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[54] **PROCESS FOR THE DETECTION AND DETERMINATION OF SPECIFIC BINDING PROTEINS AND THEIR CORRESPONDING BINDABLE SUBSTANCES**

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[57] **ABSTRACT**

The invention relates to a process for the detection and determination of a component of the reaction between a specific binding protein and the corresponding bindable substance contacting a fluid containing one of these components with a conjugate of the bindable substance with an enzyme and of antibodies against the specific binding protein which latter component is brought in an insoluble form, if necessary, in combination with the specific binding protein, and determining the activity of the liquid or solid phase of the resulting reaction mixture.

6 Claims, No Drawings

PROCESS FOR THE DETECTION AND DETERMINATION OF SPECIFIC BINDING PROTEINS AND THEIR CORRESPONDING BINDABLE SUBSTANCES

It is known that a component of the reaction between a specific binding protein and the corresponding bindable substance can be detected and determined by incubating one of the named components, labelled by a marker, in a reaction mixture which at least contains the other component, and then effecting a separation between the marked component that is and that is not bound to its binding partner, and finally determining the marker in at least one of the two fractions obtained. The distribution of the marked component over the two fractions is a measure of the amount of substance to be determined and present in the sample. Three systems can be described, in which the above method is applied.

a. antibodies as specific binding proteins, and the corresponding antigens as bindable substances. Substances which can be determined with this system are, inter alia, protein hormones and their antibodies, or virus antigens and their antibodies;

b. antibodies as specific binding proteins, and haptens as bindable substance. Here the haptens are defined as protein-free substances, which can react with antibodies, without being able to induce them. Substances which can be determined in such a system are, inter alia, steroid hormones and vitamines;

c. proteins which in the body act as receptor or transport molecules as specific binding proteins, and substances which are bound by them as bindable substances. This system is e.g., suitable for the determination of steroid hormones, but also of thyroxine and triiodothyronine, vitamine B_{12}, intrinsic factor, and adrenocorticotropic hormone.

The most important points in the methods of determination described are the application of a marker and the separation of the marked component into a fraction which is, and a fraction which is not bound to the corresponding component.

In the methods practically used up to now only radioactive atoms have been applied as marker, e.g., 131_I, 125_I, 14_C, 3_H, 57_{Co}. This method usually stands out by a high sensitivity. However, the application of this method is limited to institutes which have the required specialized apparatus available.

The separation methods can be divided as follows:

a. methods depending on the difference in physical properties between the non-bound, marked component and its complex with the binding partner, such as gelfiltration, electrophoresis, salt precipitation, and adsorption to dextran-coated charcoal;

b. the so-called solid phase methods, in which beforehand one component has already been brought into an insoluble form by cross-linking or by covalent binding or physical adsorption to a solid carrier.

c. the so-called double antibody method in which the complex formed, antigen (or hapten)-antibody, is precipitated with the help of antibodies against the antibody in the complex, which method is only known for systems in which antibodies are involved. Whilst the methods mentioned under (a) and (c) are relatively complicated to perform, those mentioned under (b) suffer from the disadvantage that as a result of bringing a reaction component in an insoluble form, its affinity for the reaction partner usually decreases. A high affinity is, however, essential for realizing a sensitive system of test.

Now a process was found for the detection and determination of a component of the reaction between a specific binding protein and the corresponding bindable substance, applying the known binding affinity of such components for one another, characterized in that use is made of a given amount of a coupling product of the combinable substance with an enzyme, and of antibodies against the specific combining protein, brought in an insoluble form, and that after the reaction the enzyme activity is determined in the liquid or solid phase of the reaction mixture, which activity is a measure of the amount of the component to be determined.

A frequent and advantageous use of this method is made when antibodies are made to act as specific binding protein and antigen or hapten as corresponding bindable substance.

In the present description the term conjugate and enzyme conjugate are used as synonyms for the coupling product of the bindable substance and the enzyme.

