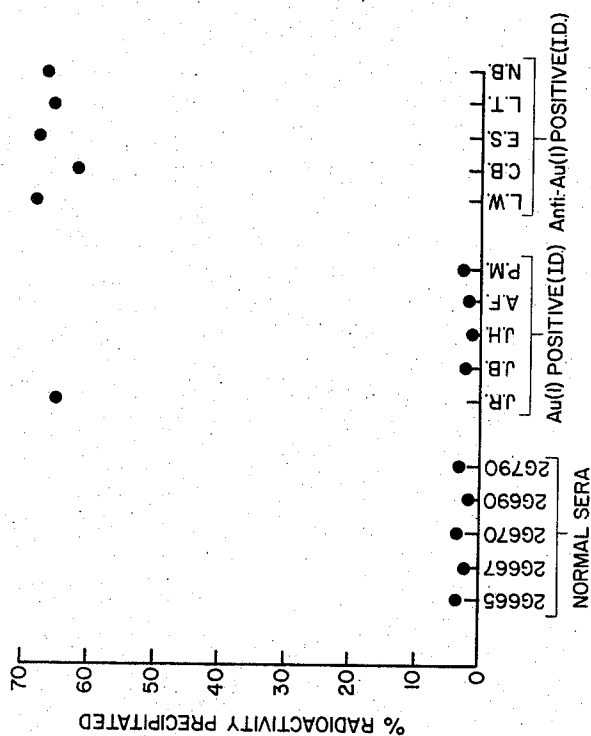


FIG. 9.

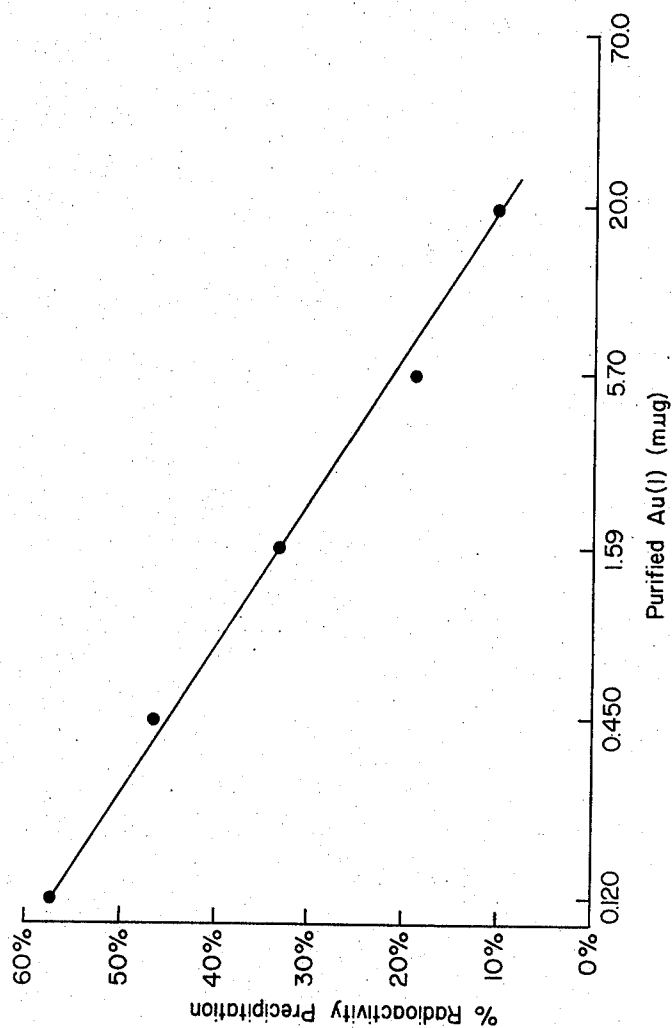
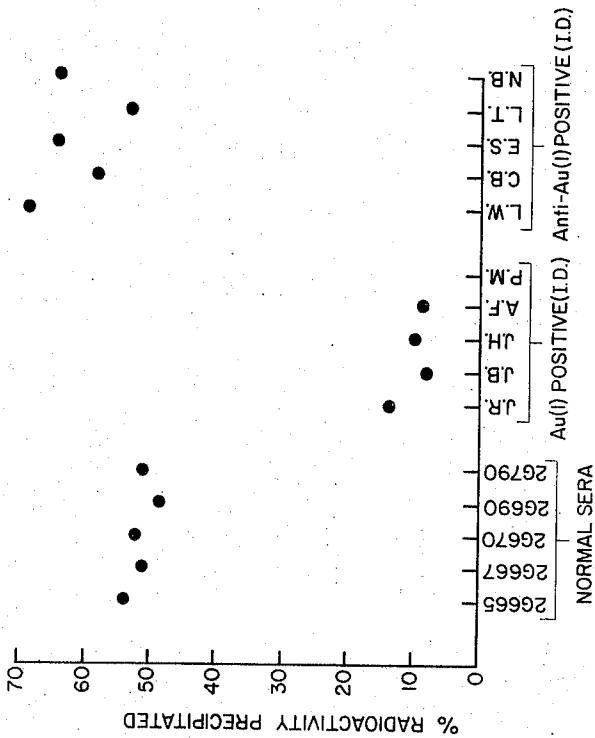


FIG. 10



PROCESS OF VIRAL DIAGNOSIS AND REAGENT RELATED APPLICATION

This application is a continuation of our U.S. patent application Ser. No. 33,327, filed Apr. 30, 1970, and now abandoned, for PROCESS OF VIRAL DIAGNOSIS AND REAGENT.

DISCLOSURE OF INVENTION

The present invention relates to clinical or experimental testing for the presence of viral moieties, either antigens or antibodies in biological fluids, including especially human sera.

A purpose of the invention is to radioactively label either antigen or its antibody, preferably with radioactive iodine, to allow this labeled moiety to react with its counterpart and to form a complex. Antibody to human gamma-globulin (preferably of another species, that is a heterologous and a human gamma-globulin) such as rabbit anti-human gamma-globulin (h-chain specific) is added and incubated. Next ammonium sulphate to a final concentration of approximately 25 percent saturation is added to facilitate precipitation of immune complexes and the precipitate is centrifuged and the radioactive content in this precipitate is measured.*

(*While precipitation is used, any other means may be employed to separate free or unbound from complexed or bound labeled antigen or labeled antibody.)

A further purpose is to determine the presence of Australia antigen Au(1)* or anti-Au(1) antibody in biological fluids, including especially human sera, by radioactively labeling Australia antigen Au(1), causing it to form a complex with anti-Au(1) antibody, precipitating the complex and measuring the radioactivity thereof.

(*Australia antigen or Au(1) is to be considered identical with hepatitis B. SH (for serum hepatitis), and to HAA (for hepatitis associated antigen). Any other suitable designation may be used.)

A further purpose is to enhance complex formation and precipitation of the complex by adding rabbit anti-human gamma-globulin and centrifuging the mixture.

A further purpose is to add to the solution in which precipitation is to take place rabbit anti-human gamma-globulin and ammonium sulphate, preferably in a final concentration which is approximately 25 percent saturated ammonium sulphate.

A further purpose is to radioactively label the Australia antigen Au(1) by first reacting with chloramine-T in the presence of radioactive iodine, then reacting with sodium meta-bisulphite solution, and then removing the unlabeled radioactive salt which has not been conjugated with the Australia antigen.

