PROCESS FOR THE DEMONSTRATION AND DETERMINATION OF LOW MOLECULAR COMPOUNDS AND OF PROTEINS CAPABLE OF BINDING THESE COMPOUNDS SPECIFICALLY

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Notice: The portion of the term of this patent subsequent to Apr. 4, 1989, has been disclaimed.

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References Cited
UNITED STATES PATENTS
3,615,222 10/1971 Mead ......................... 23/230 B
3,654,090 4/1972 Schuurs et al. ............... 195/103.5 R

OTHER PUBLICATIONS

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ABSTRACT

The invention relates to a process for the demonstration and determination of a low molecular compound, such as a hapten, or a protein or antibody capable of binding this compound specifically, contacting a fluid containing such a compound or protein to be determined with a given quantity of the coupling product of such low molecular compound and an enzyme, and with a given quantity of the component to be determined, which component is brought in an insoluble form, and determining the enzyme activity of the liquid or solid phase of the resulting reaction mixture.

13 Claims, 6 Drawing Figures
\textbf{FIG. 1}

\begin{center}
\begin{tikzpicture}
\draw[->] (0,0) -- (8,0) node[below] {CONCENTRATION ANTI-TESTOSTERONE-CELLULOSE (mg/ml)};
\draw[->] (0,0) -- (0,6) node[left] {\% ENZYME ACTIVITY IN SUPERNATANT LIQUID};
\draw (0,0) -- (0,5);
\draw (0,5) -- (8,5);
\draw (8,0) -- (8,5);
\draw (0,0) -- (8,0);
\draw (0,5) -- (8,5);
\draw (0,0) -- (0,5);
\draw (8,0) -- (8,5);
\draw (0.5,0.5) node[above] {A};
\draw (2,2) node[above] {B, D, E};
\draw (4,1) node[above] {C};
\end{tikzpicture}
\end{center}

\textbf{FIG. 2}

\begin{center}
\begin{tikzpicture}
\draw[->] (0,0) -- (8,0) node[below] {ng TESTOSTERONE IN THE SAMPLE};
\draw[->] (0,0) -- (0,6) node[left] {\% ENZYME ACTIVITY IN SUPERNATANT LIQUID};
\draw (0,0) -- (0,5);
\draw (0,5) -- (8,5);
\draw (8,0) -- (8,5);
\draw (0,0) -- (8,0);
\draw (0,5) -- (8,5);
\draw (0,0) -- (0,5);
\draw (8,0) -- (8,5);
\draw (0.5,0.5) node[above] {I};
\draw (2,2) node[above] {II};
\draw (4,1) node[above] {III};
\draw (5.5,0.5) node[above] {IV};
\end{tikzpicture}
\end{center}
PROCESS FOR THE DEMONSTRATION AND DETERMINATION OF LOW MOLECULAR COMPOUNDS AND OF PROTEINS CAPABLE OF BINDING THESE COMPOUNDS SPECIFICALLY

For the determination of low molecular substances occurring in low concentrations, such as steroid hormones in body fluids, methods have been developed using proteins capable of binding the substance to be determined specifically. These methods are based on the competition between the substance to be determined in the sample and a known quantity of the same substance which is labelled radioactive, for a limited quantity of specific binding protein. The unknown quantity of bindable substance then determines what portion of the radioactive labelled substance is bound by the specific binding protein.

It is also possible to determine by these methods an unknown quantity of specific binding protein by reacting a sample containing an unknown quantity of specific binding protein with a given quantity of bindable radioactive labelled substance.

In the literature it is common practice to distinguish these determination methods according to the nature of the specific binding protein employed, although the underlying principle of all determinations is identical. Thus, for example, they speak of "competitive protein binding assays" when they employ receptor or transport proteins occurring in the body, and of radio-immunological determinations when they employ anti-substances.

For both types of determinations radioactive labelled substances are required. The work with these substances requires the presence of precious measuring apparatuses, good laboratory facilities and a highly qualified staff. These high requirements prevent a general application of these determination, especially in smaller laboratories.

