United States Patent

Catt

[15] 3,646,346

[45] Feb. 29, 1972

[54] ANTIBODY-COATED TUBE SYSTEM FOR RADIOIMMUNOASSAY

[72] Inventor: **Kevin J. Catt,** Melbourne, Australia

[73] Assignee: **Pharmacia AB,** Uppsala, Sweden

[22] Filed: **Dec. 26, 1968**

[21] Appl. No.: **786,959**

[52] **U.S. Cl.** **250/83,** 23/230, 206/84, 250/106, 424/12

[51] **Int. Cl.** **G01t 1/16**

[58] **Field of Search** .....250/83 SA, 106 T; 23/230 B; 195/103.5; 424/12; 206/84

[56] **References Cited**

UNITED STATES PATENTS

3,372,992	3/1968	Storey	23/230 B
3,380,888	4/1968	Numerof et al.	23/230 B
3,451,777	6/1969	Di Giulio	250/83 SA X

Primary Examiner—Archie R. Borchelt

Attorney—Fred C. Philpitt

[57] **ABSTRACT**

A radioimmunological method and means for the determination of proteins in aqueous samples which includes adsorbing on the internal surface of a test tube of water-insoluble polymeric material the antibody against the protein to be determined. Suitable proteins which can be determined according to this method are plasma proteins, enzymes, and many hormones.

8 Claims, 3 Drawing Figures

FIG. 1.

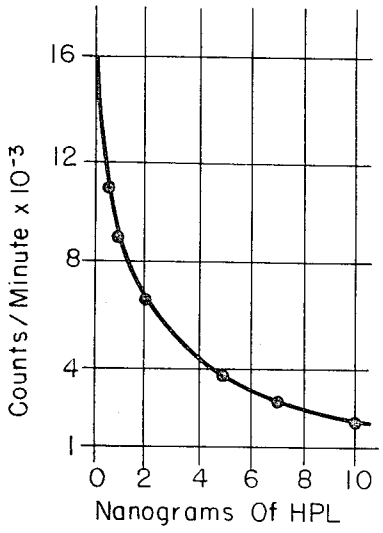


FIG. 2.

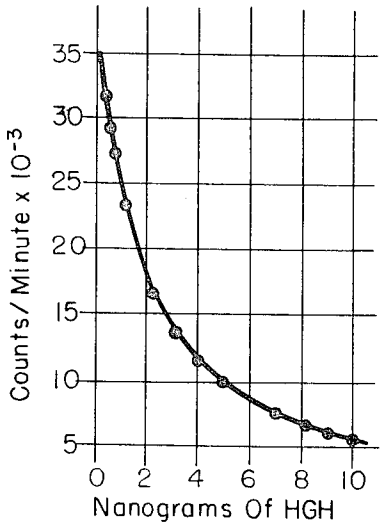
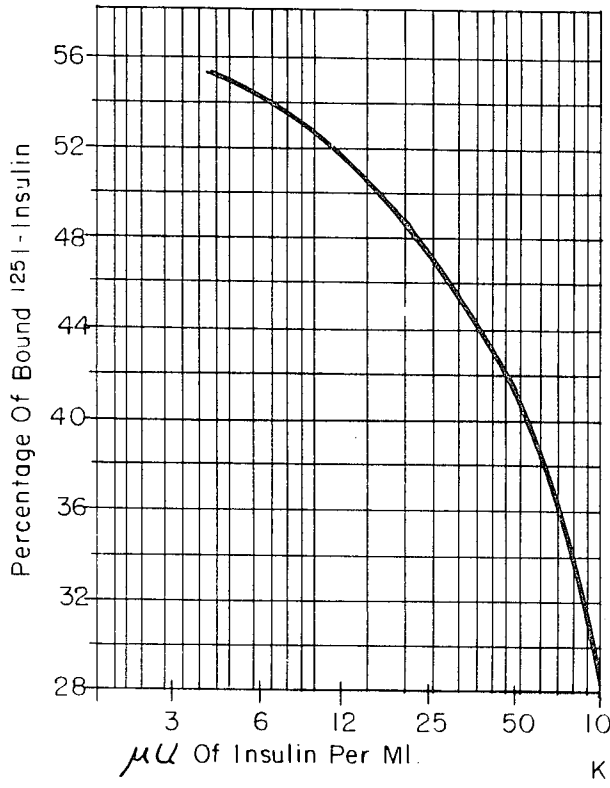


FIG. 3.