A further purpose is to incubate radioactively labeled antigen, Au(1) and an unknown sample of a biological fluid, for example especially human serum, to form a complex if the unknown sample contains human anti-Au(1) antibody, then to precipitate the complex and measure its radioactivity.

A further purpose is to determine the presence of Australia antigen, Au(1) in human sera or other biological fluids by preincubating standardized anti-Au(1) antibody with an unknown sample of human sera, adding radioactive Australia antigen Au(1) to the mixture, further incubating to form a complex whose radioactivity depends upon the composition of the unknown sample, precipitating the complex and measuring its radioactivity.

A further purpose is to utilize radioactively labeled Australia antigen Au(1), preferably radioactively labeled with ^{125}I , as a test reagent in determining the presence of Australia antigen Au(1) and anti-Au(1) antibody in human sera or other biological fluids.

A further purpose is to utilize a standard solution of anti-Au(1) antibody as a test solution to determine the presence of Australia antigen Au(1) in human sera or other biological fluids.

Further purposes appear in the specification and in the claims.

The drawings are diagrams, useful in explaining the invention.

FIG. 1 is a sucrose gradient profile, plotting Cerenkov CMP-Background against fraction number.

FIG. 2 is a density gradient showing a composite graph of the sucrose separated Au(1) fraction on cesium chloride plotting absorbancy at 290 m μ as one ordinate and Cerenkov CMP-Background as another ordinate and density in grams per ml as one abscissa and fraction number as another abscissa.

FIG. 3 plots radioactivity of 1.25 μl * aliquots of ^{125}I labeled Australia antigen Au(1) as ordinate against fraction number as abscissa, after desalting on a Sephadex G-50 column.

(* μl = μl = microliter)

FIG. 4 plots the results of cesium chloride density ultracentrifugation of ^{125}I -labeled Au(1) antigen, plotting radioactivity against fraction number, and also plotting as abscissa immunoreactivity by gel-diffusion.

FIG. 5 compares the results of immunoelectrophoresis of ^{125}I -labeled Au(1) against radioactivity as shown in FIG. 4.

FIG. 6 plots the percent radioactivity in the precipitate against 2-fold dilutions of anti-Au(1) antibody as the ordinate, after incubating with ^{125}I -labeled Au(1) antigen, rabbit anti-human gamma-globulin and centrifuging.

FIG. 7 shows the effect of 25 percent ammonium sulphate saturation on the precipitation curve.

FIG. 8 shows the percent of precipitable activity for various serum specimens, after incubation with ^{125}I -labeled Au(1), rabbit anti-human gamma-globulin, and the addition to a final saturation of 25 percent ammonium sulphate to aid precipitation.

FIG. 9 shows the effect on precipitable radioactivity of incubating purified Au(1) with a standard antibody dilution.

FIG. 10 compares the inhibition of precipitable ^{125}I -labeled Au(1) for the same normal, Au(1) positive and anti-Au(1) containing sera, as is shown in FIG. 8.

THE NATURE OF AUSTRALIA ANTIGEN

The discovery of Australia antigen, Au(1), Blumberg, Bull N.Y. Acad. Med. 40, 377 (1964) in the serum of an Australian aborigine, and the recognition of its association with viral hepatitis, Blumberg, Gerstley, Hungerford, London, and Sutnick, Ann. Int. Med. 66, 924 (1967), has advanced considerably the capacity to establish in some cases an affirmative diagnosis of viral hepatitis rather than having to rely solely on non-specific laboratory parameters to support a clinical impression. This development results from a series of systematic epidemiological and immunologic investigations culminating in the strong implication that Australia antigen is either a virus capable of producing hepatitis or a major antigenic determinant of such a virus.

More recently, the purification and physical characteristics of Au(1) has more directly confirmed the relationship between the antigen and the actual virus. Blumberg and Millman, U.S. patent application Ser. No. 864,788, filed Oct. 8, 1969 for VACCINE AGAINST VIRAL HEPATITIS AND PROCESS, now U.S. Pat. No. 3,636,191, issued Oct. 8, 1969.

Ouchterlony double gel-immunodiffusion and complement fixation are currently and most widely used method for routine detection of Au(1) and the human antibody to Au(1) in serum. As determined by this procedure, the frequency to Australia antigen among non-professional blood donors in the United States is about 0.1 percent. Sutnick, A. I. et al., JAMA 205,670 (1968). In striking contrast, Australia antigen is present in upwards of 50 percent of patients with acute viral hepatitis. In order to obtain this rate of detection, however, with Ouchterlony double gel-immunodiffusion, the serum must be tested early and frequently in the course of the disease. There is now well-documented evidence demonstrating the high frequency of transmission of hepatitis infection following the administration of Au(1) positive donor blood. Blumberg, B.S., et al., Gastroenterology 56, 1212 (1969); Gocke, D. J., Lancet 1, 1055 (1969); Okoche, et al., Vox Sang, 18, 289 (1970). However, in cases of viral hepatitis following transfusion, where donor bloods have been available for retrospective examination, the likelihood of finding an Au(1) positive donor unit is less than 20 percent.

If the agent responsible for the infection in the recipient was in fact transmitted from an Au(1) negative donor unit as determined by immunodiffusion or complement fixation, two of the obvious explanations would include: (1) the agent transmitted was immunologically unrelated to Australia antigen, or (2) if it was related, the currently employed methods are not sufficiently sensitive to permit its detection.

The investigation which resulted in the present invention was undertaken to determine the feasibility of non-destructive conjugation of a radioactive label, particularly radioactive iodine and preferably iodine-¹²⁵I to purified Australia antigen and to evaluate the capacity of isotopically labeled Au(1) to serve as a sensitive immunologic indicator for the detection of circulating Australia antigen or antibody to Australia antigen.

PURIFICATION OF AUSTRALIA ANTIGEN

The purification of Australia antigen is performed according to the procedure outlined in the Blumberg and Millman patent application aforesaid. Australia antigen is strongly resistant to enzyme digestion, whereas human plasma components can be degraded and removed without impairing the Australia antigen.

Another important aspect is that impurities can be eliminated from the Australia antigen fraction by differential density centrifugation, which can be carried out in a solution of cesium chloride, and preferably also in a separate solution of sucrose, the solvent in both cases being water.

As a result of these purification techniques, a relatively concentrated suspension of Australia antigen is produced, and free from normal human serum components as determined by immunological methods used.

PREFERRED TECHNIQUE FOR PURIFICATION

Sedimentation

The serum obtained by plasmaphoresis is subjected to high centrifugal force in an ultracentrifuge. One hundred ml of plasma is clarified by centrifugation for 1 hour at $2,000 \times g$ in a Sorvall refrigerated centrifuge and then re-centrifuged at $370,000 \times g$ for 18 hours in Spinco 65 Ti angle rotor ultracentrifuge. Only the pellet at the bottom of the tube contained Au(1). The remaining liquid was removed by decantation and the pellet was re-suspended to 10 ml of 0.85% (w/v) weight of sodium chloride solution in water.