A process has now been found for the demonstration and determination of a component of the reaction between a low molecular compound and a protein capable of binding this compound specifically using the binding affinity of such components for each other, characterized in that the determination is performed with a given quantity of the insoluble specific binding protein. The more low molecular compound the sample contains, the less opportunity the soluble enzyme conjugate of that compound has to combine with the insoluble specific binding protein and the more of the conjugate will remain in the liquid phase, in which the enzyme activity can be measured in a simple manner.

In the determination of a specific binding protein with the same reagents the soluble protein to be determined and the insoluble protein enter into competition for a given quantity of the conjugate of the corresponding low molecular compound and an enzyme. If the content of specific binding protein of the sample is higher, the insoluble protein will bind less of the enzyme conjugate, and consequently more enzyme remains in the liquid phase.

A specific binding protein having two or more binding possibilities can also be demonstrated and determined according to the invention, i.e. with the enzyme conjugate and with the low molecular compound in an insoluble form. The liquid phase of the reaction mixture then contains the conjugate bound to the specific binding protein and in the solid phase the complex of enzyme conjugate - specific binding protein - insoluble low molecular compound. The more of the protein to be determined there is in the sample, the more enzyme activity the liquid phase will possess.

By means of an assay curve for a certain system in which increasing contents of substance to be determined have been set off against the enzyme activity found, preferably in the liquid phase, the quantity of substance to be determined in the sample can be derived from a value of enzyme activity found.

The most important reagents of this determination method is the coupling product of the low molecular substance and an enzyme, for convenience hereinafter also called enzyme conjugate, which on the one hand can react with the specific binding protein, via the low molecular component and on the other hand possesses enzyme activity. This reagent is prepared by a method described for similar products. The second reagent, the insoluble component in the reaction system, serves to facilitate the separation between various enzyme-containing fractions of the reaction mixture. Addition of this reagent causes the formation of a solid phase in addition to a liquid phase. The present determinations can in principle be performed without an insoluble component of the reaction system. In that case the various fractions with enzyme activity will have to be separated by, for example, chromatographic or electrophoretic route or gelfiltration. For practical reasons such a determination is less attractive, however.

The enzyme activity of a fraction of the reaction mixture is demonstrated or measured by incubating that fraction with a substrate and other substances required for processing the relative enzyme reaction. For preference a reaction is used in which a coloured compound is formed or removed whose absorption can also be measured quantitatively in an easy manner.

Low molecular substances which are eligible for demonstration by the new method and have a molecular weight of up to about 1500 are for example: steroids, vitamin B12, folinic acid, thyroxine and triiodothyronine, releasing factors, histamine, serotonin and other biogenic amines, digoxine, digitoxin, prostaglandins, adrenalin, nor-adrenalin, vegetable hormones such as auxin, kinetin and gibberellic acid, and antibiotics such as penicillin.
The method of demonstrating specific binding proteins for low molecular substances can be employed for, for example, the determination of antibody against penicillin or for the determination of Intrinsic factor.

The preparation of conjugates of enzymes and low molecular substances can take place in various manners. Some low molecular substances may already possess groups that can be cross-linked with reactive groups at the surface of the enzyme, while other substances will have to be provided with such groups by organic chemical reactions. It stands to reason that the original binding properties of the low molecular compound and the activity of the enzyme may not change essentially during this process. The groups of the enzyme which are particularly suited for coupling reactions are amino and carboxyl groups. If the modified or unmodified low molecular substance also possesses such groups, the coupling can be performed by, for example, reactions known from the peptide synthesis. Furthermore such substances as glutaraldehyde, difluorodinitrophenylysulfon, tolune diisocyanate, di- and trichloro-s-triazine can be employed for the coupling reaction.

Specific examples of the coupling of hapten to proteins are described in, for example, Methods in Immunology and Immunochemistry, vol. I. The methods described are used for the preparation of conjugates for immunisation but they can also be used for the preparation of conjugates of the low molecular substance and an enzyme which are essential in the invention.

