

PATENT SPECIFICATION

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- (72) Inventors VINCENT MARKS
 JAMES WILFRID BRIDGES
 DAVID MORRIS
 MICHAEL JOHN O'SULLIVAN and
 ERNESTO GNEMMI



(54) IMPROVEMENTS IN AND RELATING TO ENZYME-LINKED IMMUNOASSAY

(71) We, FARMITALIA CARLO ERBA S.P.A. formerly known as Carlo Erba S.p.A., an Italian Body Corporate, of Via Carlo Imbonati 24, 20159 Milan, Italy, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to enzyme-linked immunoassay and provides a novel method for the detection and assay of antigens, and reagents for use therein. The term "antigen" as used herein means not only substances which are capable by themselves of provoking the production of antibodies in animals, i.e. immunogens, but also substances, sometimes called haptens, which, after conjugation with a carrier molecule, become capable of provoking the production of and of reaction with specific antibodies.

There is a considerable amount of theoretical and practical interest in being able to detect and measure the presence of antigens. Since such substances are often of complex, and frequently unknown, chemical structure, and occur at very low concentrations in biological and other materials it is not possible to use conventional analytical techniques. As a result, in the past few years, a number of techniques have been developed based upon the antigen-antibody reaction, in which a component of the reaction is labelled with a radio-active tracer or, for example with an enzyme, so that the products of the reaction, or in some cases the unused reagent, can be detected and/or measured. While methods based on the use of radio-active labels have certain theoretical advantages, in particular the fact that with certain types of label, notably those utilising tritium or ¹⁴C, the radio-active tracer itself does not interfere with the antigen-antibody reaction, there are

certain disadvantages inherent in the use of this technique, in particular the need to provide the necessary apparatus to measure the radio-active tracer, to protect the worker from possible adverse effects from the radio-activity and to dispose of the radio-active waste after use. Many radioactive labels must be discarded at regular intervals due to the radiochemical decomposition of the radio-active isotope. Furthermore, it may be difficult to radio-label small molecular weight antigens and retain their essential antigenic properties. Considerable interest has, therefore, been shown in methods in which the antigen-antibody reaction is followed by using a reagent in which an enzyme acts as tracer. Such methods are simple to operate using relatively simple equipment, and do not involve any health hazard. Nonetheless, many of the systems proposed hitherto have suffered from the disadvantage that it is necessary for their successful operation to prepare a conjugate between the enzyme used as tracer and the antigen to be detected. The preparation of these conjugates is not always easy, because of the need to preserve the enzymic activity of the enzyme part of the conjugate, and it is particularly inconvenient to have to prepare a separate conjugate for each individual antigen which it is proposed to detect. In the method of the present invention, on the other hand, a single enzyme-containing conjugate can be used with a wide variety of antigens. Indeed, in principle, a single such conjugate can be used with all antigens.

In one prior proposal (Engvall *et al.*, J. Immunology 109 (1972) page 129), antibodies have been determined by reaction with antigens on an insoluble support followed by reaction of the supported antigen-antibody conjugate with an enzyme-labelled anti-immunoglobulin known to be capable of reacting with the antibody of the conjugate. In this method,

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the amount of enzyme finally attached to the support depends upon the amount of antibody in the sample under test, since the enzyme becomes linked to the support by a chain of which the antibody to be determined constitutes a distinct and essential link.

The method of the present invention for the detection or assay of an antigen (as hereinbefore defined) comprises bringing into contact, simultaneously or in stages, and in a liquid medium (1) an antigen (as hereinbefore defined) on an insoluble support or in the form of an insoluble aggregate, which antigen (1) may be the same as or different from the antigen to be detected or assayed, (2) a sample containing the antigen to be detected or assayed, (3) a first antibody (as hereinafter defined) and (4) an enzyme-labelled second antibody (as hereinafter defined); separating the soluble and insoluble materials, and measuring the enzyme activity of one or other of them. The term "first antibody" as used herein means an antibody able to bind both the antigen on the insoluble support and the antigen to be assayed. For a quantitative assay the first antibody should preferably be present in an amount more than sufficient to react with all the antigen present in (2) but not more than sufficient to react with all the antigen present in (1) plus (2). This is essential in quantitative procedures involving reaction of the first antibody sequentially with the sample (2) then with the supported or insoluble antigen (1). The term "second antibody" means antibody specific to antibodies of the immunoglobulin class to which the first antibody belongs e.g. all sheep IgG, rabbit IgG or rabbit IgM. For example, if the first antibody is an immunoglobulin raised in a particular animal, e.g. a sheep, then the second antibody is an antibody against immunoglobulins of that species, regardless of their own antibody specificities, raised in a different animal, e.g. a donkey.

The new method can easily be adapted for the detection and assay of a wide variety of antigens proper and haptens. These include serum and tissue proteins e.g. α -foetoprotein, α_2 -haptoglobulin and carcinoembryonic antigen; hormones, e.g. triiodothyronine, norepinephrine, thyroid stimulating hormone, follicle stimulating hormone, lutenizing hormone, insulin and thyroxine; steroids, e.g. cortisol, progesterone, oestrone, oestradiol and testosterone, drugs, e.g. morphine, amphetamine, barbiturates, diphenylhydantoin, methotrexate and gentamycin; vitamins and food contaminants, e.g. pyridoxal-5-phosphate

and aflatoxins, herbicides, insecticides and nitrosoureas can also be used.

It will be noted that the nature of the enzyme-labelled second antibody in no way depends upon the antigen to be detected. It is only necessary to raise the first antibody in an animal of the same species as that which provided the immunoglobulin used to provoke the production in another species of the second antibody. There is, therefore, no need to provide a wide variety of enzyme labelled antibodies, and the difficulties inherent in linking enzymes to antibodies require to be solved only once.