Enzyme Digestion

To 10 ml of the Au(1) suspension just referred to in a 30 ml stoppered bottle, 400 μ l of amylase at a weight concentration of 10 mg/ml, 400 μ l of lipase at a weight concentration of 1 mg/ml and 200 μ l neuraminidase at a weight concentration of 1 mg/ml were added. This mixture was incubated at 37°C. with gentle agitation for 15 minutes, then 100 μ l of phospholipase C at a weight concentration of 1 mg/ml were added. The incubation and agitation were continued for 20 minutes. During this period it was noted that the mixture gelled. To the gel was added 2 ml of pronase at 10 mg/ml weight concentration and the mixture was incubated and agitated for one hour. At the end of this period the gel had been completely dissolved and an additional 2 ml of pronase of the same concentration was added. The incubation and agitation were continued for an additional hour and then the mixture was cooled in an ice bath.

While a specific preferred procedure is given, it will be evident that any of a wide variety of enzymes may be used in the procedure referred to above. Among the enzymes which are suitable are trypsin, pronase, lipase, phospholipase, ribonuclease, deoxyribonuclease, amylase, neuraminidase, wheat germ lipase and alpha amylase. Any other suitable enzyme may be employed if desired.

Gel Filtration

Five ml aliquots of the enzyme treated suspension above were subjected to gel filtration through a column of preferably Sephadex G-200 produced by Pharmacia, Uppsala, Sweden. This column retards the smaller units but allows the larger Au to come through quickly. Salts and inorganic materials are also retarded. The Au(1) comes through in the first peak.

The column was 650 mm long and 32 mm in diameter, and it had been previously equilibrated with 0.85% by weight sodium chloride solution in water. The elution was carried on with the same solution and all of the Au(1) positive material was eluted in the first peak.

A decrease in the number and intensity of precipitin bands of the product was noted, after separation by electrophoresis. Patterns were developed by adding horse anti-human serum antiserum to the troughs. All of the fractions which comprise the first peak contained all of the Au(1) immunoreactivity and were combined.

Sucrose Gradient

The combined fractions from peak 1 obtained from the gel filtration were dialyzed against 0.01 M potassium chloride solution in water, lyophilized (freeze dried) and subjected to sedimentation on sucrose gradients in an ultracentrifuge. In carrying out this operation the tube of the ultracentrifuge is filled with progressive increments of sucrose solution, the most concentrated being at the bottom and the least concentrated being at the top. The specimen resuspended in

distilled water is layered on top of the differential density increments. The usual range of sucrose concentration in the bottom layer is about 30% (w/v) and the sucrose concentration in the top layer is about 10% (w/v) but other differential concentrations can be used. If desired an automatic machine well known in the art can supply the sucrose gradient. For test purposes some of the Au(1) had been rendered radioactive prior to the initial operation and FIG. 1 shows an analysis of the gradient for ^{32}P incorporation and antigenicity. Those fractions 18 to 23 inclusive with major "Cerenkov" radioactivity and antigenicity were combined.

As shown in FIG. 1, the line within the radioactivity peak ^{32}P represents Au(1) positive range when assayed by immunodiffusion. The sucrose gradients were prepared and sampled by the method of Martin and Ames, J. Biol. Chem. 236, 1372 (1961). One ml of material was layered over each of the two 32.5 ml linear sucrose gradients from 10 to 30 percent by volume in 0.01 M tris maleate buffer at a pH of 7.4. The gradients were centrifuged in a SW 27 Spinco rotor at $57,000 \times g$ for 20 hours at 2° to 5°C . Forty drop fractions were removed by puncturing the bottom of the tubes and radioactivity and anti-genicity of each fraction was determined as described below.

It is preferred to use a zonal ultracentrifuge for this purpose and for other differential density centrifugations as later explained but a standard ultracentrifuge may be used if desired.

The active fraction after differential density centrifugation in sucrose solution was dialyzed and lyophilized to remove the sucrose and concentrate the Au respectively.

Centrifugation in Cesium Chloride

The Au from differential density centrifugation above was subjected to sedimentation on cesium chloride gradients. The pooled immunoreactive fractions from the sucrose density tubes of FIG. 1 were dialyzed against 0.01 M potassium chloride solution in water at 5°C . for 18 hours and lyophilized. The dried Au(1) fraction was then resuspended in a small amount of distilled water and mixed with saturated cesium chloride solution to produce a final density of 1.3 determined by a refractometer. The final volume of the mixture was 5 ml. This was centrifuged in a Spinco SW 50 rotor at $222,000 \times g$ to equilibrium in about 40 hours. Ten drop fractions were collected by puncturing the bottom of the tube.

Instead of using the technique described, a preformed linear cesium chloride density gradient either in a centrifuge tube or a zonal rotor may be used. At the end of the centrifugation the Au bands at a certain level as determined by assay. The impurities are denser and separate at a lower level.

FIG. 2 shows an analysis of the gradient for ^{32}P incorporation, 280 mu absorption, density, and immunoreactivity. Immunoreactivity [presence of Au(1)] was spread between fractions 15 and 21 with greatest activity in fraction 16. The relative activity of each fraction was determined by two-fold dilution titration. That fraction which could be diluted the farthest and produce a precipitin line was considered the peak fraction. Fraction 16 has a density of 1.21 and it falls within both the 280 mu and the Cerenkov peaks. The 280 mu peak seen in FIG. 2, fraction 8, is an unidentified moiety that does not separate on sucrose gradient alone and contains no Au(1) immunoreactivity. Fractions 15 to 18

were pooled, dialyzed against 0.01 M solution of potassium chloride in water and lyophilized.

CONJUGATION WITH RADIOACTIVE IODINE

The method used for conjugation is basically that of Greenwood, Hunter and Glover, Biochemical J. 89, 114 (1963). The conjugation was carried out in an isotope shipping vial for iodide ^{125}I . Other isotopes may also be used. ^{125}I was chosen here because of its long half life and the ease with which it can be coupled to protein moieties. 4mC ^{125}I in 0.050 ml 0.1 N NaOH diluent was partially neutralized with 0.045 ml 0.1 N HCl and buffered with 0.050 ml of 0.15 M phosphate-buffer solution consisting of 0.15 M $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ in water at pH about 7.6. Purified Au(1) was equilibrated and concentrated by ultrafiltration* against 0.05 M phosphatebuffer solution containing 0.05 M $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ in water, pH 7.6, and the protein concentration was determined by nesslerization according to the method of Lonni, Dillon and Beard, Proc. Soc. Exp. Biol. Med. 74, 4 (1950). 100 ul (150 ug—ug = μg = microgram) of purified Au(1) was added to the vial and followed by 50 ul (100 ug) of chloramine-T solution (2 mg/ml) dissolved in 0.05 M phosphate-buffer solution (freshly prepared). After 3 minutes of constant agitation, the reaction was arrested by adding 50 ul (244 ug) of sodium meta-bisulphite solution (4.88 mg/ml) dissolved in 0.05 M phosphate-buffer solution (freshly prepared). The reaction mixture was then diluted with 200 ul (2 mg) of potassium iodide solution (10 mg/ml) dissolved in 0.05 M phosphate-buffer solution and the entire contents, along with an additional 400 ul (4 mg) potassium iodide wash of the vial, was transferred to a Sephadex G-50 column.**

(*Ultrafiltration is not a requirement for this method. It was used here for convenience.)

(**These units have been successfully used. However, other amounts may be and have been used for labeling.)