The bindable substance can be detected and determined by bringing together the unknown sample or a dilution series of same with a known amount of a conjugate of the substance to be determined and an enzyme, and with an amount of specific binding protein, depending on the amount of enzyme conjugate that was added. Then an amount, preferably an excess, of the insoluble-made antibodies against the specific binding protein is added, so that all the enzyme conjugate that has reacted with the binding protein is coupled — via this protein — to these insoluble antibodies. In proportion as there is more bindable substance present in the sample, less enzyme conjugate will react with the specific binding protein and ultimately get into the insoluble phase; the result is that more unbound enzyme conjugate remains in the liquid phase, which can simply be determined there.

The specific binding protein can be determined by incubating the sample or a dilution series of same with a known amount of enzyme conjugate and with an amount of the insoluble-made antibodies against the specific binding protein. The enzyme activity can only pass into the insoluble phase if the conjugate has reacted with the specific binding protein: the more specific binding protein there is in the sample, the less of bound enzyme conjugate remains in the liquid phase.

The sensitivity of the test systems described can be varied by altering the quantities of the reagents (whether or not in the same ratio). However, the amount of enzyme conjugate that can be applied is limited at the lower end by the requirement that its enzyme activity can be measured reasonably, so that the sensitivity of the test systems has a limit. The minimum measurable enzyme activity depends inter alia on the nature of the enzyme used for the coupling and on the nature of the substrate and of the incubation period of the enzyme reaction. Further the affinity of the specific binding proteins strongly influences the sensitivity of the determination. For a highly sensitive test system specific binding proteins with a higher affinity are required.

The quanitities of reagent required per determination are established empirically.

For the determination of the bindable substance the quantity of enzyme conjugate will be determined with the help of the enzyme activity; then this quantity is incubated with a dilution series of the specific binding protein to determine the required quantity of this protein. By preference a quantity of specific binding protein is chosen, which binds 50–90% of the enzyme conjugate. Finally it is checked whether the desired sensitivity has indeed been reached, by testing a dilution series of the substance under test in the system. For the determination of the specific binding protein another consideration regarding the dosage of the enzyme conjugate is its activity, which should be reasonably determinable.

The insoluble-made antibodies against the specific binding protein are preferably added in excess in the two types of determination; the dosing of same is determined in preliminary tests. The advantages of this method over the existing are:

a. working with radioisotopes can be replaced by working with enzymes. This requires considerably less laboratory facilities and apparatus, whilst the staff can be less highly qualified. Moreover, working with radioactive isotopes is highly limited because of legal regulations. Further the reagents according to the invention will keep long and the safety is increased;

b. the combination of "Double Antibody" and "Solid Phase" methods, for ease of reference called DASP method below, offers several advantages over the existing methods. Thus the performing of the found method, viz.: addition of insoluble-made antibodies against the specific binding protein, incubation, centrifuging and measuring, is very simple. The dosing is often easier than in the solid phase methods because it suffices to add an excess of insoluble material, whereas in the solid phase methods an accurately measured amount of material has to be used. Moreover, the affinity of the binding protein for the bindable substance is not impaired by the binding to the carrier material, which can be the case in the solid phase method. A further advantage over the latter method is the rapid equilibrium adjustment of the reaction between specific binding protein and bindable substance (both in solution). A further advantage is that in the DASP method the insoluble-made antibody, called immunoadsorbent below, can be used in any system in which antibodies are used as specific binding protein, provided that these antibodies have been prepared in the same animal species. On the other hand, for each antigen or hapten to be determined with the solid phase method, the antibodies have to be made insoluble. The double antibody method is very sensitive for relatively low variations in salt concentrations, pH and the like, which makes a rigid control of the conditions necessary. Moreover, the method requires the addition of "carrier" γ-globuline and consequently much second antibody to obtain an immune precipitate. In addition to being a simpler procedure, the DASP method, which does not require "carrier" γ-globuline, thus leads to material saving. Further it be added that a double antibody-like separation applied to transport or receptor proteins is not possible, as no suitable "carrier" protein is available for this purpose. The method according to the invention will therefore offer a unique opportunity in this respect.

c. The method according to the present invention can easily be automatized. In principle it is possible to bring the reaction components together at once or to add them in any sequence. It was found, however, that the determination acquires a higher sensitivity if the immunoadsorbent is added after the incubation of the other components.