Any enzyme could be employed as a label on the second antibody. Oxidases such as horse radish peroxidase, hydrolytic enzymes such as alkaline phosphatase or dehydrogenases such as glucose-6-phosphate dehydrogenase are particularly appropriate. Ideally an enzyme used as the label should have the following properties: (1) it is available relatively cheaply in high purity; (2) it has a high activity per unit weight; (3) it is easily water-soluble and stable in ordinary storage conditions; (4) it makes it possible to use an assay procedure which is simple, rapid, sensitive and cheap; (5) it should be absent from biological fluids; and (6) biological fluids should contain no substrates, inhibitors, or activators for it; (7) it should contain suitable, chemical groups for conjugation to a second antibody, enabling conjugation to have only a minimal effect on both enzyme and antibody activity. β -galactosidase meets these requirements. Other possible but less preferable enzymes include glucose oxidase, acetylcholinesterase, glucoamylase, lysozyme, glucose-6-phosphate dehydrogenase, and malate dehydrogenase.

The enzyme used is preferably coupled to a second antibody using the coupling procedure described below, which is applicable to any combination of antibody and enzyme provided that the latter contains a mercapto group or can be modified so as to contain such a group. However, other coupling methods can be used if required. These include

a) One step glutaraldehyde linkage in which glutaraldehyde is used to link proteins in a complicated reaction to give heterogeneous conjugates of high molecular weight, (see Avrameas, S., *Immunochemistry* 6 43 (1969)).

b) Two step glutaraldehyde linkage, in which the enzyme horse radish peroxidase reacts with only one molecule of glutaraldehyde. This limits enzyme-enzyme conjugation and is an improvement on the one step method. However, this method is probably not applicable to many enzymes besides horse radish peroxidase as these will

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ectic and	65		react with more than one molecule of glutaraldehyde. (see Avrameas, S and Ternynck, <i>Immunochemistry</i> 8 1175 (1971)).	65
nature of the body in no			c) Oxidation of saccharide residues in the enzyme followed by Schiff base formation.	
lign to be	70	5	Horse radish peroxidase has several oligosaccharide groups, and their oxidation to aldehyde groups (using periodate) that can react with amino groups is the basis of	70
to raise the			this method of linkage. Other enzymes also contain oligosaccharide groups and can in principle also be used. However, large molecular weight conjugates are formed using this procedure. (Nakane P.K.+Kawaoki, A. J. <i>Histochem. Cytochem.</i> 22 1084 (1974)).	
of the same		10	d) Dimaleimide linkage using N,N'-o-phenylenedimaleimide, after introduction of -SH groups into the antibody using 2-mercaptoethylamine which reduces pre-existing -S-S- bridges to two SH groups. (Kato <i>et al</i> <i>European J. Biochem.</i> 62 285 (1976)).	
provided the			e) Other bifunctional reagents which may be used include toluene-2,4-diisocyanate, p',p'-difluoro-m,m'-dinitrophenyl sulphone, 1 - cyclohexyl - 3 - (2 - morpholinoethyl) - carbodiimide but these generally give inferior results to those described above.	
provoked the	75		f) Linkage may also be effected with hetero-bifunctional reagents, i.e. reagents containing two different functional groups in each molecule, e.g. m-maleimidobenzyl N-hydroxy-succinimide ester, which has been used in a two step reaction to couple insulin to β -galactosidase. (T. Kitagawa+T. Aikawa <i>J. Biochem.</i> 79 233-236 (1976)).	75
of the second		15	g) Noncovalent crosslinking, by for example, coupling antibodies to horseradish peroxidase to form a conjugate which is noncovalent but relatively stable in nature. This method has the disadvantages that	
no need to			(i) On storage the enzyme antibody conjugate may slowly dissociate.	
yme labelled	80	20	(ii) The antibodies may inhibit the enzymes they are raised against	80
is inherent in			(iii) The antibodies take several months to produce, and antibodies raised by different workers have different properties.	
require to be		25	Because of the greater speed and convenience associated with the handling of relatively large quantities of antibodies, it is often convenient, if the first and second antibodies are raised in relatively large animals such as horse, donkeys or sheep, rather than in small animals, such as rats, mice, guinea pigs or hamsters through the use of these is not excluded. As already indicated, the first and the second antibody must be raised in different species of animals. It has been found convenient to raise the first antibody in sheep and the second antibody in a donkey, but any other	85
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pair of different species of animals can be used if preferred.

The possibility of interference by the second antibody of the antigen-first antibody reaction is lessened if the second antibody is raised not against the whole of the first antibody, but only against the Fc fragment of the immunoglobulin constituting the first antibody.

The second antibodies can be prepared essentially in known manner, but the preferred method is to prime the animals, follow the production of antibodies and to give booster dosages when serum antibody levels are past their peak. The blood usually contains maximum levels of antibodies shortly after boosting. When a suitably high level (or a maximum level) of anti-immunoglobulin has accumulated in the blood stream of the animal the latter is bled and the serum containing the anti-immunoglobulin is separated.

The second antibody is preferably purified by incubating the donkey antiserum with an appropriate immuno-adsorbent, for example, Sepharose (Registered Trade Mark) or other insoluble support which has been activated by reaction first with a suitable bifunctional reagent, e.g. 1,4-bis(2,3-epoxypropoxy)butane, and then with a solution of the immunoglobulin (or Fc fragment thereof) used in raising the second antibody. This gives an immuno-adsorbent which is specific for the second antibody, and the latter can be purified by adsorption on the immuno-adsorbent followed by elution, before or after labelling with an enzyme.

To prepare the labelled second antibody, it is preferred to treat the immuno-adsorbent having the second antibody adsorbed thereon with a reagent such as methyl - mercaptobutyrimidate hydrochloride so as to modify the amino groups in the antibody and introduce from 3 to 5 thiol groups in each antibody molecule. The second antibody having the incorporated mercapto groups is then eluted from the immuno-adsorbent and treated so as to bind the enzyme thereto. Preferably it is treated with N,N'-o-phenylene-bis-maleimide and then reacted with purified β -galactosidase normally derived from *Escherichia coli*. The enzyme-second antibody conjugate is finally purified by chromatography on a diethylaminoethylagarose gel or other suitable adsorbent so as to separate the conjugate from unreacted enzyme and unreacted second antibody.