COLUMN CHROMATOGRAPHIC SEPARATION OF RADIOACTIVE Au(1)

* Sephadex G-50 (coarse) (Pharmacia Inc.) was swollen and equilibrated with 0.1 M borate-saline buffer. The boratesaline buffer contained 6.184 boric acid, 9.536 g sodium tetraborate (decahydrate), 4.384 g sodium chloride, diluted with water to 1 liter; having a pH of 8.5. The borate-saline buffer was at a temperature of 4°C . The slurry was poured in a column to form a 1.5×11.5 cm gel bed. One milliliter 0.1 M boratesaline buffer with bovine serum albumin (NBC 20 mg/ml) was passed through the column to permit saturation of any potential binding sites and measure the void volume as determined by ultraviolet absorbance at 278 mu. Upon completion of the conjugation reaction, the contents of the reaction vial plus the potassium iodide wash (total volume 945 ul) were transferred to the column. Elution was accomplished with 0.1 molar borate-saline buffer at a rate of 1 ml per minute. One milliliter fractions were collected in tubes, containing 1 ml of 0.1 molar borate-saline buffer each with 40 mg of bovine serum albumin. The eluted radioactivity was determined by counting 1.25 ul aliquots of each 2 ml fraction (1 ml from the column plus 1 ml borate-saline bovine serum albumin solution in the collection tube).

*This is a dextran gel used here to facilitate the separation of labeled-Au(1) from free ^{125}I -iodide. Other well known means of separation of protein in solution from ionic salts may be used for this purpose — e.g. dialysis.

MEASUREMENT OF RADIOACTIVITY

All radioactive determinations were performed on a Packard Tri-Carb, well-type, auto-gamma spectrometer, series 5000 with a sodium iodide crystal (2×2 inches). The voltage was 765 volts, discriminators 40 to 180, and gain 90 percent. The counting duration was one minute.

IMMUNODIFFUSION AND IMMUNOELECTROPHORESIS

Ouchterlony double gel-immunodiffusion as modified by Blumberg and Riddell, J. Clin. Invest. 42, 867 (1963), was performed on gel plates of 1.1% agar in phosphate-buffered-saline solution having a pH of 7.4.

Immunoelectrophoresis was performed on $3\frac{1}{4} \times 4\frac{1}{4}$ inches photographic plates with 1.1% agarose in veronal buffer, pH 8.2, ionic strength 0.038. An 80 volt potential resulted in a current of 22–24 ma.

CESIUM CHLORIDE DENSITY GRADIENT ULTRACENTRIFUGATION

A 10 ul volume of undiluted ^{125}I -labeled Au(1) having 500,000 counts per minute was mixed uniformly with 1.0 ml. (600 ug) of purified unlabeled Au(1) as a carrier and sufficient saturated cesium chloride plus water was added to give a final density of 1.3 in a total volume of 5 ml. This mixture was banded at $300,000 \times g$ for 40 hours in an SW-50 Spinco rotor. Thirty 10-drop fractions were collected by puncturing the bottom of the nitro-cellulose tube. The radioactivity and immunodiffusion reactivity were determined for each fraction.

IMMUNOPRECIPITATION OF RADIOACTIVE AU(1)

A dilution of ^{125}I -labeled Au(1) harvested from the first half of the protein peak following Sephadex G-50 column chromatography was made such that 500 ul of the dilution contained approximately 15,000 counts per minute on the day of labeling. Following dialysis this volume content approximated 12,000 cpm. Unless otherwise stated, this constant volume of dialyzed ^{125}I -labeled Au(1) was used for all immunoprecipitation procedures.

A 50 percent saturated ammonium sulphate, globulin

tions of this antibody fraction was employed in most experiments. To facilitate the precipitation of human anti-Au(1)-Au(1) complexes a second antibody, rabbit anti-human $\text{I}_\text{g}\text{G}$ (h-chain specific, Hoechst) was used in the dilution specified in the experiments.

All reactions were carried out in disposable glass culture tubes 10×75 mm (B.D.). Because of the potentially infectious nature of the materials employed, these tubes could be placed inside larger capped carrier tubes for counting. Mixing was performed following the addition of all reactants. The incubation format is detailed with each experiment. Incubations at 37°C . were carried out in a warm-air incubator. Centrifugation of immunoprecipitates was achieved at 5,000 rpm ($3300 \times G$) for 30 minutes at 25°C . or at 8,000 rpm ($8800 \times G$) for 1 hour at 25°C . in a Sorvall RC-2B centrifuge using a GSA rotor. Supernatants were removed by vacuum aspiration. Precipitates were not washed before counting.

PURITY OF AUSTRALIA ANTIGEN

An aliquot of the pooled immunoreactive fractions following cesium chloride density gradient ultracentrifugation was subjected to immunoelectrophoresis. FIG. 5 of the earlier mentioned application of Blumberg and Millman demonstrates the failure of horse anti-human total serum to display immunoreactivity with purified Australia antigen whereas human anti-Au(1) is reactive with purified Au(1), but the purified Au(1) is not reactive with horse anti-human serum.

CONJUGATION AND ISOLATION OF ^{125}I -LABELED Au(1)

Following the conjugation of purified Au(1) with ^{125}I , the reaction mixture was desalted of unconjugated ^{125}I on a short Sephadex G-50 column having a void volume of 8.5 ml. The radioactivity of 1.25 ul aliquots of each fraction was measured as shown in FIG. 3. Two peaks of radioactivity are shown, the first immediately after the void column, representing the protein peak, and the second is delayed on the column as expected for the salt peak.

Upon completion of the separation, the $4 \mu\text{C} = 4 \mu\text{C} = \text{micro Curie}$ of ^{125}I were distributed as follows:

		Counts per Minute	% of Total	
A.	Reaction vial	38.2×10^6	50.5×10^6	1.2%
	cap	12.3×10^6		
Subtotal				
B.	Remaining on column	9.8×10^6	9.8×10^6	0.2%
C.	Elution			
	Protein peak			
	fraction 7-11	$589. \times 10^6$		
	fraction 12-15	$506. \times 10^6$		
	Protein total		$1,094. \times 10^6$	26.8%
	Salt peak			
	fraction 16-40	$2,911. \times 10^6$		
	Salt total		$2,911. \times 10^6$	71.8%
	Total CPM for 4 mC		$4,066. \times 10^6$	100.0%

fraction of human serum from a patient with persistent antibody to Au(1) was made and concentrated two fold after equilibration in borate-saline buffer. This fraction failed to demonstrate immunoreactivity on Ouchterlony double gel-immunodiffusion in dilutions greater than 1:2. A constant volume (500 ul) of various dilu-

In estimating the amount of radioactivity transferred to the purified Australia antigen, it is necessary to consider not only that portion of the activity in the protein peak — 26.8% (1.077 mC) but also the amount of activity that remains in the glass vial — 0.93% (0.037 mC). This residual radioactivity in the vial was not re-

moved after repeated washings, which is indicative of adherence of labeled protein to the glass. The portion remaining on the column (0.24%) has been disregarded.

If the initial amount of purified Au(1) antigen (150 ug) is assumed to be distributed between the vial and the protein peak in proportion to the amount of radioactivity in each, then the amount of protein eluted from the column was equivalent to 145 ug of Au(1). To minimize contamination with free iodide- ^{125}I , only the first half of the protein peak was used for subsequent investigation. These first five fractions, represented in FIG. 3 at the left by cross-hatching, contain 5 ml of column eluant plus 5 ml of borate-saline buffer as above described with bovine serum albumin in the collection tubes. The pooled 5 fractions were designated stock undialyzed ^{125}I -labeled Au(1). Again, if it is assumed that the radioactivity of the protein curve as extrapolated to the base line is proportional to the protein content, the stock undialyzed ^{125}I -labeled Au(1) containing $589 \times 10^6 \text{ cpm}$ represents 52.9 percent of the eluted protein or 76.7 ug of Au(1).