The reagent required for the invention, viz. the coupling product of antigen, hapten or bindable substance with an enzyme, can be prepared in a known manner. These methods can also be used to bind a hapten or a low-molecular bindable substance to an enzyme, provided that one substance possesses one or more amino-groups and the other one or more carboxyl-groups. If the latter is not the case then it is possible to introduce the desired group into the molecule to be coupled with the help of known organo-chemical processes. Methods are also known to bind amino or carboxyl-groups together, whether or not by introducing a bridge. Finally compounds as glutaric aldehyde, difluorodinitrodiphenyl-sulphon and di- and tri-chloro-s-triazine can often be used for the coupling in question. It can be necessary to separate the prepared enzyme conjugates from non-converted substances or from substances that have become inactive. To this end the known biochemical methods can be used, such as precipitation with organic solvents, gelfiltration, and centrifugation at a density gradient.

The choice of the enzyme which is taken up in the coupling product, is determined by a number of properties of that enzyme. It is, of course, essential that the enzyme should be resistant to the coupling with another molecule, i.e., modification of one or more aminoacid side chains. Also of great importance is the specific activity of the enzyme. As less enzyme conjugate needs to be added to reach a measurable enzyme effect, the test system grows more sensitive. Further those enzymes are to be preferred, of which the determination of the activity can be made in a simple manner. In the first place those enzymes are considered that can be determined colorimetrically, spectrophotometrically or fluorimetrically. This kind of determinations is suitable for automation, which is an additional advantage.

Colorimetrically those enzymes can be determined that catalyze a reaction in which a coloured substance appears or disappears, either in the primary or in the secundary reaction.

As enzymes considered to act as enzymatically active component in conjugates, are mentioned catalase, peroxidase, β-glucoronidase, β-D-glucosidase, β-D-galactosidase, urease, glucose oxidase, galactose oxidase, and alkaline phosphatase.

At the end of the reaction between the components and the reagents according to the invention the enzyme activity of the liquid or solid phase of the reaction mixture or of the two phases can be determined. Most simple is, however, to determine the enzyme activity of the liquid phase.

The insoluble-made antibodies against the specific binding proteins, which are also an essential reagent for the process of the invention, can also be prepared in a known way. The antibodies can be prepared by taking a purified preparation of the specific binding protein,

or of proteins which have at least partly the same antigen properties as the specific binding protein, and injecting this in a known way into another animal species than from which it was obtained. The serum of the treated animal, or the gammaglobuline fraction thereof, can be made insoluble by cross-linking with compounds such as glutaric aldehyde and chloroformic acid ethyl ester, or by binding to solid carrier particles, either physically by adsorption, or chemically by the formation of covalent bonds. As solid carriers can be considered materials such as cellulose (modified or not), agarose, cross-linked dextran, polystyrene and the like. Covalent binding of the antibodies to these materials can be effected with the help of substances such as carbodiimides, di- and tri-chloro-s-triazines, glutaric aldehyde, cyanogenbromide, and e.g., by diazotation.

The advantages of the method of determination found are done full justice if an excess of insoluble antibodies is applied, so that the specific binding protein passes completely into the solid phase.

The forms in which the reagents can be used are manifold. The enzyme-conjugated component of the reaction system can be freeze-dried, or dissolved in a buffer. Also a solid carrier can be used, e.g., a strip of paper impregnated with the conjugate. This applies equally to the required specific binding proteins.

The insoluble component can be brought in the form of particles of different dimensions, such as granules, flakes, rods, or in the form of a strip of some carrier material.