The choice of the enzyme that is to be a component of the conjugate (low molecular substance-enzyme) depends on properties such as the specific activity (a high conversion rate enhances the sensitivity of the test system) and the simplicity of the determination of the enzyme. The determination of an enzyme which catalyzes a conversion involving coloured reaction components, is simple. Such colorimetric determinations can be automatized in a simple manner.

According to the invention it is also possible to use enzymes which catalyse conversions involving reaction components that can be determined spectrophotometrically or fluorimetrically. These determinations can also be automatized.

For the preparation of the conjugates enzymes such as catalase, peroxidase, β-glucuronidase, β-D-glucosidase, β-D-galactosidase, urease, glucose oxidase and galactose-oxidase are preferred, particularly the group of the oxido reductases.

The insoluble specific binding protein or the insoluble low molecular compound to be used in the present determination can be prepared by a known method, for example, by cross-linking with chloroformic acid-ethylster, by covalent binding with insoluble carriers such as agarose, cross-linked dextran or filter paper, or by physical coupling to insoluble carriers such as plastic objects.

The form in which the reagents can be used are manifold. The component of the reaction system conjugated with an enzyme can be freeze-dried or dissolved in a buffer. Furthermore a solid carrier, for example, a strip of paper impregnated with the conjugate, can be employed.

The insoluble component can be brought in the form of particles of various sizes, such as grains, platelets and rods, or in the form of a strip of some other carrier material.

For the performance of the process according to the invention a test pack in preferably employed, chiefly composed of:

a. a given quantity of the coupling product of the low molecular compound and an enzyme;  
b. a corresponding quantity of one or more of the components of the reaction system in an insoluble form;  
c. a substrate for the determination of the activity of the enzyme employed.

If required, the test pack can also contain the necessary auxiliaries for making a dilution series of the sample to be examined for a quantitative determination, such as test tubes, pipettes and flasks of diluent. For the determination of a hapten or its antibody the test pack contains at least:

a. a given quantity of the coupling product of this hapten and an enzyme;  
b. a corresponding quantity of a component of the reaction system in an insoluble form, hapten-antibody;  
c. a substrate for the determination of the enzyme activity.

The invention is illustrated further by the following examples, which are not to be construed as limiting.

Example I

Determination of testosterone

A. Preparation of testosterone-3-HRP

One hundred milligrams of testosterone-3-(O-carboxymethyl)-oxim and 0.143 ml of tri-n-butylamine were dissolved in 5 ml of dioxan. The solution was cooled down to 2°C and then 0.03 ml of isobutylchloroborate were added. After 30 minutes the solution was added to 100 mg of HRP (horse radish peroxidase) in a mixture of 9 ml of water and 6 ml of dioxan, and adjusted to pH 9 with 0.1 N NaOH. This solution was stirred for 4 hours at 2°C and dialysed overnight. The precipitate obtained after the dialysate had been adjusted to pH 4.6 was centrifuged after having been left to stand overnight, suspended in 10 ml of water and dissolved by means of caustic soda solution. The material was precipitated three times with 15 ml of acetone at pH 4.5, dissolved in 15 ml of water which had been adjusted at pH 7.8 with caustic soda solution, dialysed and finally lyophilized.

B. Preparation of testosterone-3-BSA

This conjugate was prepared in the same manner as the testosterone-3-HRP, but the starting materials were 50 mg of testosterone-3-(O-carboxymethyl)-oxim and 150 mg of BSA (bovine serum albumin).

C. Preparation of antibodies against testosterone-3-BSA

Five rabbits were injected intramuscularly with increasing dosages of testosterone-3-BSA in complete Freund’s adjuvant (0.5, 1 and 2 mg) at intervals of 3 weeks. Two weeks after the last injection the animals were injected intravenously with 2 mg of antigen dissolved in physiological salt. One week after that blood was taken from the animals. The antibodies formed against BSA were removed by treating the serum portionwise with BSA-maminobenzyloxymethyl cellulose, prepared in accordance with Gurvich’s method (see D).