When the undialyzed stock was dialyzed against borate-saline solution containing bovine serum albumin as above described, there was a rapid loss to the dialysate of 28.0 percent of the radioactivity. Ninety-five percent of the non-dialyzable activity was precipitable in 2.5 percent trichloroacetic acid. It was therefore concluded that the 76.7 ug of Au(1) was conjugated with 0.416 mC ($425 \times 10^6 \text{ cpm}$) iodide- ^{125}I . This represents a labeling efficiency of 20.3 percent and a specific radioactivity of 5.4 uC/ug Au(1) . Assuming that the molecular weight of purified Au(1) is approximately 3,000,000 (this is a rough approximation based on physical/chemical data), there would be an average of 7.4 atoms of iodide- ^{125}I per molecule of Au(1).

For immunoprecipitation experiments, a dilution of the pooled dialyzed ^{125}I -labeled Au(1) was used such that a 500 ul volume would have approximately 12,000 cpm on the day of labeling.

PHYSIOCHEMICAL AND IMMUNOLOGIC CHARACTERIZATION OF ^{125}I -LABELED Au(1)

The results of cesium chloride density gradient ultracentrifugation of a volume of ^{125}I -labeled Au(1) containing 500,000 counts per minute with 600 ug of purified unlabeled Au(1) as carrier is shown in FIG. 4. Aliquots of the 30 resultant fractions were tested for radioactivity and immunoreactivity by gel-diffusion. The distribution of radioactivity and immunoreactivity is parallel, and is similar to that observed for ^{32}P labeled-Au(1). Blumberg and Millman patent application aforesaid.

For immunoelectrophoresis of ^{125}I -labeled Au(1), equal volumes of ^{125}I -labeled Au(1) and purified unlabeled Au(1) were mixed and run in each of two parallel patterns on the same agarose plate. At the completion of electrophoresis the gel from one pattern was immediately cut into 16 equal segments and the radioactivity of each gel-segment was measured. To the trough of the second pattern, human serum containing antibody to Au(1) was added and immunoprecipitation was allowed to develop in the routine manner. The relationship of the radioactivity to the precipitin band is shown in FIG. 5.

IMMUNOPRECIPITATION OF RADIOACTIVE IODINE LABELED Au(1)

A 50 percent saturated ammonium sulphate globulin fraction was made from a human serum having anti-Au(1) antibody as demonstrated by immunodiffusion. This preparation was designated as the standard antibody and used in several subsequent experiments. Serial dilutions of the standard antibody from 1:40 to 1:1,280,000 in borate-saline solution containing bovine serum albumin (400 mg/liter) were made. 500 ul of each dilution was incubated with 500 ul of standard ^{125}I -labeled Au(1) for 2 hours at 37°C . and for 22 hours at 4°C . 50 ul of a 1:10 dilution of rabbit anti-human IgG (h-chain specific) antibody was then added followed by a 2 hour incubation at 37°C . and 18 hours incubation at 4°C . The reaction mixture was then centrifuged at 8,000 rpm for 1 hour and the supernate was separated from the nonvisible precipitate by vacuum aspiration. The radioactivity of both the precipitate and the supernate was measured. The percentage of precipitable ^{125}I -labeled Au(1) for each dilution is shown in FIG. 6.

An area of equivalence is observed between an antibody dilution of 1:10,000 to 1:20,000 with significant precipitable radioactivity present at up to 1:160,000 dilution. This is contrasted to the lack of significant precipitable activity (<3 percent) when similar dilutions of a non-anti-Au(1) containing human serum (immunodiffusion) were substituted for the antibody dilutions. If more concentrated rabbit anti-human IgG (50 ul of 1:2 dilution) was used, a broader (but no higher) zone of equivalence was obtained with the anti-Au(1) preparation while the non-anti-Au(1) preparation persisted at a non-significant level of precipitable activity. If the second antibody was not added, there was no significant precipitable activity with either preparation. The 1:2 dilution or rabbit anti-Human IgG was used for all subsequent immunoprecipitation experiments.

In the area of standard antibody excess and constant rabbit anti-human IgG, there is failure to precipitate the same degree of radioactivity as is obtained at equivalence. If this non-precipitating zone is the result of soluble antigen-antibody complex formation, then the selective precipitation of gamma-globulin should also render insoluble any bound ^{125}I -labeled Au(1).

It has been possible in the case of many polypeptides and glycoproteins, where the purified antigen is soluble in 50 percent ammonium sulphate to use this salt to precipitate the gamma-globulin moiety and thereby coprecipitate any bound labeled antigen. Farr, RS, J. Infect. Dis. 103, 239 (1958). This partition, however, is not so clearly obtainable with Australia antigen since a significant portion of Au(1) is precipitable in concentrations of ammonium sulphate which insure quantitative precipitation of gamma-globulin. It was found, however, that a satisfactory partition of bound and unbound ^{125}I -labeled Au(1) can be obtained with ammonium sulphate solutions of lesser concentration if critical attention is given to the definite but narrow limits of optimal concentration.

FIG. 7 illustrates the effect of a final ammonium sulphate saturation of 25 percent on the precipitable activity of the standard antibody dilutions and on similarly prepared dilutions from a non-anti Au(1) containing sera. In this procedure after completing the incubation with rabbit anti-human IgG, a volume of 60.9 percent saturated ammonium sulphate sufficient to give a

final saturation of 25 percent was added to each tube. The tubes stood at room temperature for 30 minutes and were then centrifuged at 5000 rpm for 30 minutes. The amount of precipitable radioactivity in the area of anti-Au(1) excess is found to equal the precipitable activity observed at equivalence. In contrast, there is no significant precipitable activity at any dilution of the non-anti Au(1) containing preparation. If, however, the final ammonium sulphate saturation is 30 percent approximately 10 percent of the radioactivity in tubes containing the non-anti Au(1) preparation is precipitated irrespective of the dilution. At 40 percent saturation, this non-specific precipitation increases to 60 percent. Consequently, by limiting the final ammonium sulphate saturation to 25 percent, it is possible to demonstrate anti-Au(1) activity without the necessity of being in the area of equivalence. Therefore qualitative determination of anti-Au(1) activity can be made without the necessity of being in the range of equivalence, reserving the need to perform a full precipitin curve to those specimens that demonstrated initial activity and on which knowledge of a titer is desired.

Using 5 known Ouchterlony double gel-immunodiffusion Au(1) positive sera, 5 anti-Au(1) antibody containing sera, and 5 normal sera, preliminary evaluation of this method of qualitative antibody detection was made. The incubation format was as follows:

10 ul of test sera is incubated with 500 ul of ^{125}I labeled Au(1) at 37°C. for 2 hours and at 4°C. for 22 hours. 50 ul of 1:2 dilution rabbit anti-human IgG is added and incubated at 37°C. for 2 hours and at 4°C. for 18 hours. A volume of 60.9 percent saturated ammonium sulphate at 22°C. is added to produce a final saturation of 25 percent. After 30 minutes the reactant mixture is centrifuged at 5000 rpm for 30 minutes. The radioactivity of both precipitate and vacuum aspirated solution is measured.