The first antibody is raised in an appropriate animal using known techniques. Where, as is often the case, the antigen to be detected or assayed is a hapten of relatively simple chemical structure e.g.

triiodothyronine, oestradiol, gentamycin, phenytoin or morphine, it is not capable by itself of provoking the formation of the first antibody. It is therefore necessary to conjugate the antigen with a carrier molecule to produce a conjugated molecule capable of provoking the production of antibody. For this purpose, it is convenient to conjugate the antigen with a readily available protein foreign to the animal in which the first antibody is to be raised, e.g. bovine serum albumin (BSA). The conjugation may be carried out by reaction of the albumin or other protein with, for example, 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide, followed by reaction of the product with the antigen, which must, for this reaction, contain an amino or carboxylic group. Other known methods of conjugate can be used if desired, ideally about 3 to 30 molecules of the antigen should be incorporated into each molecule of the albumin or other high molecular weight protein.

The first antibody may be raised by injecting into a suitable animal, e.g. a sheep, the antigen or antigen conjugate, repeatedly over a period of weeks or months until a sufficiently high antibody titre with the required binding properties has accumulated in the animal's blood stream. The animal is then bled and the anti-serum separated.

The first antibody is preferably then purified by contact with the antigen against which the antibody was raised or a related compound on an insoluble support. After absorption, the antibodies can be eluted, e.g. with guanidinium hydrochloride solution at high or low pH with solutions of high salt concentration, or with non-aqueous solvents, and then dialysed against an appropriate buffer to remove the eluting reagent.

The insoluble support used to carry the antigen or related compound both in the purification procedure just mentioned and in the method of the invention may be any suitable water-insoluble material to which the antigen or related compound can conveniently be bound sufficiently to ensure that it remains attached during the reactions involved in the purification or assay method as the case may be. While it is possible to use a support which simply adsorbs the antigen, as in the system described by Engvall *et al* in the paper already mentioned, it is preferred to bind the antigen chemically to the support, and this means that it is necessary for the support to contain functional groups through which the necessary reactions can take place. Supports including glass, nylon polyacrylamide, cellulose or dextran may be used. Certain types of immuno-adsorbents

containing hydroxyl groups can be reacted with 1,4-bis(2,3-epoxypropoxy)butane and the product reacted with the antigen. This method has been found to be simple, safe, effective and give a stable covalent linkage provided the antigen or related compound contains hydroxyl, amino or thiol groups. The requirements for an immunoadsorbent for purifying antibodies are not necessarily the same as those for the immunoadsorbent in the actual assay.

The method of the present invention can be carried out in a variety of different ways. The quantity of first antibody and hence label attached to the insoluble antigen bears an inverse relationship to the amount of antigen in the sample to be assayed. The enzyme activity, either free or attached to the insoluble or supported antigen is measured. Some of the specific ways of carrying out the new method are as follows.

In a first method, the first antibody is incubated with a sample containing the antigen to be determined. After the reaction is complete, the supported antigen is added, and the mixture again incubated. The solid phase is then separated from the liquid phase, washed, and mixed with excess of the enzyme-labelled second antibody. After incubation, the solid phase is again separated and washed. The amount of activity found in the insoluble fraction (solid phase) is inversely proportional to the amount of antigen present in the sample, that in the supernatant is directly proportional to it. Either fraction may be measured.

In a second method, the first antibody is reacted with the enzyme-labelled second antibody to form a complex which is then added to excess to the sample containing the antigen to be determined and incubated. The insoluble support carrying the antigen is then added, and, after incubation, the solid and liquid phases are separated. Determination of the enzyme activity of the liquid phase gives a result which is directly proportional to the amount of antigen in the sample.

In a third method, the complex of first and second antibodies, the sample containing the antigen to be determined, and the support carrying the antigen are incubated simultaneously and, after separation of the solid phase, the enzyme activity of the liquid phase is determined. Again, the result is directly proportional to the concentration of antigen in the sample.

As already indicated, for a quantitative assay by the first and second methods above, the amount of first antibody used must be in excess of that required to react with all the antigen in the sample, and the support carrying the antigen must be added in a sufficient excess to ensure adsorption of

all the first antibody which has not reacted with antigen in the sample. In practical terms, the easiest way of ensuring this is by carrying out a series of determinations with different concentrations of first antibody in geometric or arithmetic series. If this is done, it is a simple matter to find an appropriate concentration of the first antibody which enables an accurate assay to be carried out.

The following Examples illustrate the invention.

EXAMPLE 1

1. Production and Labelling of Second Antibody

(a) Preparation of F_c fragment of sheep immunoglobulin (IgG)

The method employed for the preparation of sheep F_c fragment is based on that of Porter (R. R. Porter, 1959, *Biochem J.* 73, 119).

320 ml. of sera were collected from 28 sheep. 150 ml. of serum were incubated with stirring in an ice-bath with 30 g. of moist diethylaminoethyl (DEAE)-A 50 Sephadex (Registered Trade Mark) previously equilibrated with 10 mM phosphate buffer at pH 6.5. After one hour the gel was filtered on a coarse sintered glass funnel and washed with 50 ml. of 10 mM phosphate buffer pH 6.5. The filtrate plus washings were incubated once more with 30 g. of moist DEAE-Sephadex, filtered and washed with 100 ml. of the phosphate buffer. The filtrate and washings (300 ml.) were treated with 150 ml. of saturated $(NH_4)_2SO_4$ solution which were slowly added to the continuously stirred solution. The precipitate was collected and washed with 100 ml. of 20% $(NH_4)_2SO_4$ solution. The final precipitate was dissolved in 50 ml. of 50 mM phosphate buffer of pH 8.0 and dialysed overnight at 4°C, against 5 l. of distilled water. The solution was then freeze-dried.