D. Preparation of anti-testosterone cellulose
This substance was prepared in accordance with Gurvich's method as described in Biokhimiya 26, 934 (1961):

1. Preparation of "aminocellulose":
Fifty grams of Whatman cellulose, which had been frequently washed and decanted, were suspended in 100 ml of a 0.7% sodium acetate solution containing 2 gm of (N-nitrobenzoxyl)methylpyridine. The mixture was dried at 60-80°C and heated for 40 minutes at 125°C.

The resulting product was thoroughly washed with distilled water, dried at 80°C, washed with benzene and dried again. Fifty grams of the dried product were reduced by suspension in 300 ml of a 15% NaS2O3 solution and stirred for 30 minutes at 50-60°C. The product was filtered and washed with successively distilled water, 30% acetic acid and again with distilled water.

2. Treatment with ammoniacal copper solution:
Forty millilitres of 10% sulphuric acid, 20 ml of 50% nitric acid and 140 ml of distilled water were heated, while stirring, to 90°C, after which 5.9 gm of CuO were added in small portions. The solution was boiled for 2 hours and completed to 500 ml with distilled water. Eighty millilitres of this solution were transferred into an ice bath and added to 160 ml of cold 4 N NaOH, while stirring. After being stirred for 30 minutes, the precipitate was washed twice with distilled water and dissolved in 80 ml of 25% ammonia. To this solution was gradually added 1 gm of aminocellulose. The mixture was stirred for 1.5 hours, after which 40 ml of boiling water were added, whereupon the solution was quickly cooled down to 0°C. The solution was neutralized with 10% sulphuric acid, after which the aminocellulose flocculated. It was washed with cold distilled water.

3. Preparation of γ-globulin:
To rabbit anti-testosterone serum were added 180 mg of Na2SO4 per ml of serum. The mixture was stirred for 1 hour at room temperature, after which the resulting precipitate was centrifuged, washed twice with an 18% Na2SO4 solution and taken up in so much 0.05 M sodium borate of pH 8.6 that the protein concentration was about 10 mg/ml.

4. Binding γ-globulin to aminocellulose
Aminocellulose (350 mg) was suspended in distilled water (50 ml). The suspension was cooled down to 0°C. Ten millilitres of a 36% hydrochloric acid were added and after that dropwise 10 ml of a 10% NaNO2 solution. The suspension was centrifuged, washed with cold distilled water and then with 0.05 M sodium borate of pH 8.6. The cellulose was suspended in 43 ml of 0.05 M sodium borate of pH 8.6. To this suspension were added 7 ml of the γ-globulin solution prepared. The mixture was stirred for 26 hours at 4°C, centrifuged and washed with 0.02 M phosphate buffer of pH 6.0. From the antisemirum of each of the 5 immunized rabbits a cellulose suspension was prepared (A – E respectively).

E. Determination of testosterone by means of testosterone-3-HRP and anti-testosterone cellulose
The following test system was built up:

1. Immunoreaction

0.5 ml of a sample containing testosterone, 0.2 ml of testosterone-3-HRP (100 ng/ml) and 0.3 ml of an anti-testosterone-cellulose suspension were rotated at room temperature for 2 hours and then centrifuged for 5 minutes at 1000 g.

The immunoreaction took place in 0.02 M phosphate buffer of pH 6.0 and containing 2% sheep serum.

II. Enzyme reaction
0.5 ml of the supernatant liquid was incubated at room temperature with 1.5 ml of substrate for 30 minutes. The extinction was measured at 460 nm.

The enzyme substrate contained 10 μl of 30% hydrogen peroxide and 20 mg of 5-aminosalicylic acid in 150 ml of 0.02 M phosphate buffer of pH 6.2.

FIG. 1 shows the measure in which testosterone-3-HRP is bound by the anti-testosterone-cellulose preparations made. In this case only buffer was added as a sample in the test system. If cellulose is added instead of anti-testosterone-cellulose, more than 95% of the enzyme activity will remain behind in the supernatant liquid. The preparations B, D and E proved to bind hardly any testosterone-3-HRP, but A and C did.