The percent of precipitable activity is shown in FIG. 8; the mean precipitable activity for the five normal sera was 2.8 percent $\pm$ 0.5 (1 SD). The precipitable activity of the five antibody containing specimens was from 64 to 68 percent. One of the Au(1) positive specimens (J.R.) demonstrated significant anti-Au(1) activity. This specimen revealed only antigen by Ouchterlony double gel-immunodiffusion and complement-fixation.

DETECTION AND QUANTITATION OF AU(1): DETECTION AND QUANTITATION OF PURIFIED AU(1)

The detection of Au(1) is based on the capacity of added unlabeled Au(1) in the test specimen to compete with labeled Au(1) for a predetermined amount of the standard anti-Au(1) preparation. The selection of the standard antibody dilution to be used is dependent upon the strength of the particular antibody preparation and the amount and nature of the test specimen employed. This determination has to be made for each new preparation of standard antibody or labeled Au(1). In the case of purified Au(1), normal serum components have been excluded, thus permitting the selection of standard antibody to be taken directly from the standard antibody curve. (FIG. 7) A standard antibody dilution is selected such that it is somewhat into the area of labeled antigen excess, therefore insuring

maximal antibody binding and utilization. (See A on FIG. 7.)

If the standard antibody is incubated with unlabeled purified Au(1) prior to the addition of the standard ^{125}I -labeled Au(1), a portion of the available antibody will be bound to unlabeled Au(1). The amount of antibody so bound is a function of the amount of Au(1) added. The prior binding to Au(1) will be reflected by less antibody available to bind ^{125}I -labeled Au(1). Consequently, the amount of precipitable radioactivity will be inhibited as a function of the amount of unlabeled Au(1) added.

The format used to relate the inhibition of precipitable activity to the amount of purified Au(1) added is as follows:

A 10 ul volume in a series of increasing dilutions of purified Au(1) is in each case incubated with 500 ul of 1:20,000 standard antibody at 37°C. for 2 hours, then 4°C. for 22 hours. In the case of each one of the series, the further procedure is as follows: 50 ul ^{125}I -labeled Au(1) is added and the incubation repeated. 50 ul rabbit anti-human IgG is then added followed by incubation at 37°C. for 2 hours, then 4°C. for 18 hours. A volume of 60.9 percent saturated ammonium sulphate at 22°C. is added to give a final saturation of 25 percent. After 30 minutes the reactant mixture is centrifuged at 5000 rpm for 30 minutes. The radioactivity of both the precipitate and the vacuum aspirated supernatant is measured. FIG. 9 demonstrates the relationship between the degree of inhibition of precipitable activity and the amount of purified Au(1) added. With the particular reagent preparations used for the inhibition curve illustrated in FIG. 9, if no purified Au(1) was added, 62 percent of the radioactivity was precipitated. In FIG. 9, the presence of purified Au(1) in the range of 120 uug* was detectable.

(* uug — $\mu\mu\text{g}$ — micromicrogram)

DETECTION AND QUANTITATION OF AU(1) IN UNFRACTIONATED HUMAN SERA

Unlike the detection of purified Au(1), the detection of Au(1) in human sera may be complicated by the possible simultaneous presence of anti-Au(1) antibody, and other serum components. Compensation for the normal serum components can be accomplished by using a sufficient dilution of the test specimen, with or without a constant amount of normal human serum.

If 10 ul of a 1:10 or greater dilution of normal human sera is incubated with the standard antibody dilutions prior to the addition of ^{125}I -labeled Au(1), the resultant curve of precipitable activity is little changed from that which is observed in the absence of normal human sera, or in other words, for the standard antibody curve (FIG. 7). That is, the antibody dilution corresponding to the area of slight ^{125}I -labeled Au(1) excess is still in the range of 1:20,000 to 1:40,000. If however, in the place of normal human sera, a similar dilution of Au(1) positive (by immunodiffusion) sera is added, the precipitable radio-activity for a given dilution of standard antibody will be inhibited as a function of the amount of unlabeled Au(1) present.

The format used for the detection of Au(1) in human serum is the same as that which was used above for the detection of purified Au(1) except that 10 ul of a 1:10 dilution of sera is substituted for the 10 ul dilutions of purified Au(1).

FIG. 10 compares the inhibition of precipitable ^{125}I -labeled Au(1) for the same normal, Au(1) positive and anti-Au(1) containing sera that were qualitatively examined for anti-Au(1) activity in the preceding section. The patients who are Au(1) positive by immunodiffusion exhibit inhibition, including patient J.R. in whose sera antibody activity had previously been demonstrated (FIG. 8).

The quantitation of Au(1) in human sera is accomplished by determining how much purified Au(1) is required to give the same amount of inhibition that is given by the serum being tested. Greatest accuracy is obtained by using a dilution of the test serum such that the degree of inhibition falls along the mid-range of inhibition as observed from purified Au(1) (20 percent to 40 percent on FIG. 9). Following this procedure, the concentration of Au(1) in human serum can be determined. For example, patient J.B. (FIG. 10) has 140 ug Au(1) per ml serum.

TESTING FOR VIRAL SYSTEMS

The present invention provides a general method for testing in viral systems, not limited to hepatitis nor to Au(1). Viral, or viral-associated antigens and/or the antibodies to such antigens can be labeled with radioactive iodine or other suitable isotopes by the procedure here described or by other appropriate procedure, and then used in the method of detection. Examples of other viral systems to which this invention may be applicable would include influenza (flu), mumps, rubella, chicken pox/Herpes zoster, etc. The nature and proportion of the reactants and the manner of separation of bound and unbound complexes requires standardization for each system employed. The method of testing is not limited to serum or plasma or a fraction thereof, since other materials can be tested, for example, urine, cerebral spinal fluid, feces, tissue culture fluid, water supplies, tissue homogenates, etc.

OPTIMAL TESTING PROCEDURE

Specifically to detect the presence of antibody in an unknown sample of serum, radioactive Au(1) antigen is incubated with a certain amount of an unknown sample which may contain anti-Au(1) antibody. Further incubation with rabbit anti-human gamma-globulin is followed by ammonium sulphate in a final concentration of approximately 25 percent saturation. Precipitation is accomplished by centrifugation. The amount of precipitable radioactivity is determined. If antibody is present, radioactivity will be precipitated in proportion to the quantity of antibody. If no antibody is present, insignificant levels of radioactivity will be precipitated.

In testing for Au(1) antigen the preferable technique is to incubate a measured quantity of a standardized anti-Au(1) antibody with the unknown sample. If Au(1) antigen is present in the unknown sample, this will form a complex with the antibody. The mixture is then incubated with radioactively labeled Au(1) antigen. Further incubation with rabbit antihuman gamma-globulin is followed by ammonium sulphate preferably to a final concentration of approximately 25 percent saturation. Precipitation is accomplished by centrifugation and the amount of precipitable radioactivity is determined. If Au(1) antigen was present in the unknown sample, then there will be less precipitable radioactivity because the Au(1) antigen from the unknown will compete with the radioactively labeled Au(1) antigen for

the limited amount of available anti-Au(1) antibody. The degree of competition or inhibition of precipitable activity will be a function of the amount of Au(1) in the test specimen. On the other hand, if the unknown sample did not contain Au(1) antigen, then the precipitable radioactivity will be that expected for the combination of standard anti-Au(1) antibody used and the specific amount of ^{125}I -labeled Au(1) added.