INVENTOR
Kevin J. Catt

BY Fred Philpott

ATTORNEY

ANTIBODY-COATED TUBE SYSTEM FOR RADIOIMMUNOASSAY

The present invention relates to a method and means for the determination of proteins, for instance protein hormones, in aqueous samples, e.g., from body fluids such as blood serum or urine, but also from other sources such as different types of gland extracts. An essential factor of the method is that the substance to be determined is capable of acting as an antigen, i.e., is capable of causing the formation of antibodies against itself in animals.

In the following specification and in the annexed claims the term "protein" is intended to include proteins, polypeptides and peptides.

The invention is based partly upon the knowledge that under certain circumstances proteins are generally able to act as antigens, i.e., able to cause the formation of antibodies, and partly on the fact that radioimmunological methods are very sensitive and well suited for determining different proteins present in a very low concentration in body fluids.

Radioimmunological methods are in general based on the ability of an antibody to bind its protein antigen irrespective of whether the latter is labeled with, for example, a radioactive isotope, or not. The binding of labeled and unlabeled protein antigens takes place in proportion to the concentration of labeled and unlabeled, respectively, proteins. The radioactivity of the labeled protein which is bound to the antibodies, and/or of the free, labeled protein in the sample liquid is measured. The amount of unlabeled competing protein can be determined from the obtained values by calculation or by direct comparison with a standard curve.

In principle, radioimmunological methods can be applied to proteins which are antigenic, capable of being purified and labeled with a radioactive isotope or a fluorescent group. The antibody bound protein has to be separated from the unbound protein.

The present invention is based on the ability of a polymeric material coated, by adsorption, with antibodies against the protein to be determined to bind specifically the same protein labeled with a radiation emitting atom or group such as a radioactive isotope. The use of antibodies in this form allows rapid removal of the free labeled protein by washing of the solid phase with water on completion of the immune reaction. The solid-phase material, that is the labeled protein bound to the antibodies on the polymer, can then be counted for quantitation of the bound labeled substance, which varies inversely with the total quantity of protein in the original sample. This simple and sensitive procedure can be used to measure very small quantities of the protein in, for example, plasma.

In connection with the conception of the present invention it has been found that various polymeric materials present applicability to the above determination method based on solid-phase radioimmunoassay. It has been apparent that certain polymers may adsorb antibodies that can then bind an adequate quantity of labeled protein or polypeptide for use in the assay. In contrast, adsorption of antibodies to glass is negligible. The adsorption of antibodies by polymeric surfaces, from antiserums preassume that the antiserum is of moderately high titer.

The adsorption process has been applied to the immunoassay above mentioned by coating of the interior of plastic tubes with uniform quantities of specific antibodies. The excellent practical results and advantages obtained with the invention is ascribed to the use of the above plastic tube for adsorbing the antibody material.

The method according to the invention comprises contacting the internal surface of a test tube of water-insoluble polymeric material capable of adsorbing antibodies against the protein to be determined, at least part of said surface being coated with a layer of said antibodies by adsorption, with the aqueous sample containing the protein and with the same protein in labeled form capable of emitting radiation to bind part of said labeled and unlabeled protein to said antibodies adsorbed on the surface of the polymeric material to produce a two-phase system comprising a solid phase comprising said bound part of labeled and unlabeled protein and a liquid phase comprising unbound labeled and unlabeled protein, separating said two phases from each other, and measuring the emitted radiation of at least one of said solid and said liquid phases, the value of said radiation being each a function of the concentration of the protein in the aqueous sample.

The labeled protein can be labeled with, for example, a radioactive isotope or a fluorescent group.

The method can be used for qualitative and quantitative determination.