For the performing of the procedure according to the invention a test pack is applied by preference. This consists mainly of:

1. a known amount of a conjugate of an antigen, hapten or low-molecular bindable substance with an enzyme,
2. a corresponding amount of specific binding protein (antibodies or transport or receptor proteins),
3. a known amount of insoluble-made antibodies directed against the specific binding protein used.

The test pack can further contain the reagents required for the enzyme determinations, and also auxiliary means for conducting the test, such as test tubes, pipettes and bottles with dilution liquid. Such a test pack is suitable for the determination of a bindable substance, but also for the determination of a specific binding protein, in which case the specific binding protein contained in the test pack need not be used.

The test packs are especially frequently and with advantage used for the detection and determination of an antigen or hapten and to this end they contain mainly:

a. a known amount of a conjugate of the antigen or hapten and an enzyme,
b. a corresponding amount of corresponding antibodies,
c. a known amount of insoluble-made antibodies directed against the antibodies used. When applying such test packs for the detection and determination of antibodies the antibodies mentioned under (b) are not required.

An important embodiment of a test pack according to the present invention is a test pack to be used for the determination of gonadotropic hormones, and particularly for the determination of HCG (Human Chorionic Gonadotropin) as a means to diagnose pregnancy already in a very early stage, which test pack consists of an ampoule, tube, bottle or other container containing as essential ingredients, in separate lyophilized layers, pre-determined amounts of:

a. a conjugate of HCG with an enzyme, e.g., HCG-peroxidase.
b. anti-HCG.
c. antibodies against anti-HCG, made insoluble.
d. possibly other ingredients like a buffer.

By adding a certain amount of urine of a possibly pregnant woman to this test kit and to incubate the urine with the components of the kit a mixture of insoluble material is formed whereas the supernatant contains the remaining soluble HCG-enzyme conjugate. The amount of the latter is dependent on the amount of HCG in the urine to be tested. By determining the enzyme activity of this remaining HCG-enzyme conjugate it can be ascertained whether the urine is coming from a pregnant woman or not.

A preferably applied method for the determination of the enzyme activity consists in contacting an indicator-paper impregnated with enzyme reagents, e.g., in case use is made of a peroxidase, a H_2O_2-supplier like urea-H_2O_2, and a colour-reagent like o-tolidine.

By a correct choice of the amounts of each of the reagents it will be possible to ascertain pregnancy already in a very early stage and in a simple, rapid and very reliable way to be performed even by unskilled persons.

Example I

Determination of human choriongonadotrophine (HCG)

a. Preparation of HCG-HRP. 5 mg HCG and 20 mg horse radish peroxidase (HRP) were dissolved in 2 ml 0.05 M phosphate buffer of pH 6.2. After addition of 40 μl 25% glutaric aldehyde solution the mixture was shaken for 2 hours at room temperature. After 5 minutes centrifugation at 250 g, the liquid was fractionated over Sephadex G-200 in 0.05 M phosphate buffer of pH 6.2. The fractions of which the highest percentage enzyme activity was bound by antibodies against HCG were used in the test system.
b. Preparation of antibodies against HCG. Antibodies against HCG were induced in rabbits as described by Schuurs et al. Acta Endocr. (Kbh.) 59, 120 (1968).
c. Preparation of antibodies against rabbit-γ-globuline. Rabbit-γ-globuline was isolated from normal rabbit serum by precipitation with 18% w/v solid sodium sulphate. Antibodies against this were prepared by immunizing a sheep according to the following schedule:

day	amount		Freund's adjuvans	injection manner
0	0.5	mg	+	intramuscular
14	0.5	mg	+	intramuscular
28	1	mg	+	intramuscular
42	1	mg	–	intravenous
56	1	mg	–	intravenous

On day 70 the sheep was bled.

