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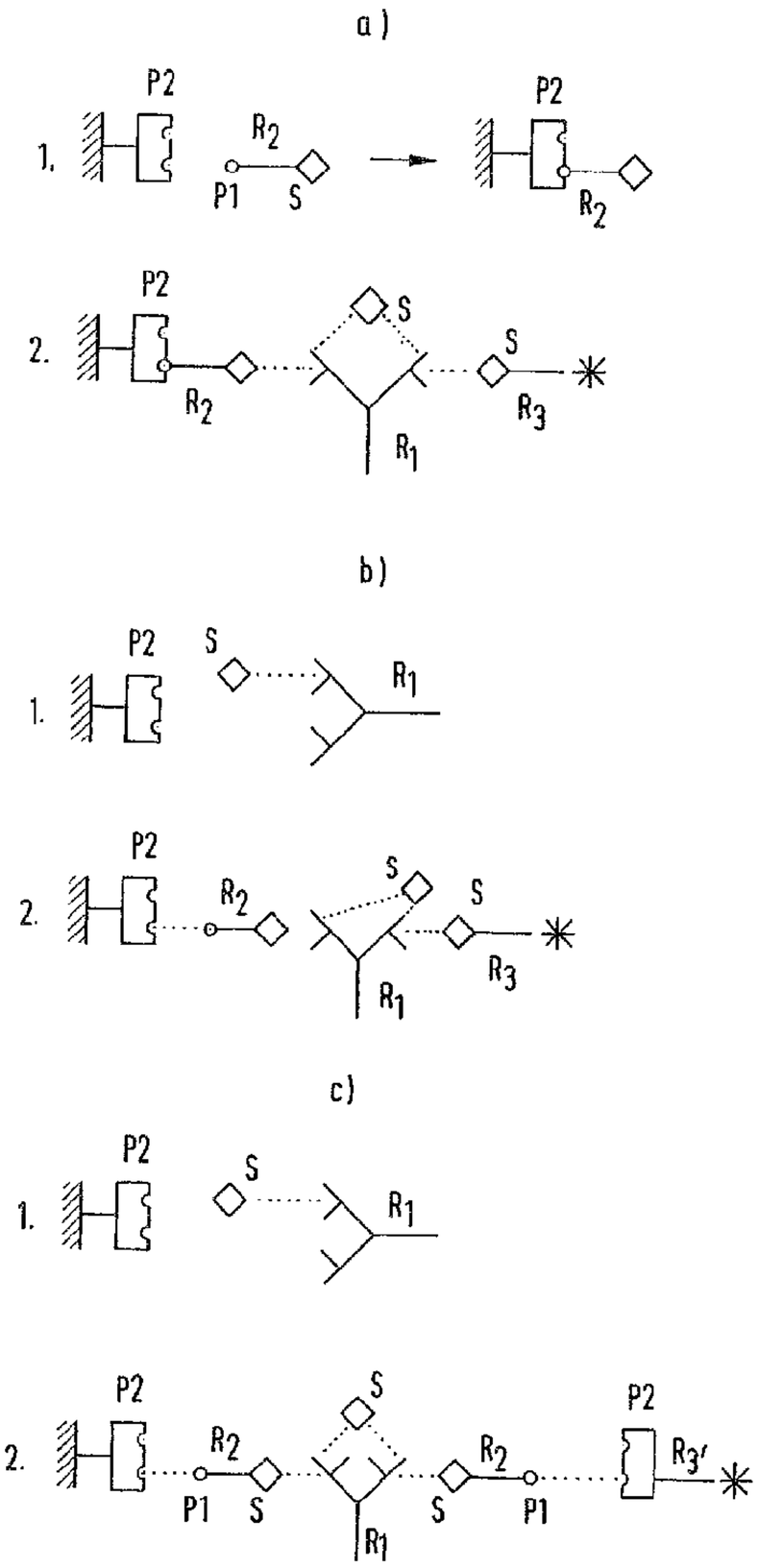
(54) **PROCEDE ET REACTIF POUR LA DETERMINATION D'UNE SUBSTANCE POUVANT ETRE PARTICULIEREMENT LIAISONNEE**