A method is previously known in which the antibodies are attached to particles of a water-insoluble carrier by covalent bonds. The labeled protein reacting with the antibodies in the determination can thus be readily separated from the unbound labeled protein, the separation being insensitive to variations in the salt and protein concentration of the liquid within physiological limits.

In comparison with the above-mentioned excellent method the present one offers several additional advantages. Thus, it will be very easy to prepare the reagent comprising antibodies in that a solution of said antibodies only need to be contacted with the inside of the tube of polymeric material. After the removal of the liquid containing the antibodies from the surface of the test tube the antibodies will be bound by adsorption with sufficiently high firmness to render it possible to transport the tube without any special precautions. (If particles are used as in the previously described method steps must be taken so that they retain in the tube during transportation.) The steps of the determination will be very easy to carry out which is of value in routine analysis carried out in hospital laboratories. The separation of the solid and liquid phases, which is the essence of the method before measuring the radiation can be carried out by simply removing the liquid from the test tube rather than separating by such methods as filtration and centrifugation, said latter methods being also connected with risks that the separation be incomplete.

The method requires access to the protein to be determined for producing antibodies and for preparing radioactive labeled proteins, and suitably also for obtaining standard solutions, for instance, for obtaining standard curves.

Examples of proteins against which antibodies can be obtained are plasma proteins, enzymes and many hormones. Examples of such hormones are insulin, gonadotropins, growth hormone, ACTH, thyrotropin and parathormone.

The antibodies against the protein can be prepared by any method known per se, by immunizing animals used for experiments, by, for instance, repeated subcutaneous injections of small amounts of the antigenic protein possibly combined with a so-called adjuvant such as Freund's mineral oil emulsion, into the animal. The antibodies produced in the animals can be recovered from the blood serum of the same. The protein fraction, which contains the antiserum, can be recovered by conventional methods, e.g., by precipitating the serum with suitable amounts of a saturated aqueous solution of ammonium sulphate.

Labeling of the protein with a radioactive isotope can be effected in a conventional manner, a suitable isotope for the purpose being selected, e.g., I^{125}, I^{131}, C^{14} or H^3. A particularly suitable isotope is a radioactive isotope of iodine such as I^{125}, as labeling with this isotope is simple and as many hospital laboratories now have the equipment necessary to measure this isotope.

As test tubes of polymeric materials may be mentioned ordinary plastic test tubes for laboratory purposes. As polymeric materials may be mentioned polystyrene, polyethylene, polypropylene, nitrocellulose, copolymers of acrylnitril with styrene such as poly(styrene-co-acrylonitril).

The radioactivity determinations can be effected by common methods, e.g., by means of scintillation detectors.

The amount of labeled protein, e.g., I^{125} hormone, added in the reaction is chosen so that, for instance, 20–60 percent of the labeled hormone can be bound to the antibodies when no competing unlabeled hormone is present. The incubation is

preferably made at temperatures between +4° and 37° C. and commonly at room temperature. It is not necessary for the reaction between the antigen and the antibodies to go to completion. The reaction is interrupted after, for instance, 24 hours, but may also be stopped earlier, for instance, after 2–4 hours. It is important that the reaction time and temperature are the same for the sample solutions and standard solutions.

Because the method is simple, rapid and practical, and gives accurate analysis results it is well suited for quantitative determinations also for routine usage and permits determination of even very small amounts of sample substances.

The invention also encompasses a means for carrying out the above-mentioned method. This means comprises a first reagent comprising a test tube of polymeric material capable of adsorbing antibodies against the protein to be determined, the internal surface of said test tube having at least part thereof coated by adsorption with said antibodies, and a second reagent comprising labeled protein capable of emitting radiation, said first reagent being intended to be contacted with the sample containing the protein and with the second reagent to bind part of the said labeled and unlabeled protein to the adsorbed antibodies thereby to produce a two-phase system comprising a solid phase comprising said bound part of labeled and unlabeled protein and a liquid phase comprising unbound labeled and unlabeled protein, the emitted radiation being each a function of the concentration of the protein.

According to a preferred embodiment of the invention the means is in the form of a test pack.