d. Preparation of the immunoadsorbent [sheep-anti-(rabbit-γ-globuline)] cellulose. The γ-globuline fraction of the sheep serum described under 1 c)

was prepared by precipitation with 16% w/v solid sodium sulphate. After washing, the precipitate was taken up in so much 0.05 M borate buffer of pH 8.6 that the resulting protein concentration amounted to 10 mg/ml. 350 mg m-aminobenzyloxymethylcellulose was suspended in 50 ml distilled water, and diazotized by adding 10 ml 36% hydrochloric acid and, dropwise, 10 ml 10% $NaNO_2$ solution at 0°C. The suspension was centrifuged, washed and the precipitate re-suspended in 43 ml 0.05 M sodium borate of pH 8.6. Then 7 ml of the prepared γ-globuline solution was added. The mixture was stirred for 26 hours at 4°C, then centrifuged and washed with 0.02 M phosphate buffer of pH 6.0.

e. Determination of HCG. A dilution series (32-16-8-4-2-1-0.5-0 IU/ml) of HCG in 0.02 M phosphate buffer with pH 6.0 was prepared, which contained 2% v/v normal sheep serum. 0.5 ml of each of the HCG-containing samples was incubated with 0.1 ml rabbit-(anti-HCG) serum and 0.1 ml HCG-HRP conjugate, both in suitable dilution, for half an hour at room temperature. Then 0.3 ml of the immunoadsorbent (10 mg/ml) prepared according to d) was added, and the resulting mixture was rotated at room temperature for 1 hour. After centrifugation the enzyme activity in the supernatant was measured by mixing 0.5 ml of this liquid with 1.5 ml substrate (10 μl 30% H_2O_2 and 20 mg 5-aminosalicylic acid in 150 ml 0.02 M phosphate buffer of pH 6.0) and after 30 minutes at 25°C measuring the extinction at 460 nm. In this way it proved possible to detect a HCG concentration from 0.5 to 1 IU/ml in the sample. With this method also urine samples could be tested; the test is therefore suitable for a pregnancy check. The correlation with an existing method of test, a haemagglutination inhibiting test, was good. It proved possible to raise the sensitivity of the system by the application of a pre-incubation. Here, first the sample only was incubated with the antiserum, and then the HCG-HRP conjugate was added.

Example II

Determination of insuline and anti-insuline.

a. Preparation of insuline-(glucose oxidase). 5 mg pig insuline and 25 mg glucose oxidase were dissolved in 2 ml 0.05 M phosphate buffer of pH 6.5. To this, 5 μl 25% glutaric aldehyde solution was added, after which the mixture was shaken for 90 minutes at room temperature. The mixture was fractionated over Sephadex G-200 in 0.05 M phosphate buffer of pH 6.5. The fractions of which the highest percentage of enzyme activity could be bound by antibodies against insuline, were used in the test system.

b. Preparation of antibodies against insuline. 10 guinea pigs were given a weekly intramuscular injection with 1 mg pig insuline in complete Freund's adjuvans over a period from 4–8 weeks. After two weeks' rest the animals were given 1 mg additional insuline by intravenous injection without adjuvans. 2 weeks after that the animals were bled. Hypoglycaemia occurring at times was counteracted by intraperitoneal administration of glucose.

c. Preparation of antibodies against guinea pig γ-globuline. Guinea pig γ-globuline was prepared by adding 1 volume saturated ammonium sulphate solution to 2 volumes guinea pig serum. The precipitate formed was twice washed with 33% saturated ammonium sulphate solution, and then taken up in a physiological salt solution. A sheep was immunized with increasing doses of the prepared γ-globuline: 0.5, 1 and 2 mg. The injections were given every two weeks, whilst the immunogen was mixed with complete Freund's adjuvans. Two weeks after the last injection an additional 2 mg γ-globuline in a physiological salt solution were given, and 1 week later the animal was bled.