FIG. 2 shows the results of incubation of a testosterone dilution series with testosterone-3-HRP and four different concentrations of anti-testosterone-cellulose C: 1 mg/ml (I), 2 mg/ml (II), 4 mg/ml (III) and 16 mg/ml (IV). It is obvious that with this system a quantity of about 10 ngm of testosterone can be demonstrated.

EXAMPLE II

Determination of oestradiol

A. Oestradiol-17-succinyl-HRP was prepared by the mixed anhydride method described in example I A, using 50 mg of oestradiol-17-hemisuccinate and 50 mg of HRP as starting materials.

B. Oestradiol-17-succinyl-BSA was prepared by the mixed anhydride method described in example I A, using 100 mg of oestradiol-17-hemisuccinate and 150 mg of BSA as starting materials.

C. For the preparation of antibodies against oestradiol-17-succinyl-BSA five rabbits were immunized in accordance with the scheme described in example I C. The sera were absorbed with BSA-m-amino-benzoyloxymethyl-cellulose.

D. Anti-oestradiol-cellulose was prepared in the same manner as the anti-testosterone-cellulose described in example I D. From each of the immunized rabbits a cellulose preparation was made, numbered 16 up to 20 inclusive.

E. The test was performed analogous to that for testosterone as described in example I E.

FIG. 3 and 4 show a few results. FIG. 3 shows that the immunization yielded 3 usable antiseras, of which As 17 has the highest titre.

FIG. 4 shows the test system in which anti-oestradiol-cellulose 17 is used in a concentration of 8 mg/ml. The system does not discriminate between oestrone and 17β-oestradiol. 17α-oestradiol and especially oestriol show a smaller cross-reaction. Testosterone and progesterone influence the system only in very high concentrations.
Example III
Determination of antibodies against penicillin

Penicilloyl-catalase
Thirty milligrams of benzyl penicillic acid were dissolved in 5 ml of 96% ethanol and added dropwise to 200 mg of catalase in 45 ml of 0.1 M phosphate buffer of pH 7.5. The reaction process was continued for 2 hours, the pH being maintained between 7.2 and 8.2 with 0.5 N NaOH. The reaction mixture was dialysed against 6 x 3 l of 0.02 M phosphate buffer of pH 7.0.

In the same manner 250 mg of benzyl penicillic acid were coupled to 5 gm of m-aminobenzylxymethyl-cellulose prepared by Gurvich’s method, Biokhimija 26, 934 (1961). The coupling product was not dialysed however, but washed on a glass filter.

It proved to be possible to demonstrate over-sensitivity to penicillin in humans in the following manner.

0.2 ml of a sample of non-haemolysed serum were mixed with 0.5 ml of a solution of penicilloyl-catalase (1 : 800). After 30 minutes 10 mg of the penicilloyl-m-aminobenzylxymethyl-cellulose were added. The mixture was rotated, for 30 minutes after which the enzyme activity in the supernatant liquid was determined by adding 0.02 ml of it to 2.8 ml of 0.05 M phosphate buffer of pH 6.8, which contained 1.2 μl of 30% H2O2, and following the decrease in the extinction at 240 nm.

In the serum from patients who were hyper-sensitive to penicillin less enzyme activity was found in the liquid than in checking with rabbit serum. The serum from normal human beings in these tests did not deviate considerably from normal rabbit serum.

Example IV
Determination of folic acid

A. Preparation of folate-glucose-oxidase
Two hundred milligrams of glucose-oxidase (140 IU/mg) were dissolved in 10 ml of PBS (phosphate buffered saline, a phosphate-containing physiological salt solution) of pH 7.0. Thirty milligrams of 1-cyclohexyl-3-(2-morpholinol-ethyl)-carbodiimide (MCDI) were added and then 24 mg of folic acid. The reaction process lasted 2 hours, after which a thorough dialysis was performed against PBS of pH 7.0.