In the accompanying drawings there are shown examples of standard curves which were used in connection with practicing the present invention. The following is a brief description of the various figures:

FIG. 1 shows a standard curve obtained by plotting radioactivity (measured as counts per minute $\times 10^{-3}$) along the ordinate against amount of human placental lactogen in nanograms along the abscissa (see Example 1 below),

FIG. 2 shows a standard curve obtained by plotting radioactivity (measured as counts per minute $\times 10^{-3}$) along the ordinate against amount of human growth hormone in nanograms along the abscissa (see Example 2 below), and

FIG. 3 shows a standard curve obtained by plotting percentage of bound ^{125}I-insulin (based on the total amount of ^{125}I-insulin added) along the ordinate against concentration of insulin in μU per ml. along the abscissa (see Example 6 below).

The invention will be more closely illustrated in the following with reference to detailed examples.

EXAMPLE 1

Determination of Human Placental Lactogen (HPL) in plasma

a. Preparation of antibodies

Rabbits were injected with 5 mg. quantities of purified human placental lactogen. The placental lactogen was emulsified in a 1:1 mixture of saline and complete Freund's adjuvant, and injected subcutaneously at intervals of 3 weeks for a period of 3–6 months. After this time, two of four rabbits were found to have a satisfactory titre of antibodies to placental lactogen, showing reactions of partial identity with human growth hormone on gel diffusion. These antisera were used to coat tubes for the assay without further fractionation.

b. Preparation of antibody-coated tubes

Polystyrene blood collection tubes were coated with 1.0 ml. of a 1 in 500 dilution of anti-HPL serum in 0.05 M bicarbonate buffer pH 9.6. After incubation for 2–16 hours at room temperature, the contents were aspirated and the tubes washed with saline and 10 percent aged human serum as described above.

c. Preparation of labeled HPL

Purified HPL was labeled with ^{125}I by the chloramine-T procedure as described above for human growth hormone. The resulting iodinated peptide had a specific activity of 100–120 mμc/mμg, and was stored frozen in 0.5 percent bovine serum albumen in 0.15 M NaCl until used for the assay. This material was stable for up to 6 weeks when stored frozen.

d. Measurement of HPL in plasma and urine

Standard HPL solutions in 20 percent horse serum and a final volume of 1.0 ml. were incubated in the coated tubes with 0.25 ml. of buffer containing 100,000 counts per minute of ^{125}I human placental lactogen. Plasma or urine samples were similarly diluted with a solution of 20 percent horse serum in 0.15 M NaCl containing 0.01 M phosphate buffer pH 7.4 and 0.01 percent merthiolate. An identical aliquot of ^{125}I-human placental lactogen was added to the sample tubes. After incubation of standards and samples at 37° for 16 hours, the contents of the tubes were aspirated and washing was performed twice with tap water followed by counting for 1 minute in an automatic gamma counter. A standard curve was constructed by plotting bound counts versus added HPL, and as with the HGH assay described above, a linear form of the standard curve could be obtained by plotting a reciprocal function of the bound count against added HPL. The concentration of HPL in plasma and urine samples was estimated by reading off the standard curve, which was performed with each assay. HPL was not detected in the plasma or urine of normal subjects, and was found to be present at a level of 36 nanograms/ml. at the ninth week of pregnancy, rising to 33,000 ng./ml. at the 26th week.

On accompanying drawing FIG. 1, there is shown a standard curve obtained with tubes coated with 2.0 ml. of antiserum to human placental lactogen (HPL) diluted 1:250. Incubation has been performed in quadruplicate for 64 hours at 37° C. with 0 to 10 ng. of HPL and 118,000 counts per minute ^{125}I-HPL in 2.2 ml. of 20 percent horse serum.

In this connection, the tube count (counts per minute) $\times 10^{-3}$ has been plotted along the ordinate as a function of the amount of HPL measured in nanograms along the abscissa to obtain the standard curve as shown.

The curve can be used as a basis for determining the amount of HPL in unknown samples in the range of from 0 to 10 nanograms.