(54) **PROCESS AND REAGENT FOR THE DETERMINATION OF A SPECIFICALLY BINDABLE SUBSTANCE**



(57) The present invention provides a process for the determination of a specifically bindable substance by incubation of a sample solution with at least three receptors R_1, R_2 and R_3, of which R_1 is specifically bindable with R_2 and R_3,

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as well as with the substance to be determined, R_2 brings about the binding to a solid phase and R_3 carries a labelling, separation of bound labelling from unbound labelling and measurement of the labelling in one of the two phases, wherein, as receptor R_1, a receptor is used which has at least two binding positions which bind specifically with an epitope of the substance to be determined, as R_2 a conjugate of a partner P_1 of a specifically binding pair and of a substance S which corresponds to the substance to be determined or is a derivative thereof and has at least one epitope of the substance to be determined, the partner P_1 thereby either being bound to a solid phase or being immobilized, and as R_3 a complex is used which contains at least the substance S and a labelling. The present invention also provides a reagent for carrying out this process.

ABSTRACT

The present invention provides a process for the determination of a specifically bindable substance by incubation of a sample solution with at least three receptors R_1, R_2 and R_3, of which R_1 is specifically bindable with R_2 and R_3, as well as with the substance to be determined, R_2 brings about the binding to a solid phase and R_3 carries a labelling, separation of bound labelling from unbound labelling and measurement of the labelling in one of the two phases, wherein, as receptor R_1, a receptor is used which has at least two binding positions which bind specifically with an epitope of the substance to be determined, as R_2 a conjugate of a partner P_1 of a specifically binding pair and of a substance S which corresponds to the substance to be determined or is a derivative thereof and has at least one epitope of the substance to be determined, the partner P_1 thereby either being bound to a solid phase or being immobilized, and as R_3 a complex is used which contains at least the substance S and a labelling. The present invention also provides a reagent for carrying out this process.

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The present invention is concerned with a process for the determination of a specifically bindable substance by incubation of a sample solution with at least three receptors R_1, R_2 and R_3, of which R_1 is specifically bindable with R_2 and R_3, as well as with the substance to be determined, R_2 brings about the binding to the solid phase and R_3 carries a labelling, separation of bound and unbound labelling and measurement of the labelling in one of the two phases. The present invention also provides a reagent kit for carrying out this process.

Very many substances occur in body fluids and tissues which are immunologically active, i.e. they are bindable with a specific binding partner and serve as parameters for certain diseases or for the state of health of the human body. These include, inter alia, haptens, for example hormones, proteins, such as tumour labels, protein hormones and viral proteins, as well as antibodies. For the supervision of a medical treatment, the determination of the medicaments in the blood is frequently also necessary. Examples therefor include anti-epileptics, antibiotics, digitalis and opiates. Since these substances often only occur in very small amounts, processes according to the immunoassay principle are used for the detection thereof. There are many variants of this. The different immunological processes of determination can be divided into

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homogeneous and heterogeneous processes. In the case of heterogeneous processes, a solid phase reaction always participates in order to be able to separate the bound part of the labelled components from the unbound ones. In the case of the homogeneous process variant, no separation of bound labelling and unbound labelling takes place so that a differentiation of bound and unbound labelling must take place by other methods.

For carrying out heterogeneous immunoassays, there are essentially two variants in which, on the one hand, an antibody against the substance to be determined is immobilised and, on the other hand, the substance to be determined is itself immobilised. In the first variant, a sample solution which contains the substance to be determined and a conjugate of the substance to be determined and a labelling are incubated with an immobilised antibody, the substance to be determined and the labelled substance thereby competing for the binding to the antibody. The more substance to be determined is present in the solution, the less labelled substance can be bound. Therefore, the amount of labelled substance is an indirect measure for the amount of substance to be determined. After separation of solid and liquid phase, the labelling can be determined in one of the two phases.