B. Preparation of folate-MBSA (methylated bovine serum albumin)
Folate-MBSA was prepared by Ricker and Stollar’s process described in Biochemistry 6, 2001 (1967).

Twenty-five milligrams of MCDI were added to 50 mg of MBSA in 5 ml of water and then 20 mg of folic acid. Two hours later a yellow precipitate had formed. Finally the whole reaction mixture was dialysed against physiological salt for a considerable time.

C. Preparation of anti-serum against folate-MBSA
On the days 0, 21 and 42 four rabbits were each injected intramuscularly with 2 mg of folate-MBSA in complete Freund’s adjuvant and on day 35 intravenously with 2 mg of folate-MBSA in physiological salt. On day 49 the animals were exsanguinated.

D. Anti-folate cellulose was prepared by the process described in example I D.
E. Test system for folic acid

Example V
Determination of digoxin

A. Preparation of digoxin-HRP
To 22 mg of digoxin, suspended in 1 ml of absolute ethanol, was added dropwise, while stirring, 1 ml of 0.1 M sodium metaperiodate. After 25 minutes 0.3 ml of 0.1 M ethyleneglycol was added. Five minutes later this mixture was added dropwise, while stirring, to a solution 32 mg of horse radish peroxidase (HRP) in 1 ml of distilled water, which had been adjusted to pH 9.5 with a 5% K2CO3 solution. During the reaction the pH was maintained at 9–9.5 with 5% K2CO3. When the pH had become stable 15 mg of NaBH4 in 1 ml of distilled water was added. After 3 hours the pH was adjusted to 6.5 with 1 M formic acid. One hour later 1 M NH2OH was added till a pH of 8.5 had been reached. The mixture was dialysed overnight against cold running water. Finally the pH was adjusted at 4.5 with 0.1 N hydrochloric acid. The mixture was left to stand at room temperature for 1 hour and 4 hours at 4°C to obtain a precipitate which was centrifuged for 1 hour at 1000 g. The precipitate was dissolved in 5 ml of 0.1 M NaHCO3, thoroughly dialysed and freeze-dried.

B. Preparation of digoxin-BSA
Digoxin bovine serum albumin (BSA) was prepared in the same manner as the above digoxin-HRP, but the starting materials were 436 mg of digoxin and 560 mg of BSA, the quantities of the other reagents having been raised in the same ratio as the digoxin.

C. Preparation of antibodies against digoxin
Five rabbits were each injected with 400, 800 and 1600 μg of digoxin-BSA respectively, at fortnightly intervals. The immunogen was mixed with complete Freund’s adjuvant and administered intramuscularly. A fortnight after the last injection the animals were injected intravenously with 800 μg of digoxin-BSA in physiological salt. Ten days later the animals were bleded. The serum was adsorbed with BSA-m-aminobenzylxymethyl-cellulose.

D. Preparation of anti-digoxin-cellulose
Anti-digoxin-cellulose was prepared by Gurvich’s method as described in example I D.
E. Determination of digoxin
A dilution series was prepared of digoxin in 0.1 M phosphate buffer of pH 7.5 containing 0.9% NaCl, 0.5% Tween-20 and 1.0% of BSA. The dilution series was of from 0.1–100 ng/ml. One millilitre of a digoxin solution was mixed with 0.1 ml of digoxin-HRP in a suitable dilution, after which 2 mg of antidigoxin-cellulose suspended in 0.4 ml of buffer were added. The mixture was rotated for 6 hours at room temperature, after which it was centrifuged and the enzyme activity in the supernatant liquid determined.

Addition of 0.8 ng/ml of digoxin proved to cause a measurable increase of enzyme activity in the supernatant liquid. Digitoxin only showed a slight cross-reaction in the system whereas cholesterol, cortisone, oestradiol, testosterone and progesterone did not show any cross-action in the system.

Example VI

Determination of cortisol

A. Preparation of cortisol-21-galactose-oxidase

Fifty mg of cortisol-21-hemisuccinate and 100 mg of galactose-oxidase were coupled by the mixed hydride technique as described in example I A.