EXAMPLE 2

Determination of Human Growth Hormone (HGH) in blood plasma

a. Preparation of antibodies

Rabbits were injected subcutaneously with 2.5 mg. human growth hormone in saline: complete Freund's adjuvant: 1:1 at intervals of 3 weeks for a total period of 3–5 months. Blood from the animals with the highest antibody titre was then harvested over the following several weeks and the serum stored frozen. These antisera were used to coat plastic tubes without further fractionation.

b. Preparation of antibody-coated tubes

Polystyrene blood collection tubes (90×15 mm.) were used for most of the assays. These polystyrene tubes were coated with antibody by adding 1.0 ml. of a 1 in 500 dilution of anti-HGH serum in 0.05 M bicarbonate buffer pH 9.6, then standing the tubes for 2–24 hours. After this period, the contents were aspirated and the tubes washed out three times with 0.15 M NaCl and once with 10 percent aged human plasma in 0.15 M in NaCl containing 0.01 percent merthiolate.

The antisera obtained from various rabbits were diluted over a range 1:500–1:25,000 to determine the maximum dilution which could satisfactorily be used for the assay. As expected, the most diluted solutions gave somewhat more sensitive assays, though the relationship between dilution and sensitivity was much less direct than that observed in the conventional liquid-phase assay. For most assays, a standard antiserum was used at a dilution of 1:10,000, giving very satisfactory results over the range 0.4–10 nanograms/ml. plasma.

c. Preparation of labeled human growth hormone

Human growth hormone was labeled with ^{125}I by a modification of the technique of Hunter & Greenwood. In this

procedure, 5–10 μg. of purified HGH was reacted with 2 millicuries of ^{125}I by the Chloramine T method, using a specially designed disposable radioiodination pipette. The labeled HGH was then isolated by fractionation of the reaction mixture on a column of Sephadex G 75, or on a column of cellulose. This procedure uniformly gave labeled HGH of specific activity of 100–150 microcuries per microgram, a level found to be satisfactory for use in this form of radioimmunoassay. The labeled hormone was collected into 5 percent bovine serum albumen in 0.15 M NaCl, and stored frozen for up to 3 weeks for use in the radioimmunassay.

d. Measurement of HGH in plasma

To estimate HGH levels in human plasma, specimens were diluted 1 in 5 with a diluent solution consisting of 5 percent aged human plasma in 0.15 M NaCl containing 0.01 M phosphate buffer pH 7.4 and 0.01 percent merthiolate. Standard HGH solutions were prepared in a solution consisting of 20 percent serum in a similar diluent solution, and levels of 0.25–5 nanograms/ml. were set up in each standard curve. The total volume of standards and diluted serums was 1.0 ml., to this was added 0.25 ml. of diluent containing 10^5 c.p.m. of ^{125}I HGH. The final incubation volume then exceeded the coated volume of the tube by 0.25 ml. The tubes were incubated overnight at 37° C., then the contents were aspirated and each tube was washed out twice with tap water and counted for 1 minute in an automatic gamma counter. The counts obtained for the standard HGH solutions were used to construct a standard curve from which the quantities of HGH present in the unknown samples could be read. It has been shown that the relationship between any reciprocal function of the bound count, i.e., the count attached to each tube is proportional to the quantity of growth hormone present in the tube, so that a straight line can be obtained by plotting the levels of the standard growth hormone versus any reciprocal function of the bound count. Alternatively, the bound count can be plotted directly against added HGH concentration, giving a hyperbolic standard curve.

The FIG. 2 shows a standard curve obtained in analogy with FIG. 1 with tubes coated with 1.0 ml. of antiserum to HGH, diluted 1:500. Incubation has been performed for 20 hours at 37° C. with 0 to 10 ng. of HGH and 95,000 counts per minute ^{125}I - HGH in 1.2 ml. of 20 percent horse serum.

By this method, the levels of HGH present in normal plasma were found to be between 1 and 10 nanograms/ml. The level fluctuates considerably in normal subjects, sometimes reaching spontaneous peaks of up to 25 nanograms per ml. In hypopituitary individuals, such peaks are not observed, and the basal level of less than 1 nanogram/ml. does not rise during insulin hypoglycaemia as it does in the normal subject. In acromegalic subjects, the level of HGH was between 12 and 200 nanograms/ml., and these levels were not suppressed following glucose ingestion, unlike the elevated levels sometimes observed in the normal individual.