d. Preparation of insoluble antibodies against guinea pig γ-globuline. 10 g microcrystalline cellulose was activated by adding it whilst stirring to 400 ml 2.5% w/v CNBr-solution, after which the pH was brought to 10.5 with 1 N NaOH solution and maintained thus for 2 minutes. Then the cellulose was washed with ice water and with 0.1 M $NaHCO_3$. To 10 ml sheep-anti(guinea pig γ-globuline) serum 1.6 g Na_2SO_4 was added. After 1 hour stirring at room temperature the precipitate was centrifuged, twice washed with 20 ml 16% w/v Na_2SO_4 solution, and subsequently taken up in 10 ml 0.1 M $NaHCO_3$. The activated cellulose was mixed with 40 ml 0.1 M $NaHCO_3$ solution and the 10 ml γ-globuline solution. This suspension was rotated for 40 hours at 4°C, and in succession washed with twice 500 ml 0.5 M $NaHCO_3$, twice 500 ml 0.05 M citrate pH 1.1, and twice 500 ml 0.05 M phosphate of pH 6.2.

e. Determination of antibodies against insuline. 0.1 ml insuline-(glucose oxidase), in a suitable dilution, was incubated with 0.4 ml of a dilution series of a guinea pig anti-insuline serum for 4 hours. The dilution series was made with 0.05 M phosphate buffer of pH 6.0. Then 0.3 ml immunoadsorbent (15 mg/ml) and 0.2 ml buffer were added and the mixture was rotated during the night at 4°C. After centrifuging the enzyme activity of the supernatant was determined by incubating 0.5 ml of same with 2.5 ml substrate for 30 minutes, and then measuring the extinction at 460 nm. The substrate contained 50 mg glucose, 10 μg peroxidase and 1 mg 5-aminosalicyclic acid per 2.5 ml 0.05 M phosphate buffer of pH 6.0. By means of this system the antibody content of the different serums could be intercompared. As reference point was chosen the serum dilution at which 50% of the total combinable enzyme activity is bound.

f. Determination of insuline. 0.2 ml of a dilution series of insuline was incubated for 2 hours with 0.4 ml anti-insuline serum in such a dilution that it could bind 60% of the enzyme conjugate to be added. Then 0.1 ml insuline-(glucose oxidase) was added in the corresponding dilution, and incubated for 4 hours. Finally 0.3 ml immunoadsorbent (15 mg/ml) was added. The mixture was rotated during the night at 4°C. After centrifuging the enzyme activity of the supernatant was measured as described under (e). The sensitivity of the determination, which depends on the antiserum used, lies in the nanogram range: 20–100 ng/ml, i.e., 0.5–2.5 mU/ml.

Example III

Determination of oestradiol.

a. Preparation of oestradiol-17-succinyl-HRP. 50 mg oestradiol-17-hemisuccinate and 0.08 ml tri-n-butylamine were dissolved in 2.5 ml dioxane. To the cold (2°C) solution 15 μl isobutylchlorocarbonate was added. After 30 minutes this solution was mixed with 100 mg horse radish peroxidase (HRP) in 7.5 ml of a dioxane/water mixture (2:3) which had been adjusted to pH 9.5 with sodium hydroxide. The solution was stirred for 4 hours at 2°C, and then dialyzed for 18 hours. The precipitate formed after the pH of the dialysate had been adjusted to 4.6, was centrifuged, washed and taken up in 5 ml distilled water that had been adjusted to pH 8. The material was further purified by twice precipitating with 10 ml acetone. The finished product was taken up in 10 ml 0.05 M phosphate buffer of pH 7.8.

b. Preparation of oestradiol-17-succinyl-BSA. The preparation was prepared according to the mixed anhydride method, as described in Example IIIa. This preparation was made starting from 100 mg oestradiol-17-hemisuccinate and 150 mg bovine serum albumine (BSA).

c. Preparation of antibodies against oestradiol. A sheep was injected once in four weeks with 4 mg oestradiol-17-succinyl-BSA in complete Freund's adjuvans. At regular intervals blood was taken from the sheep. The serum was absorbed with BSA that was made insoluble.

d. Preparation of antibodies against sheep-γ-globuline. Sheep-γ-globuline was prepared as described in example I, but now with 16% w/v sodium sulphate. Rabbits were immunized with this sheep-γ-globuline according to the following schedule:

day	amount	Freund's adjuvans	injection manner
0	200 μg	+	intramuscular
14	400 μg	+	intramuscular
28	800 μg	+	intramuscular
42	800 μg	−	intravenous