B. Preparation of insoluble transcortine

One hundred milligrams of transcortine, purified by DEAE, cellulose, and hydroxylapatite chromatography respectively, were coupled to 3 gm Sepharose 4 B by the CNBr method, as follows. Three grams of Sepharose 4 B suspension were activated by mixing it with 4 ml of a 2.5% (w/v) CNBr solution in distilled water, after which the pH was adjusted between 10 and 11 with 1 N NaOH, at which value it was maintained for 6 minutes. The Sepharose was washed with ice-water and with 0.1 M NaHCO₃. Then 100 mg of transcortine in 20 ml of 0.1 M NaHCO₃ were added and the suspension was shaken for 24 hours at 4°C. After being washed with successively 0.5 M NaHCO₃, 0.05 M citrate buffer of pH 4.5 and 0.05 M phosphate buffer of pH 6.0, the Sepharose was kept in the last buffer, to which 0.1% mercaptoethanol had been added.

C. Determination of cortisol

Of a cortisol-containing sample (standard, plasma or urine) 0.5 ml were extracted twice with methylene chloride (2 × 3 ml). The combined extraction liquids were evaporated to dryness. The residue was taken up in 0.5 ml of physiological salt solution and mixed with 0.2 ml of cortisol-21-galactose-oxidase in a suitable concentration and 0.3 ml of a transcortine-Sepharose suspension (5 mg/ml). The mixture was rotated for 15 minutes at 4°C and centrifuged, after which the enzyme activity in the supernatant liquid was determined by adding 0.5 ml of it to 1.5 ml of substrate consisting of: 100 mg of D-galactose, 20 mg of 5-aminosalicylic acid and 10 μg of peroxidase in 150 ml of 0.02 M phosphate buffer of pH 6.0. Thirty minutes later the extinction was measured at 460 nm.

Five ng/ml of cortisol in the sample caused a measurable increase of the enzyme activity in the supernatant liquid. Corticosterone and progesterone only influenced the system when larger quantities were added. Testosterone and aldosterone had hardly any influence.

Determination of transcortine

The reagents used for the determination of cortisol, as described in Example IV, were also employed for the determination of transcortine.

Of a dilution series of transcortine of from 0 – 1280 ng/ml 0.5 ml was incubated for 15 minutes at 4°C with 0.2 ml of cortisol-21-galactose-oxidase in a suitable dilution. To this dilution series 0.3 ml of transcortine-Sepharose (15 mg/ml) were added and the mixture was rotated for 15 minutes at 4°C. The activity in the supernatant liquid was measured as described in example VI.

A sample containing 40 ng/ml of transcortine proved to cause a measurable increase of enzyme activity in the supernatant liquid, whereas in the presence of 320 ng/ml all the enzyme activity was found in the supernatant liquid.

What is claimed is:

1. Process for the demonstration and determination of a low molecular weight organic compound by means of an antibody against said low molecular weight compound, comprising the steps of:
   a. providing a given quantity of the coupling product of said low molecular weight organic compound with an enzyme;
   b. providing a corresponding given quantity of an insolubilized antibody against said low molecular weight organic compound;
   c. contacting a sample of a fluid containing the low molecular weight organic compound to be determined with said components (a) and (b) to form a reaction mixture; and
   d. determining the enzyme activity of the liquid or the solid phase of the resulting reaction mixture, which activity is a measure of the quantity of low molecular weight organic compound to be determined.

2. The process of claim 1 in which said low molecular weight organic compound is a hapten.

3. The process of claim 1 in which said enzyme is an oxido-reductase.

4. Process for the demonstration and determination of a low molecular weight organic compound by means of a protein capable of reacting to bind said low molecular weight organic compound specifically, comprising the steps of:
   a. providing a given quantity of the coupling product of said low molecular weight organic compound with an enzyme;
   b. providing a corresponding given quantity of an insolubilized specific binding protein having more than one valency capable of reacting to bind said low molecular weight organic compound specifically;
   c. contacting a sample of a fluid containing the low molecular weight organic compound to be determined with said components (a) and (b) to form a reaction mixture; and
   d. determining the enzyme activity of the liquid or the solid phase of the resulting reaction mixture, which activity is a measure of the quantity of low molecular weight organic compound to be determined.