EXAMPLE 3

Measurement of Luteinizing Hormone (LH) and Human Chorionic Gonadotropin (HCG) in human plasma and urine

Tubes were coated with anti-HCG serum (1:2000) and used to measure LH and HCG in human plasma and urine, employing ^{125}I labeled LH as tracer and purified LH as standards. Levels of 1–2 ng./ml. were found in plasma in normal males and females rising to 7–13 ng./ml. at the time of ovulation, and following the menopause.

EXAMPLE 4

Estimation of Luteinizing Hormone (LH) in the plasma of the sheep and cow

Antisera to purified ovine and bovine LH prepared in the horse and rabbit were used to coat polystyrene tubes, at dilution of 1:2,000–1:10,000. Such coated tubes were used to establish an assay for estimation of LH in sheep and cow plasma. Basal levels in female animals were 1–4 ng./ml., rising to high levels (up to 200 ng./ml.) at the time of ovulation.

EXAMPLE 5

Estimation of Tetanus Toxin by radioimmunoassay

Tubes were coated with anti-tetanus serum diluted 1:1000, and purified tetanus toxin was labeled with ^{125}I by the chloramine-T method. By this procedure, small concentrations of tetanus toxin, down to the range of 10–100 nanograms/ml. could be estimated.

EXAMPLE 6

Determination of insulin in blood plasma

a. Preparation of antibodies

Guinea pigs were each injected subcutaneously with 0.1 mg. of pig insulin in 1 ml. of Freund's adjuvant. Immunization was repeated every week for 4 weeks. After further 2 weeks, blood was drawn off from the guinea pigs and antiserum recovered from the blood by allowing the same to coagulate, and removing the clots of blood.

The antibody fraction was precipitated from this antiserum by treatment with 18 percent sodium sulphate.

The precipitate was separated by centrifugation. The precipitate was washed two times with a 18 percent sodium sulphate solution. Subsequent to the last washing the precipitate was dissolved in original serum volume of an aqueous solution of sodium hydrogen carbonate, after which dialysis took place against 0.05 M sodium hydrogen carbonate solution. This antibody fraction was used for the preparation of antibody coated tubes.

b. Preparation of antibody coated tubes

Polystyrene test tubes (55×10 mm.) were used as the starting material. To the tubes was added 1 ml. of a 5,000 times diluted gammaglobulin solution of the anti-insulin serum, in 0.05 M carbonate-bicarbonate buffer of pH 8.3. The tubes were incubated at 37° C. for 1 hour and then carefully washed two times with 0.9 percent sodium chloride solution containing 1 percent human serum albumen and finally with 0.05 M phosphate buffer, pH 7.4 containing 0.05 percent NaN_3, 0.9 percent NaCl, 0.3 percent human serum albumen and 0.05 percent Tween 20. (This buffer will be called "incubation buffer.")

c. Preparation of labeled insulin

Pig insulin was labeled with ^{125}I according to the following method: 5 mC ^{125}I in the form of NaI was oxidized with Chloramine T in the presence of 5 μg. of insulin in accordance with a method described by Hunter and Greenwood (ref. Nature/London/ , volume 194/1962/, page 495). Subsequent to the labeling sodium dithionite was added to convert the remaining amount of iodine to soluble iodide. The obtained insulin labeled with ^{125}I was mixed with human plasma-albumen and separated from low molecular weight products and from denaturation products of insulin bound to the plasma-albumen by gel filtration on a copolymer of dextran with epichlorohydrin (Sephadex G-50). The insulin labeled in this way has a specific activity of 100–200 mC per mg. The second peak of the labeled protein fraction was collected in a small vessel containing ½ ml. of a solution of human plasma-albumen containing 50 mg. per ml. The labeled hormone was stored in cold surroundings and diluted before being used.

d. Determination

The analyses were performed in the antibody coated test tubes.