1.50 g. of freeze-dried sheep IgG so obtained was dissolved in 50 ml. of 0.1 M Tris-HCl buffer at pH 7.2 containing 10 mM cysteine and 2 mM EDTA. The buffer was prepared immediately before use. 0.6 ml. of a crystalline suspension of papain (Sigma, twice crystallised) in 50 mM-Acetate buffer at pH 4.5 was added. The solution was incubated at 37°C., for 4 hours with occasional shaking. 0.222 g. of iodoacetamide was then added and the solution immediately applied to a column (80x5 cm.) of G.100 Sephadex equilibrated with 10 mM-Acetate buffer at pH 5.6 at 4°C.

The second peak eluted from the column with 10 mM-Acetate buffer at pH 5.6 contains a mixture of F_c and F_{ab} fragments. The first peak contains undigested IgG. The

second peak was applied to a column (40x2.5 cm.) of carboxymethylcellulose (Registered Trade Mark) Whatman CM 32) equilibrated with 10 mM-Acetate buffer at pH 5.6 at 4°C. A peak was eluted from the column on washing with 10 mM-Acetate buffer pH 5.6 and several minor peaks and a major peak were eluted when a gradient from 10 mM-to 500 mM-Acetate pH 5.6 was applied (1000 ml. in each compartment of a gradient mixer). The major peak was collected and rechromatographed on the carboxy-methyl-cellulose column. This time a gradient from 10 mM- to 300 mM-Acetate buffer at pH 5.6 with 1000 ml. in a mixing vessel was applied. A single peak was eluted. This was collected, dialysed exhaustively against distilled water, and freeze-dried.

b) Preparation of Second Antibody (Donkey Anti-sheep immunoglobulin)

A stable emulsion was prepared by mixing 1 part of saline containing sheep F_c fragment (0.75 mg/ml) and *Bacillus Calmette-Guerin* (1.5 mg/ml) with 2 parts of Marcol 52 adjuvant. Six intramuscular injections (1 ml) of the emulsion were given in the lower leg. The production of donkey anti-sheep antibodies was monitored and the animal was ready for boosting after two months. An emulsion consisting of 2 parts adjuvant to 1 part saline (sheep F_c 0.75 mg/ml) was injected intramuscularly into six sites (0.5 ml) in the lower legs. The bleeds ten days after and for the next month contained high level of antibodies.

c) Preparation of Immunoabsorbent for Purification of Donkey Anti-Sheep Immunoglobulin

Sepharose 4B (Registered Trade Mark) was activated with 1,4-bis(2,3-epoxypropoxy)butane by incubating equal volumes of the gel, the bisphoxide and 0.6 M NaOH containing 2 mg. per ml. sodium borohydride with continuous stirring at 25°C., for 8 hours, and then washed. Sheep IgG was prepared from a pool of normal serum by precipitation with 33% ammonium sulphate solution. A solution of the sheep IgG in 0.1 M $NaHCO_3$ buffer at pH 10 was added using 10 mg. of sheep IgG per ml. of gel. The activated gel was incubated with the IgG for 48 hours and the immunoabsorbent finally washed and treated with 0.1 M ethanolamine at pH 10 for 24 hours.

d) Purification and labelling of donkey anti-sheep second antibody

Donkey anti-sheep immunoglobulin was purified by incubating donkey anti-serum (prepared as described above) with the immunoabsorbent. Thiol groups were

incorporated into the immunoglobulin while it was adsorbed in the immunoabsorbent using methyl-4-mercapto-butyrimidate/HCl to modify the amino groups of the immunoglobulin. The extent of incorporation was limited to give between 3 and 5 thiol groups per globulin molecule.

The immunoabsorbent antibody complex was washed and suspended in 0.1 M N-ethylmorpholine/HCl at pH 7.5 at a concentration of 1 ml. immunoabsorbent in a total volume of ten mls. A solution of methyl-4-mercaptobutyrimidate/HCl in 0.2 M Na_2CO_3 (10 mg./ml) was prepared immediately before use. 0.1 ml. of this solution was then added for every 10 ml. of suspension. The suspension was stirred for 30 minutes at room temperature and then washed with 0.1 M N-ethylmorpholine/HCl at pH 7.5 containing 1 mg. dithiothreitol per 10 ml. of buffer. The gel was then thoroughly washed with HCl containing 0.1 M NaCl to pH 3.5.

The immunoglobulin containing incorporated thiol groups thus prepared was eluted with HCl containing 0.1 M NaCl at pH 2.5. The most convenient method for achieving this was to pack the immunoabsorbent in a small column and collect the effluent in tubes containing buffer. Since the next stage of the preparation is performed in 0.1 M Acetate buffer at pH 5.0, the effluent was collected in sufficient 0.1 M Acetate at pH 6.0 to give a final pH of 5.0.

2 ml. of 0.1 M Acetate buffer at pH 5.0 containing 2.5 mg. of the immunoglobulin modified with thiol groups were cooled on an ice-bath. This solution was then slowly added drop by drop to a 1 ml. saturated solution of N,N'-o-phenylene-bis-maleimide in 0.1 M Acetate buffer at pH 5.0 on an ice-bath. The mixture was next incubated at 30°C., for 20 minutes. The immunoglobulin was then separated from excess N,N'-o-phenylene-bis-maleimide by passing the solution through a column (20x1.5 cm.) of G. 25 Sephadex equilibrated at room temperature with 0.01 M phosphate buffer containing 0.01 M MgCl_2 at pH 6.0.

5.0 mg. of ammonium sulphate precipitated β -galactosidase from *Escherichia coli* were dissolved in the purified activated immunoglobulin solution and incubated at 30°C., for 4 hours. Sufficient N NaOH to adjust the pH of the reaction mixture to 7.5 and sufficient 2 M 2-mercapto-ethanol to give a final concentration of 10 mM was added.