In testing for antigen or antibody, it is possible to add two or more of the appropriate reagents, that is, the ^{125}I -labeled Au(1) antigen, the standard anti-Au(1) antibody, rabbit anti gamma-globulin and the unknown simultaneously or after shorter incubation periods. However, sensitivity is enhanced by performing appropriate sequential additions and allowing suitable intervening incubation conditions and periods.

In order to perform the test for Au(1) antigen in a simple manner, the dilution of standard antibody chosen is approximately one-half way down the right hand side of the curve. (See A of FIG. 7.) A dilution of antibody from this particular part of the curve can be used as the standard antibody dilution for the detection and quantitation of Au(1).

Under the conditions prescribed, the addition of an appropriate dilution of a normal human sera would result in no significant change in precipitable radioactivity. However, the addition of a similar dilution of any serum containing an amount of antigen within the limit of sensitivity of this test would result in a decrease or inhibition of the precipitable radioactivity. The decrease or inhibition is a function of the amount of Au(1) antigen in the unknown. On the other hand, if the unknown contains anti-Au(1) antibody, the additional antibody in combination with the added standard antibody can result in an increased amount of precipitable radioactivity which may be described as "coprecipitation augmentation" or "coprecipitation enhancement." It is, therefore, possible when employing the conditions most desirable for the detection of Au(1) antigen to also be able to detect anti-Au(1) antibody. However, the simultaneous existence of both Au(1) antigen and anti-Au(1) antibody activity in the same specimen may interfere, one with the other, in either the qualitative or quantitative determination of Au(1). For accurate interpretation therefore, this may necessitate the separate determination of anti-Au(1) antibody activity specifically as outlined in the section on detection of anti-Au(1) antibody.

SEPARATION OF BOUND AND UNBOUND LABELED AU(1)

A prerequisite of the radio-immunoprecipitation detection of Au(1) antigen and/or anti-Au(1) antibody is the capacity to separate or otherwise differentiate between antibody-bound labeled Au(1) and unbound labeled Au(1). The method of separation or differentiation employed will depend on the desired purpose, rapidity, sensitivity and convenience. In the method of separation most completely described, a liquid system is used and separation is accomplished by precipitation. The method of separation of antibody-bound from unbound labeled antigen is facilitated by addition of a second antibody directed against human gamma-globulin. The second antibody is produced in rabbits. However, other preparations, (e.g. horse anti-human serum antiserum) can be and have been successfully employed. Similarly, heterologous antibody against a human gamma-

globulin moiety other than IgG (e.g. IgM) can, has, and may be more desirable to use when certain human antibody types or preparations are being determined. A heterologous anti-Au(1) antibody may be used as the first antibody and with it an appropriate second antibody, selected from another species when the heterologous anti-Au(1)-bound labeled Au(1) complex fails to be easily differentiated from unbound labeled Au(1).

Furthermore, the separation, as by precipitation has been facilitated by the addition of ammonium sulphate to a final saturation of approximately 25 percent. Certain other salts or ions can and have been employed (e.g. sodium sulphate) to attain adequate separation, or cause a desired influence on the dynamics or kinetics of the reaction. Furthermore, separation of precipitable activity can be attained by means other than the described centrifugation (e.g. porous gels, membranes, filters, etc.).

By appropriate manipulation, separation or differentiation of antibody-bound labeled antigen from unbound labeled antigen can be achieved by means other than precipitation in a liquid system. In particular, the capacity of some molecules to adhere to particular surfaces or particles without significant depreciation of immunoreactivity can be utilized to facilitate the separation of antibody-bound labeled antigen from unbound labeled antigen. The particular reactant that is made adherent is effectively then a part of a solid surface and can be easily separated from a liquid member of the reactants. Separation by this method is applicable if either the viral antigen or antibody is made adherent to the particle or surface. In the case of the detection of Au(1) it is preferable to permit the anti-Au(1) antibody to adhere to the particle or surface. Adherence, coupling, or linkage to certain particulate substances (e.g. agarose, cellulose, bentonite, etc.) is often dependent upon a specific chemical reaction. However, adherence to some materials, e.g. polystyrene, polypropylene, can be effectively achieved with minimal chemical manipulation. Polystyrene, polypropylene, or similar material may be used in the form of particulate beads, spheres, fragments, etc., or as a preformed test tube, cylinder, or flat surface. Selective adsorption of anti-Au(1) antibody is most satisfactory if other proteins have been removed from the solution containing the antibody. The anti-Au(1) antibody preparation in a neutral or somewhat alkaline buffer is incubated in contact with the polystyrene or polypropylene surface to which adherence is desired. Following incubation, the residual antibody solution is washed from the tube and incubation is performed with an unrelated protein solution, e.g. BSA, to insure surface coverage by protein. If anti-Au(1) antibody has been adsorbed, then subsequent incubation of labeled Au(1) antigen in the presence of the surface will result in the binding of labeled Au(1) to the adsorbed antibody in proportion to the amount of antibody adsorbed. The previous exposure to an excess of unrelated antigen, e.g. BSA, effectively blocks the non-immune adherence of labeled Au(1) to the surface. If prior to the addition of labeled Au(1), the surface with adsorbed anti-Au(1) antibody was incubated with a specimen containing unlabeled Au(1) then a portion of the adsorbed antibody binding sites will bind the unlabeled Au(1), decreasing the sites available to bind labeled Au(1) and therefore inhibiting the amount of radioactivity that can bind to the adsorbed anti-Au(1).

A modification of this purpose can be used to obtain the "purification" of anti-Au(1) antibody for adsorption to the adhering surface, as described above. Purified Au(1) is incubated in contact with the surface and in the absence of other proteins. If labeled Au(1) is used for this purpose, the degree of adherence and any subsequent loss can be monitored. Following incubation and removal of excess purified Au(1), protein saturation of the surface is insured with an unrelated protein solution (e.g. BSA). Human or other serum with anti-Au(1) activity is then incubated in contact with the adhered purified Au(1). Following incubation, the excess serum is thoroughly washed from the surface. The surface is now composed of adherent purified Au(1) and an unrelated adherent protein (e.g. BSA). A portion of the anti-Au(1) antibody from the serum will be immunologically bound to the purified Au(1), while the balance of the proteins from the serum will have been washed out. Elution and harvest of the anti-Au(1) antibody can now be accomplished by washing the surface with an acid buffer, e.g. glycine-HCl, thereby dissociating the antigen-antibody complex while the antigen remains adherent to the surface. If labeled purified Au(1) was used, the degree of contamination of eluted antibody with Au(1) can be determined. If small quantities of purified antibody are sought, it is convenient to perform the procedure with preformed tubes, e.g. polystyrene test tubes. If large quantities are desired, it is convenient to form a column with the surface material as packing.

It will be evident that other methods of separating the complex from non-reacting constituents may be employed although the separation by centrifugation in some cases is preferred. Examples of other ways of separation that may be used include electrophoresis, chromatography, chromatoelectrophoresis and ion exchange resin.

All percentages are by weight unless the context indicates otherwise, as in percentage radioactivity precipitated. Ammonium sulphate solutions are expressed as volume percent saturation.