2 weeks after the last injection the animals were bled.

e. Preparation of the immunoadsorbent [rabbit-anti(-sheep-γ-globuline)]-cellulose. The γ-globuline fraction of the antiserums, described under (d), was prepared by precipitation with 18% w/v Na_2SO_4. The product obtained was coupled to cellulose according to the Gurvich method, described in example I.

f. Determination of oestradiol. The immune reaction was made in 0.02 M phosphate buffer of pH 6.0, which contained 2% BSA: 0.5 ml sample was mixed with 0.1 ml of the sheep-anti-oestradiol serum in the desired dilution. After 30 minutes incubation at room temperature, 0.1 ml oestradiol-17-succinyl-HRP was added in a suitable dilution, after which another 30 minutes incubation at room temperature followed. Then 0.3 ml immunoadsorbent suspension (30 mg/ml) was added and the mixture was rotated for 2 hours at room temperature. Then the liquid and solid phases were separated from each other by centrifuging. The enzyme activity in the supernatant was measured as described in example I. The sheep-anti-oestradiol serum could be used in dilutions from 1:1600 to 1:12,800, depending on the quality of the oestradiol-17-succinyl-HRP used. With a dilution of 1:12,800 of the antiserum an oestradiol concentration of 10 ng/ml could be detected in the sample. Oestriol and oestron showed a cross reaction in this system.

Example IV

Determination of cortisol and corticoid-binding globuline.

a. Preparation of cortisol-21-(galactose oxidase). 50 mg cortisol-21-hemisuccinate and 100 mg galatose oxidase were coupled by means of the mixed anhydride technique, as described in example III*a*.

b. Corticoid-binding globuline (CBG) was isolated from the serum of men by means of chromatography in succession over DEAE-cellulose and hydroxyl apatite. Antibodies against this were prepared by injecting rabbits at 14-day intervals with 500 μg CBG in complete Freund's adjuvans. After 3 months the animals were injected with 1 mg CBG and 2 weeks later they were bled.

c. The γ-globuline fraction of anti-CBG serum was coupled to m-aminobenzyloxymethylcellulose, as described in example III.

d. Determination of cortisol. 0.5 ml of a cortisol-containing sample (standard solution) was extracted with 2 × 3 ml methylenechloride. The combined extraction liquids were evaporated to dryness. The residue was taken up in 0.5 ml 0.05 M phosphate buffer of pH 6.2, then mixed with 0.1 ml of a solution of CBG in the same buffer in a suitable concentration, and incubated for 30 minutes at 4°C. Then 0.1 ml cortisol-21-(galactose oxidase), also in a suitable dilution, and 0.3 ml of the immunoadsorbent prepared under (c) with a concentration of 5 mg/ml were added. The mixture obtained was rotated for 2 hours at 4°C and then centrifuged, after which the enzyme activity was measured in the supernatant. To this end 0.5 ml of same was added to 1.5 ml substrate consisting of 100 mg D-galactose, 20 mg 5-aminosalicyclic acid and 10 μg peroxidase in 150 ml 0.02 M phosphate buffer of pH 6.0. After 30 minutes the extinction was measured at 460 nm. When applying a CBG concentration of 0.4 μg/ml and so much cortisol-21-(galactose oxidase) that without the addition of steroids 80% of the enzyme conjugate was bound to the immunoadsorbent, it proved possible to determine amounts from 3–30 ng cortisol.

e. Determination of CBG was also possible with the reagents described. From a dilution series of transcortine, ranging from 0–1280 ng/ml, 0.5 ml was incubated for 15 minutes with 0.2 ml cortisol-21-(galactose oxidase) in a suitable dilution. Then 0.3 ml immunoadsorbent suspension (5 mg/ml) was added, and the mixture rotated for 15 minutes. The two incubations were conducted at 4°C. Then the enzyme activity in the supernatant was measured as under d). The sensitivity of the test system proved to be 50 ng/ml.