The immunoglobulin-enzyme conjugate solution was applied to a column (60x0.9 cm.) of DEAE-Agarose (Biogel) equilibrated at 4°C., with 10 mM Tris-HCl buffer at pH 7.5 containing 10 mM 2-mercapto-ethanol, 10 mM MgCl_2 and 50 mM sodium chloride.

The solution was washed onto the column with the equilibrating buffer. A gradient from 50 mM to 200 mM sodium chloride in the same buffer was applied with 250 ml. in the mixing vessels. Immunoglobulin unconjugated with enzyme fails to adsorb to the column and is eluted before the gradient is applied. When the gradient is applied immunoglobulin labelled with enzyme is eluted before free enzyme. The eluted fraction containing the labelled immunoglobulin is freeze dried to provide the enzyme-labelled second antibody for use in the invention.

2. Production of first antibody

(a) Preparation of triiodothyronine (T_3) conjugate

250 mg. of bovine serum albumin (BSA) were dissolved in 25 ml. of distilled water and 1000 mg. of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were dissolved in 50 ml. of distilled water. Both solutions were cooled to 4°C., and two-thirds of the EDC solution slowly mixed with the BSA solution. 150 mg. of purified T_3 were dissolved in 25 ml. of ethanol:2M- NH_4OH (25:1 v/v) and the solution, cooled to 4°C., was added dropwise to the mixture with continuous stirring the pH being maintained at 7.0 by dropwise addition of 0.25 M-HCl. The reaction mixture was stirred for a further 10 minutes and the remaining EDC solution added dropwise maintaining the pH at 7.0.

The mixture was stirred overnight at 4°C. In the morning the solution was centrifuged and the supernatant dialysed against three changes of 0.1 M $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ at pH 11.0 and then distilled water. The preparation was finally freeze-dried. The incorporation of T_3 into BSA was determined to be 4.3 moles of T_3 per mole of BSA.

(b) Preparation of first antibody (T_3 -antibodies)

A stable emulsion was prepared by mixing 1 part of saline containing Tween 80 (Registered Trade Mark) (50%), T_3 bovine serum albumin (2 mg./ml) and *Bacillus Calmette-Guerin* (BCG 1 mg./ml) with 2 parts of marcol S2 adjuvant. Six doses (1 ml each) of the emulsion were injected subcutaneously in the back, and four intramuscular injections (0.75 ml) were given in the legs. The antibody titres were monitored and after 36 weeks the animals were boosted. For the boost, six intramuscular injections (0.5 mls) were given in the legs. The emulsion consisted of 2 parts of adjuvant to 1 part of saline containing T_3 -BSA conjugate (2 mg./ml). The bleeds ten days after boosting and for

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the next two months contained high levels of T₃ antibodies.

(c) Production of Immunoabsorbent for the purification of first antibody

Sepharose 4B was activated with 1,4-bis-(2,3-epoxy-propoxy)butane as described previously. The activated Sepharose (4 ml) was added to phosphate buffer (pH 12.0 0.025 M, 20 ml.) containing T₃ (50 mg). The buffer was stirred for 45 hours at room temperature. The gel was then washed with phosphate buffer (pH 12.0, 0.025 M, 250 ml.) sodium chloride solution (150 ml, 0.1 M) and distilled water (100 ml). 9 mg. of T₃ were incorporated per ml. of swollen gel. Any remaining oxirane groups were blocked by suspending the gel in four times its volume of aqueous ethanolamine (1 M, pH 11) and shaking for 4 hours. The gel was then extensively washed with distilled water to remove excess ethanolamine.

(d) Purification of First Antibody

Anti-T₃ serum (prepared as described above, 20 ml) was mixed for one hour at room temperature with T₃-substituted Sepharose (1 ml). Non-specifically absorbed proteins were removed from the immunoabsorbent by extensive washing with phosphate buffered saline (0.05 M, pH 7.0, 0.2 M NaCl). The gel was packed in a syringe as described previously and washed with hydrochloric acid (pH 3) to remove the phosphate buffer. T₃-antibodies were eluted by washing with guanidinium hydrochloride (4 ml., 6 M, pH 2) and collected into barbitone buffer (1 ml, 0.05 M, pH 8.6, 0.1% bovine serum albumin). The T₃-antibodies were extensively dialysed against this buffer to remove the guanidinium hydrochloride.

3. Production of supported antigen (T₃) for use in the assay

The procedure is as described in the production of immunoabsorbent for the purification of the first antibody (2 c above) except that the concentration of T₃ is less. This decreases the concentration of T₃ substituted into the gel.

Assay for T₃ using the reagents whose preparation is described above is carried out in the manner already described.

The 'Universal Reagent' may be used in several different protocols to determine antigens. These include:—

Protocol 1a

Incubation of first antibody and free antigen followed by addition of immunoabsorbent, and after washing, addition of the label.

Protocol 1b

A simultaneous incubation of first antibody, free antigen and immunoabsorbent. The label is added to the washed immunoabsorbent.

Protocol 2a

An incubation of the first antibody, free antigen and label. This is followed after a suitable time by the addition of the immunoabsorbent.

Protocol 2a may be modified by incubating the first antibody with the enzyme label before addition of the free antigen (Protocol 2b).

Protocol 3

Protocol 3 involves the simultaneous addition of first antibody, label, free antigen and immunoabsorbent. This protocol may also be slightly modified by the preformation of the label-first antibody complex.

The following Examples describe the results of assays performed using the 'Universal Reagent' following the protocols described above.

Enzyme-immunoassay for triiodothyronine (T₃) using the Universal Reagent

The assay for T₃ was performed using several different procedures.