5. The process of claim 4 in which said enzyme is an oxido-reductase.
6. Process for the demonstration and determination of a specific binding protein having two or more binding sites by means of a low molecular weight organic compound capable of reacting to bind said protein specifically; comprising the steps of:
   a. providing a given quantity of the coupling product of said low molecular weight organic compound and an enzyme;
   b. providing a corresponding given quantity of an insolubilized specific binding protein having two or more binding sites capable of binding said low molecular weight organic compound;
   c. contacting a sample of a fluid containing the protein to be determined with said components (a) and (b) to form a reaction mixture; and
   d. determining the enzyme activity of the liquid phase of the resulting reaction mixture, which activity is a measure of the quantity of the specific binding protein to be determined.
7. The process of claim 6 in which said protein is an antibody.
8. A diagnostic pack for the demonstration and determination of a low molecular weight organic compound by means of an antibody against said low molecular weight organic compound, comprising:
   a. a given quantity of the coupling product of said low molecular weight organic compound with an enzyme;
   b. a corresponding given quantity of an insolubilized antibody against said low molecular weight organic compound; and
   c. a substrate for the determination of the enzyme activity of said enzyme.
9. The diagnostic pack of claim 8 in which said enzyme is an oxidoreductase.
10. A diagnostic pack for the demonstration and determination of a low molecular weight organic compound by means of a protein capable of reacting to bind said low molecular weight organic compound specifically, comprising:
    a. a given quantity of the coupling product of said low molecular weight organic compound with an enzyme;
    b. a corresponding given quantity of an insolubilized specific binding protein capable of reacting to bind said low molecular weight organic compound specifically; and
    c. a substrate for the determination of the enzyme activity of said enzyme.
11. The diagnostic pack of claim 10 in which said enzyme is an oxidoreductase.
12. A diagnostic pack for the demonstration and determination of a hapten by means of an antibody against said hapten, comprising:
    a. a given quantity of the coupling product of said hapten with an enzyme;
    b. a corresponding given quantity of an insolubilized antibody against said hapten; and
    c. a substrate for the determination of the enzyme activity of said enzyme.
13. The diagnostic pack of claim 12 in which said enzyme is an oxidoreductase.

* * * * *
UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 3,850,752
DATED : November 26, 1974
INVENTOR(S) : Schuurs et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the front page of the patent (disclosing the inventors, assignee, Abstract, References Cited, under "[21] Appl No.: 193,702" the following heading and contents thereunder:

Related U.S. Application Data

Continuation-in-part of Ser. No. 762,120, filed September 24, 1968, issued as U.S. Pat. 3,654,090 on April 4, 1972

In column 1, beneath the title "PROCESS FOR THE DEMONSTRATION AND DETERMINATION OF LOW MOLECULAR COMPOUNDS AND OF PROTEINS CAPABLE OF BINDING THESE COMPOUNDS SPECIFICALLY", Insert the following paragraph:

This application is a continuation-in-part of Ser. No. 762,120, filed September 24, 1968, issued as U.S. Pat. 3,654,090 on April 4, 1972.

In column 4, line 25, change "." to ":".

In column 5, lines 31 - 32, place "aminocellulose" in quotation marks.

In column 5, line 48, place "aminocellulose" in quotation marks.

In column 5, line 49, place "aminocellulose" in quotation marks.
UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 3,850,752
DATED : November 26, 1974
INVENTOR(S) : Schuurs et al.

It is certified that error appears in the above—identified patent and that said Letters Patent is hereby corrected as shown below:

In column 7, line 20, change ":" to ",:"

Signed and Sealed this Twelfth Day of July 1983

GERALD J. MOSSINGHOFF
Attest:

Attesting Officer          Commissioner of Patents and Trademarks