1. 0.5 ml. of "incubation buffer" was added to the tubes.
2. 0.1 ml. of the plasma to be tested was added to quadruplicate tubes.
3. 0.1 ml. of a solution containing different concentration of the hormone, e.g., 200, 100, 50, 25, 10, 5 and 2.5 μU insulin/ml. and 0 μU insulin/ml. were distributed to different tubes in quadruplicates.
4. 0.1 ml. of a solution containing ^{125}I-insulin (approx. 1 nanogram per ml.) was added to all tubes.
5. 0.5 ml. of "incubation buffer" was added to each tube.
6. Incubation took place at +4° C. for 64 hours.
7. The tubes were either washed two times with the "incubation buffer" or twice with distilled water. After the last

removal by suction of the washing liquid, the tubes were placed in a counter for estimating the gamma radiation from the antibody bound labeled hormone.

The number of "counts" per time of standard tubes was determined and converted into per cent of bound ^{125}I - insulin based on the total amount of ^{125}I - insulin added to the tubes. The percentages obtained were plotted along the ordinate against the concentration of insulin in μU per ml. along the abscissa to form the curve as shown in diagram 3. From this diagram the concentration of insulin in the unknown sample could be easily determined.

What I claim is:

1. A method for the determination of proteins in aqueous samples wherein said proteins are capable of acting as antigens which comprises:

a. coating by adsorption at least part of the internal surface of a test tube of water-insoluble polymeric material with antibodies against the protein to be determined;
b. said water-insoluble polymeric material being capable of adsorbing said antibodies;
c. contacting the internal surface of said test tube with the aqueous sample containing the protein;
d. contacting the internal surface of said test tube with the same protein in labeled form capable of emitting radiation;
e. contacting steps (c) and (d) causing the bonding of part of the labeled protein and part of the unlabeled protein to said antibodies adsorbed on the surface of the polymeric material;
f. producing a two-phase system;
g. said two-phase system comprising a solid phase and a liquid phase;
h. said solid phase comprising the bound part of the labeled and unlabeled protein;
i. said liquid phase comprising the unbound part of the labeled and unlabeled protein;
j. separating said two phases from each other;
k. and measuring the emitted radiation of at least one of said phases,

wherein the value of said radiation being a function of the concentration of the protein in the aqueous sample.

2. The method of claim 1 wherein the protein is labeled with a radioactive isotope of iodine.

3. The method of claim 2 wherein said radioactive isotope of iodine is I^{125}.

4. The method of claim 1 wherein said water-insoluble polymeric material is selected from the group consisting of polystyrene, polyethylene, polypropylene, nitrocellulose, and copolymers of acrylonitrile with styrene.

5. The method of claim 1 wherein said concentration of the protein is obtained by direct comparison with a standard curve.

6. The method of claim 1 wherein said protein is selected from the group consisting of human placental lactogen, human growth hormone, luteinizing hormone, human chorionic gonadotropin, tetanus toxin, and insulin.

7. Means for the determination of proteins in aqueous samples, comprising:

a. a first reagent and a second reagent;
b. said first reagent including a test tube of a polymeric material which is capable of adsorbing antibodies against the protein to be determined;
c. the internal surface of the test tube being at least partially coated with the antibodies;
d. said second reagent comprising labeled protein capable of emitting radiation;
e. the first reagent being intended to be contacted with the sample containing the protein and with the second reagent to bind part of the labeled and unlabeled protein to the adsorbed antibodies to produce a two-phase system;
f. the two-phase system comprising a solid phase and a liquid phase, wherein said solid phase includes the bound part of the labeled and unlabeled protein and the liquid phase includes the unbound labeled and unlabeled protein; and
g. the emitted radiation of each phase being a function of the concentration of the protein.

8. Means according to claim 7 in the form of a test pack.

* * * * *

Disclaimer

3,646,346.—*Kevin J. Catt*, Melbourne, Australia. ANTIBODY-COATED TUBE SYSTEM FOR RADIOIMMUNOASSAY. Patent dated Feb. 29, 1972. Disclaimer filed Oct. 31, 1974, by the assignee, *Pharmacia AB.*

Hereby enters this disclaimer to all claims of said patent.

[*Official Gazette May 27, 1975.*]