EXAMPLE 2

Protocol 1a

T₃ antisera (sheep, 1:2000 dilution) in barbitone buffer (0.1 ml, 0.05 M, pH 8.6, 0.1% w/v BSA) was mixed with T₃ standards prepared in barbitone buffer (0.1 ml) and held at 4°C for 24 hours. Barbitone buffer (0.1 ml, 0.3% w/v Tween 80) containing T₃ immunoabsorbent (50 µg) was added, mixed, held at 4°C for 1 hour, washed twice with barbitone buffered saline (5% w/v NaCl) and once with barbitone buffer. Enzyme label (100 µl, donkey anti-sheep IgG-galactosidase) was mixed with the washed immunoabsorbent, held at 4°C for 24 hours, washed as described previously and the enzyme activity bound to the immunoabsorbent was measured by the usual methods.

Results

T3 concen- tration (nmol/l)	ΔOptical density (2 hours)	Percentage of zero binding*
0	0.29	100
1	0.17	50
4	0.16	47
32	0.135	35

Non-specific binding of label to immunoabsorbent=0.05

*Binding calculated after subtracting NSB.

A similar assay was performed using donkey anti-sheep Fc-galactosidase as label. The procedure was as described above except that (1) the antisera and standards were held together for 8 hours, (2) the antisera dilution was 1:1600

added to the tubes and incubated at 4°C for 1 hour. The immunoabsorbent was washed in turn with barbitone buffer containing 5% then 1% sodium chloride. Enzyme activity bound to the immunoabsorbent was assayed by the usual methods.

	T3 concentration (nmol/l, ng/ml)		ΔOptical density (2 hours)	Percentage of zero binding
10	0	0	0.43	100
	0.42	0.25	0.35	81
	1.56	1.0	0.28	65
	6.25	4.0	0.16	37
	25	16	0.15	35
15	NSB 0.025			

Results		
T3 concentration (nmol/l)	ΔOptical density (1 hour)	Percentage of zero binding*
0	0.44	100
0.125	0.40	83
0.25	0.36	69
0.5	0.37	74
1	0.29	46
2	0.24	27
4	0.20	15
8	0.19	12
NSB	0.16	

EXAMPLE 3

Protocol 2a

Purified T3 antisera (sheep, 1:1500 dilution, 0.1 ml) and T3 standards (0.1 ml) in barbitone buffer (0.05 M, pH 8.6, 0.1% w/v BSA) were mixed with label (25 μl, donkey anti-sheep IgG-enzyme) and held at 4°C for 24 hours. Barbitone buffer (0.1 ml, 0.3% w/v Tween 80) containing T3 immunoabsorbent (50 μg) was added with mixing and held at 4°C for 1 hour. The immunoabsorbent was washed and the bound enzyme activity was assayed as described in Example 2.

Results			
T3 concentration (nmol/l)	ΔOptical density (1 hour)	Percentage of zero binding*	
0	0.47	100	
0.5	0.42	90	
1	0.35	70	
2	0.24	45	
4	0.21	38	
8	0.20	36	
16	0.18	33	
32	0.17	31	
64	0.14	22	
NSB	0.05		

*Calculated after subtracting NSB.

EXAMPLE 4

Protocol 2b

Purified T3 antibodies (1:1500 dilution) in barbitone buffer (0.05 M, pH 8.6, 0.1% w/v BSA, 8.5 ml) were mixed with the enzyme label (donkey anti-sheep IgG-galactosidase conjugate, 5.1 ml) and stored at 4°C until required.

T3 standards in barbitone buffer (50 μl) were mixed with the label-first antibody complex (200 μl) and additional barbitone buffer (0.5 μl), then held at 4°C for 4 hours. T3 immunoabsorbent (50 μg) in barbitone buffer containing Tween 80 (0.4%) was

*Binding calculated after subtracting NSB.

This experiment was repeated with a donkey anti-sheep F_c-enzyme label. The results were similar to those shown above.

EXAMPLE 5

Protocol 3

T3 standards in barbitone buffer (100 μl) were mixed with the label-first antibody complex (200 μl) and T3 immunoabsorbent (25 μg) in barbitone buffer (100 μl) containing Tween 80 (0.4% w/v) and incubated at 4°C for 2 hours. The immunoabsorbent was washed and the bound enzyme was assayed as described for Example 4.

Results		
T3 concentration (nmol/l)	ΔOptical density (1 hour)	Percentage of zero binding*
0	0.33	100
0.5	0.32	94
1	0.29	83
2	0.28	77
4	0.24	60
8	0.21	42
16	0.18	32
NSB=0.114		

*Binding calculated after subtracting NSB.

EXAMPLE 6

Protocol 2b

The procedure was as described previously for Example 4 except that the standards were made up in stripped plasma. Thyroid binding globulin was destroyed by heating the plasma samples in a water bath at 70°C for 1 hour. The results obtained were similar to that described previously in Example 4.

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EXAMPLE 7

Enzyme-immunoassay for human growth hormone (HGH) using the Universal Reagent

5 Protocol 1a

HGH antisera (rabbit, 1:20,000 dilution) in barbitone buffer (0.1 ml, 0.05 M, pH 8.6, 0.65 M NaCl, 0.5% w/v BSA) was mixed with HGH standards dissolved in barbitone buffer (0.1 ml) and held at 4°C for 24 hours. HGH immunoabsorbent in barbitone buffer (0.1 ml, 10 µg) was then added, mixed, held at 4°C for 1 hour and washed once with barbitone buffer (2 ml, 0.05 M, pH 8.6) containing 5% NaCl and once with buffer containing 1% NaCl. Enzyme label (50 µl, donkey anti-rabbit Fc-galactosidase) was added to the washed immunoabsorbent, mixed, held at room temperature for 24 hours, washed as described above and enzyme activity bound to the immunoabsorbent was measured by the usual methods.

Results

25	HGH concentration (ng/ml)	ΔOptical density (1 hour)	Percentage of zero binding*
	0	0.64	100
	0.39	0.61	95
30	0.78	0.60	93
	1.56	0.51	75
	3.125	0.43	63
	6.25	0.38	53
	12.5	0.31	41
35	25	0.26	33
	30	0.22	24
	100	0.18	18

Non-specific binding of label to immunoabsorbent=0.08

40 *Binding calculated after subtracting NSB.