It will be evident that while it is preferred to radioactively label Au(1) antigen with appropriate modification the test procedure of the invention could be accomplished by radioactively labeling anti-Au(1) antibody. In order to obtain satisfactory specificity, however, anti-Au(1) antibody would have to be purified.

As has already at least partly been indicated in the Abstract, in carrying out the present invention we use a radioactive element. Others can be used, but radioactive iodine is preferred and most desirably we use ¹²⁵I, this last being, all things considered, the best such thing now available for our purposes. ¹²⁵I has a longer half life than ¹³¹I, for example, which is an advantage in most cases.

From the preceding part of this description, the utility of the present invention in diagnosis relative to a particular type of viral hepatitis, as for example especially in the case of human beings, will be evident. The same is true especially as to the detection of a particular type of viral contamination in blood plasma and the like for use in humans. However, the present invention will be useful in detecting the presence of such an antigen or its antibody if it should happen to be present in other biological fluids and not merely in human sera, as for example the body fluids of the lower animals, tissue culture fluids, water supplies, etc.

When in the claims we mention an antibody-bound labeled antigen, this is well-known terminology for a complex made up of labeled antigen and the antibody which has associated itself with the labeled antigen to form the particular complex.

We refer to the procedure involving the separation of antibody-bound labeled Au(1) complexes from unbound labeled Au(1) by centrifugation as "radioimmunoprecipitation." It is recognized that the procedure described, mainly because of the use of a radioactive isotope, may also be referred to as "radioimmunoassay". This latter term, although acceptable from the standpoint of common usage, has been resisted because it is felt that radioimmunoprecipitation more closely describes the nature of the process, and in the more strict sense, except in arbitrary relative terms, an actual "assay" is not always performed. An exception to this is when an appropriate degree of precipitation-inhibition due to unlabeled Au(1) in the test specimen is equated to the amount of purified Au(1) by weight, the results in an identical degree of inhibition under the same conditions.

In view of our invention and disclosure, variations and modifications to meet individual whim or particular need will doubtless become evident to others skilled in the art, to obtain all or part of the benefits of our invention without copying the process and composition shown, and we therefore claim all such insofar as they fall within the reasonable spirit and scope of our claims.

Having thus described our invention, what we claim as new and desire to secure by Letters Patent is:

1. A process of determining the presence of human anti-Au(1) antibody to Australia antigen Au(1) in human serum, which comprises incubating radioactively labeled Australia antigen Au(1) and an unknown sample of human serum to form antibody-bound labeled antigen if the unknown sample of human serum contains human anti-Au(1) antibody, adding heterologous anti-human gamma globulin, then centrifuging to precipitate the radioactive complex and thus getting separation between the antibody-bound labeled antigen and unbound materials, and measuring the radioactivity of at least one of the materials mentioned above as separated from each other.

2. The process of claim 1, which comprises also adding ammonium sulphate before centrifuging.

3. The process of claim 2, in which the anti-human gamma globulin is rabbit anti-human gamma globulin.

4. A process of claim 3, in which the concentration of ammonium sulphate at the time of precipitation is about 25 percent saturation.

5. A process of claim 4, in which the Australia antigen Au(1) is labeled with radioactive iodine.

6. A process of claim 5, which comprises labeling the Australia antigen Au(1) by reacting it with chloramine-T in the presence of radioactive iodine and then reacting it with sodium meta-bisulphite.

7. A process of determining the presence of Australia antigen Au(1) in human serum, which comprises incubating a predetermined amount of anti-Au(1) antibody, an unknown sample of human serum, and radioactively labeled Australia antigen Au(1) to form antibody-bound labeled antigen whose radioactivity depends upon the composition of the unknown sample, adding heterologous anti-human gamma globulin, then centrifuging to precipitate the radioactive complex and thus getting separation between the antibody-bound

labeled antigen and unbound materials and measuring the radioactivity of at least one of the materials mentioned above as separated from each other.

8. The process of claim 7, which comprises also adding ammonium sulphate before centrifuging.

9. The process of claim 8, in which the anti-human gamma globulin is rabbit anti-human gamma globulin.

10. A process of claim 9, in which the concentration of ammonium sulphate at the time of precipitation is about 25 percent saturation.

11. A process of claim 7, in which the Australia antigen Au(1) is radioactively labeled with radioactive iodine.

12. A process of claim 11, which comprises labeling the Australia antigen Au(1) by reacting it in the presence of radioactive iodine with chloramine-T and then reacting it with sodium meta-bisulphite.

13. A process of determining the presence of Australia antigen Au(1) in human serum, which comprises incubating a mixture of standardized anti-Au(1) antibody and an unknown sample of human serum, adding radioactive Australia antigen Au(1) to the mixture, further incubating to form a complex whose radioactivity will depend upon the composition of the unknown sample, adding heterologous anti-human gamma globulin, then centrifuging to precipitate the complex and measuring its radioactivity.

14. The process of claim 13, which comprises also adding ammonium sulphate before centrifuging.

15. The process of claim 14, in which the anti-human gamma globulin is rabbit anti-human gamma globulin.

16. A process of claim 15, in which the concentration of ammonium sulphate is about 25 percent of saturation.

17. A process of claim 16, in which the Australia antigen Au(1) is labeled with radioactive iodine.

18. A process of claim 17, which comprises labeling the Australia antigen Au(1) by reacting it in the presence of radioactive iodine with chloramine-T and then reacting it with sodium meta-bisulphite.

19. A process for detecting Australia antigen Au(1), which comprises depositing on an antibody-attaching surface an antibody which will adhere to the surface and incubating it in contact with the surface, introducing an unknown sample and incubating it, removing the liquid portion, introducing and incubating a radioactively labeled antigen, removing any excess of unreacted radioactive component, and measuring the radioactivity that adheres to the surface.

20. A test reagent for determining the presence of Australia antigen Au(1) or anti-Au(1) antibody in human sera, which essentially consists of Australia antigen Au(1), a viral moiety, conjugated with a radioactive element.

21. A test reagent for determining the presence of Australia antigen Au(1) or anti-Au(1) antibody in human sera, which essentially consists of Australia antigen Au(1), a viral moiety conjugated with radioactive iodine.

22. A test reagent for determining the presence of Australia antigen Au(1) or anti Au(1) antibody in human sera, which essentially consists of anti Au(1) antibody conjugated with a radioactive element.

23. A test reagent for determining the presence of Australia antigen Au(1) or anti Au(1) antibody in human sera, which essentially consists of anti Au(1) antibody conjugated with radioactive iodine.

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24. The process of making material for use in testing for Australia antigen Au(1) or anti Au(1) antibody which comprises taking purified Australia antigen Au(1) and labeling it with radioactive material.

25. The process of claim 24, in which the radioactive material is radioactive iodine.

26. The process of claim 25, in which the radioactive iodine is ^{125}I .

27. The process of making material for use in testing for Australia antigen Au(1) or anti Au(1) antibody which comprises purifying anti Au(1) antibody by binding purified Australia antigen Au(1) to a solid sur-

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face, adding an antiserum containing anti-Au(1) antibody as part thereof, allowing this antibody to react with Australia antigen Au(1) which has adhered to the surface, and dissociating the antibody from the antigen by acid buffer wash, and labeling the anti-Au(1) antibody with radioactive material.

28. The process of claim 27, in which the radioactive material is radioactive iodine.

29. The process of claim 28, in which the radioactive iodine is ^{125}I .

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