Example V

In a bottle the following reagents were subsequently lyophilized in separate layers:

1. 0.3 ml of the immunoadsorbent suspension (10 mg/ml) as described in Example I a).
2. 0.1 ml of a 1% mannitol solution.

3. 0.1 ml of HCG-HRP as described in Example I (a).

4. a second layer of 0.1 ml of a 1% mannitol solution.

5. 0.1 ml of rabbit (anti-HCG) serum as described in Example I (b).

To this lyophilized mixture 0.5 ml of a urine sample and subsequently 0.5 ml distilled water was added. After 10 minutes the enzyme activity in the supernatant was measured by means of a slip of paper impregnated with urea-hydrogenperoxide and o-tolidine.

If the urine sample was coming from a pregnant woman (>2 IU HCG/ml) a blue colour appeared within 5 minutes, whereas in case of urine of a non-pregnant woman no discoloration took place within the same period.

We claim:

1. Process for the detection and determination of a bindable substance selected from the group consisting of an antigen and a hapten, comprising the steps of:

a. providing a given quantity of a conjugate of said bindable substance with an enzyme;

b. providing a corresponding given quantity of an antibody against said bindable substance;

c. admixing a sample of a fluid containing the bindable substance to be determined with the reactants of steps (a) and (b) to form a reaction mixture and allowing the reaction to go to completion;

d. separating the resulting mixture into a liquid phase and a solid phase by adding an insolubilized antibody against the antibody of step (b); and

e. determining the quantity of the bindable substance from the measure of enzyme activity of either separated phase.

2. The process of claim **1** in which the bindable substance is determined by first adding thereto the antibody, then the enzyme conjugate, and finally the insoluble antibody against the antibody.

3. Process for the detection and determination of an antibody in a fluid sample containing the same utilizing the reaction between said antibody and a bindable substance selected from the group consisting of an antigen and a hapten having an affinity therefor, comprising the steps of:

a. providing a given quantity of a conjugate of said bindable substance and an enzyme;

b. providing a given quantity of an insolubilized antibody against the antibody to be determined;

c. admixing said given quantities of steps (a) and (b) with said sample, and allowing the components to react;

d. separating the reaction mixture into a liquid phase and a solid phase; and

e. determining the quantity of the antibody from the measure of enzyme activity of either separated phase.

4. A test pack for the detection and determination of a bindable substance selected from the group consisting of an antigen and a hapten in a fluid sample, comprising:

a. a known amount of a conjugate of said bindable substance with an enzyme;

b. a corresponding known amount of an antibody against said antigen or hapten;

c. a known amount of an insolubilized antibody against said antibody;

d. a stabilizer; and

e. a substrate for the determination of the enzyme activity and thus of the quantity of the antigen or hapten to be determined.

5. A test pack for the detection and determination of an antibody in a fluid sample, utilizing the reaction between said antibody and an antigen or hapten to said antibody, comprising:

a. a known amount of a conjugate of the antigen or hapten with an enzyme;

b. a corresponding known amount of an insolubilized antibody against the antibody to be determined;

c. a stabilizer; and

d. a substrate for the determination of the enzyme activity and thus of the quantity of antibody to be determined.

6. A test pack for the determination of pregnancy by the detection and determination of human chorionic gonadotropin in a sample of urine, utilizing the reaction between human chorionic gonadotropin and an antibody therefor, comprising, in separate lyophilized layers:

a. a predetermined amount of a conjugate of human chorionic gonadotropin and an enzyme;

b. a predetermined amount of an antibody against human chorionic gonadotropin;

c. a predetermined amount of an antibody against the human chorionic gonadotropin antibody, in insolubilized form;

d. a stabilizer; and

e. a substrate for the determination of the enzyme activity and thus of the quantity of human chorionic gonadotropin enzyme conjugate and human chorionic gonadotropin to be determined.

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