EXAMPLE 8

Enzyme-immunoassay for insulin using the Universal Reagent

45 Protocol 1a

Insulin antisera (guinea pig, 1:10,000 or 1:40,000 dilution) in phosphate buffer (0.1 ml, 0.04 M, pH 7.4, 0.5% w/v BSA) was mixed with bovine insulin standards prepared in phosphate buffer (0.1 ml), and held at 4°C for 24 hours. Insulin immunoabsorbent in phosphate buffer (0.1 ml, 25 µg) was added, mixed and the tubes held at 4°C for 30 minutes. The immunoabsorbent was then washed with phosphate buffer (2 ml) containing 5% and then 1% sodium chloride. Enzyme label (50 µl, donkey anti-guinea pig IgG-galactosidase conjugate) was added to the tubes, mixed and held at room temperature for 24 hours. The immunoabsorbent was washed and as described previously and the

galactosidase activity bound to the immunoabsorbent was measured by the usual methods.

Results

Antisera dilution 1:10,000.

Insulin concentration (ng/ml)	ΔOptical density (90 minutes)	Percentage of zero binding
0	0.62	100
0.96	0.50	83
4.8	0.34	55
24	0.26	42

Antisera dilution 1:40,000

Insulin concentration (ng/ml)	ΔOptical density (90 minutes)	Percentage of zero binding
0	0.36	100
0.96	0.18	50
4.8	0.17	47
24	0.16	44

Nonspecific binding=0.05 of enzyme label in immunoabsorbent.

EXAMPLE 9

Enzyme-immunoassay for cortisol using the Universal Reagent

Protocol 1a

Cortisol antisera (sheep, 1:20,000 dilution) in phosphate buffer (0.1 ml, 0.1 M, pH 7.4, 0.1% w/v gelatine, 0.83% w/v NaCl, 0.1% thiomersal), was mixed with cortisol standards prepared in phosphate buffer (0.1 ml), and held at 4°C for 24 hours. Phosphate buffer (0.3% w/v Tween 80) containing cortisol immunoabsorbent (0.1 ml, 25 µg) was then added, mixed, held at 4°C for 1 hour, washed once with phosphate buffer saline (3% w/v) and once with phosphate buffer. Enzyme label (25 µl, donkey anti-sheep IgG-galactosidase conjugate, 0.5% w/v Tween 80) was added to the washed immunoabsorbent, mixed, held at 4°C for 24 hours, washed as above and assayed (as usual) for enzyme activity bound to the solid phase.

Results

Cortisol concentration (ng/ml)	ΔOptical density (1 hour)	Percentage of zero binding*
0	0.33	100
125	0.29	87
250	0.26	74
500	0.21	59
1000	0.18	47
2000	0.15	37
4000	0.11	24
8000	0.11	24

Non-specific binding of label to immunoabsorbent=0.04

*Calculate after subtracting NSB.

It is convenient to provide kits of materials for use in performing the method according to this invention on particular antigens, such a kit comprising an insoluble support having bound thereto an antigen which may be the same as or different from that which the kit is intended for detecting or assaying, an appropriate first antibody, and an appropriate enzyme-labelled second antibody, and means for determining the enzyme activity as required in performing the method, and preferably including for calibration purposes a solution containing a known amount of the antigen which the kit is intended for detecting or assaying.

It may be advantageous to have the solid phase attached to a rod which can be inserted into and removed from the other components of the assay mixture. This would facilitate washing of the solid phase and increase the precision of the assay.

For example, a kit for T_3 assay could consist of the following components:

1. an insoluble support, either as a suspension in a buffer (e.g. barbitone) or in a lyophilized form, having bound thereto the antigen T_3 .

2. the first antibody (anti- T_3 raised in sheep) together with the second antibody (raised in donkey anti-sheep IgG Fc, the second antibody being labelled with β -galactosidase, this component preferably being in a lyophilized form.

3. a substrate (e.g. nitrophenyl galactoside) at a final concentration of about 10^{-3} M in a buffer (e.g. barbitone) preferably having a pH from 7 to 9 and a molarity of 0.010 to 0.200, this component preferably being in a lyophilized form.

4. a solution containing a known amount of antigen T_3 (e.g. 10 nanomoles per litre), this component preferably being in a lyophilized form.

WHAT WE CLAIM IS:—

1. A method for detection or assay of an antigen (as hereinbefore defined), comprising bringing into contact in a liquid medium, simultaneously or in stages, (1) an antigen (as hereinbefore defined) on an insoluble support or in the form of an insoluble aggregate which antigen (1) may be same as or different from the antigen to be detected or assayed, (2) a sample containing the antigen to be detected or assayed, (3) a first antibody (as hereinbefore defined), and (4) an enzyme-labelled second antibody (as hereinbefore defined), the second antibody (4) thereby becoming attached to the insoluble material in inverse relationship to the amount of the antigen to be detected or assayed; separating the soluble and insoluble materials; and determining the enzyme activity either in the soluble or in the insoluble separated

materials, thereby to determine the presence or amount of the antigen in the sample (2).

2. A method according to claim 1, wherein the first antibody (3) is reacted with (1) and (2), the solid phase is separated from the liquid phase, and all of the first antibody attached to the separated solid phase is thereafter reacted with the second antibody (4) in a second liquid medium free from (2) and (3), followed by separation of the solid phase from the second liquid medium and detection or measurement of the enzyme activity in the solid phase.

3. A method according to claim 1, wherein the second antibody (4) is reacted with the first antibody (3) to form a complex of the two antibodies, which complex is subsequently reacted with the antigen of (1) and (2).

4. A method according to Claim 2 or 3, wherein said first antibody or complex thereof is reacted with (2) and thereafter with (1).

5. A method according to Claim 2 or 3, wherein said first antibody or complex is reacted with (1) and (2) simultaneously.

6. A method according to any one of the preceding claims, wherein the amount of said first antibody is more than sufficient to react with all the antigen present in (2) but not more than sufficient to react with all the antigen in (2) plus all the antigen in (1).

7. A method according to any one of the preceding claims, wherein the antigen in (1) is the same as the antigen in (2) and is carried on said insoluble support.

8. A method according to any one of Claims 3 to 7, wherein the enzyme activity is determined in the liquid separated phase to determine the presence or amount of the antigen in the sample (2).

9. A method according to any one of the preceding claims, wherein the first antibody (3) is any one of those hereinbefore indicated.

10. A method according to any one of claims 1 to 8, wherein the second antibody (4) is any one of those hereinbefore indicated.

11. A method according to any one of the preceding claims, wherein the second antibody (4) is labelled with any one of the enzymes hereinbefore indicated.

12. A method according to Claim 1 substantially as described in any one of the foregoing Examples 2 to 9.

13. A method according to Claim 7 substantially as described in the foregoing Example 1.

14. A kit of materials for use in performing a method according to any one of the preceding claims, comprising an insoluble support (1) having bound thereto

(19)



It is convenient to provide kits of materials for use in performing the method according to this invention on particular antigens, such a kit comprising an insoluble support having bound thereto an antigen which may be the same as or different from that which the kit is intended for detecting or assaying, an appropriate first antibody, and an appropriate enzyme-labelled second antibody, and means for determining the enzyme activity as required in performing the method, and preferably including for calibration purposes a solution containing a known amount of the antigen which the kit is intended for detecting or assaying.

It may be advantageous to have the solid phase attached to a rod which can be inserted into and removed from the other components of the assay mixture. This would facilitate washing of the solid phase and increase the precision of the assay.

For example, a kit for T_3 assay could consist of the following components:

1. an insoluble support, either as a suspension in a buffer (e.g. barbitone) or in a lyophilized form, having bound thereto the antigen T_3 .

2. the first antibody (anti- T_3 raised in sheep) together with the second antibody (raised in donkey anti-sheep IgG Fc, the second antibody being labelled with β -galactosidase, this component preferably being in a lyophilized form.

3. a substrate (e.g. nitrophenyl galactoside) at a final concentration of about 10^{-3} M in a buffer (e.g. barbitone) preferably having a pH from 7 to 9 and a molarity of 0.010 to 0.200, this component preferably being in a lyophilized form.

4. a solution containing a known amount of antigen T_3 (e.g. 10 nanomoles per litre), this component preferably being in a lyophilized form.

WHAT WE CLAIM IS:—

1. A method for detection or assay of an antigen (as hereinbefore defined), comprising bringing into contact in a liquid medium, simultaneously or in stages, (1) an antigen (as hereinbefore defined) on an insoluble support or in the form of an insoluble aggregate which antigen (1) may be same as or different from the antigen to be detected or assayed, (2) a sample containing the antigen to be detected or assayed, (3) a first antibody (as hereinbefore defined), and (4) an enzyme-labelled second antibody (as hereinbefore defined), the second antibody (4) thereby becoming attached to the insoluble material in inverse relationship to the amount of the antigen to be detected or assayed: separating the soluble and insoluble materials: and determining the enzyme activity either in the soluble or in the insoluble separated

materials, thereby to determine the presence or amount of the antigen in the sample (2).

2. A method according to claim 1, wherein the first antibody (3) is reacted with (1) and (2), the solid phase is separated from the liquid phase, and all of the first antibody attached to the separated solid phase is thereafter reacted with the second antibody (4) in a second liquid medium free from (2) and (3), followed by separation of the solid phase from the second liquid medium and detection or measurement of the enzyme activity in the solid phase.

3. A method according to claim 1, wherein the second antibody (4) is reacted with the first antibody (3) to form a complex of the two antibodies, which complex is subsequently reacted with the antigen of (1) and (2).

4. A method according to Claim 2 or 3, wherein said first antibody or complex thereof is reacted with (2) and thereafter with (1).

5. A method according to Claim 2 or 3, wherein said first antibody or complex is reacted with (1) and (2) simultaneously.

6. A method according to any one of the preceding claims, wherein the amount of said first antibody is more than sufficient to react with all the antigen present in (2) but not more than sufficient to react with all the antigen in (2) plus all the antigen in (1).

7. A method according to any one of the preceding claims, wherein the antigen in (1) is the same as the antigen in (2) and is carried on said insoluble support.

8. A method according to any one of Claims 3 to 7, wherein the enzyme activity is determined in the liquid separated phase to determine the presence or amount of the antigen in the sample (2).

9. A method according to any one of the preceding claims, wherein the first antibody (3) is any one of those hereinbefore indicated.

10. A method according to any one of claims 1 to 8, wherein the second antibody (4) is any one of those hereinbefore indicated.

11. A method according to any one of the preceding claims, wherein the second antibody (4) is labelled with any one of the enzymes hereinbefore indicated.

12. A method according to Claim 1 substantially as described in any one of the foregoing Examples 2 to 9.

13. A method according to Claim 7 substantially as described in the foregoing Example 1.

14. A kit of materials for use in performing a method according to any one of the preceding claims, comprising an insoluble support (1) having bound thereto

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an antigen (as hereinbefore defined) which may be the same as, or different from the kind which the kit is intended for detecting or assaying, or comprising said antigen (1) in the form of said insoluble aggregate first antibody (3) (as hereinbefore defined), an enzyme labelled second antibody (4), (as hereinbefore defined) and means for determining the enzyme activity as required in performing said method.

15. A kit according to Claim 14, which includes a solution containing in known amount of the antigen which the kit is intended for detecting or assaying.

J. A. KEMP & CO.,
Chartered Patent Agents,
14, South Square,
Gray's Inn,
London, W.C.1.

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