In another variant, the sample solution which contains the substance to be determined is incubated

with a labelled antibody specific for it, as well as the immobilised sample substance, immobilised sample substance and the substance to be determined present in the solution thereby competing for the binding to the antibody. The more substance to be determined is present in the solution, the less labelled antibody is bound by binding to the immobilised sample substance on the solid phase. The amount of bound labelled antibody is thus also an indirect measure for the amount of substance to be determined in the sample solution. Here, too, after separation of the solid phase from the liquid phase, the amount of bound labelling is determined.

It is a disadvantage of these processes that modified antibodies must always be used. In the first case, the antibody is bound to a solid phase and, in the second case, the antibody is bound to a labelling substance. This modification is laborious and can lead to undesired changes of the properties of the antibody, for example a reduction of the specificity and affinity. This is the reason why polyclonal antibodies often cannot be used but rather, with great expense, there must be used monoclonal antibodies selected for low cross-reactivity and high affinity which, in addition, must mostly be purified in a laborious manner. Furthermore, for each individual determination, all reagents must be specifically selected, which is also unfavourable. Admittedly it is already known from Federal

Republic of Germany Patent Specification No. 31 38 489 to carry out the immobilisation of formed labelling complexes without the participation of antibodies but also in the case of this process a labelled and thus modified antibody is necessary.

Therefore, it is an object of the present invention to provide a process for the determination of immunologically active substances which can be widely used, in which antibodies can be employed without modification and in which it is not absolutely necessary to use monoclonal antibodies.

Thus, according to the present invention, there is provided a process for the determination of a specifically bindable substance comprising: a) an incubation of a sample solution with at least three receptors R_1, R_2 and R_3 or R_1, R_2 and R_3', wherein; R_1 is specifically bindable with R2 and R3, as well as with the substance to be determined; R_2 brings about the binding to a solid phase or to R_3'; and R_3 or R_3' carries a labelling; b) a separation of bound labelling from unbound labelling; and c) a measurement of the labelling in one of the two phases, wherein; as receptor R_1 a receptor is used which has at least two binding positions which bind specifically with an epitope of the substance to be determined; as R_2 a conjugate of a partner P_1 of a specifically binding pair and of a substance S which corresponds to the substance to be determined or is a derivative thereof

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and has at least one epitope of the substance to be determined, the partner P_1 thereby being bound to a second partner P_2 of said specifically binding pair; as R_3 a complex is used which contains at least the substance S and a labelling; or as R_3' a complex is used which contains at least one labelling and the partner P_2.

The process according to the present invention can be used for the determination of practically all substances which are to be determined in body fluids or tissue extracts and are capable of a specific binding. Substances present in low concentration can be detected just as well as substances present in high concentration. The present invention provides the possibility of being able to carry out determinations quickly and dependably with simple reagents.

The process is especially useful for the determination of haptens, i.e. substances which possess only one binding position for a specifically bindable partner. As examples therefor. there can be mentioned hormones and medicaments, such as anti-epileptics, antibiotics, digitalis and opiates.

Surprisingly, we have found that, in the case of carrying out the process according to the present invention, in contradistinction to the known processes, high demands do not have to be made on the quality of the antibodies so that polyclonal antibodies can here readily be used. Furthermore, we have ascertained that the blank values obtained are very low and thus the sensitivity is increased in comparison with known processes.

By epitope in the description of the present invention, there is to be understood a binding position which can enter into a specific binding with another substance. Examples of epitopes are antigenic determinants on antigens and haptens and also specific binding positions on protein.

Figure 1 shows three reaction schemes for preferred embodiments of the process according to the present invention. In all of the variants shown, receptor R_1 is an antibody.

Figure 1a shows a variant in which first a solid phase to which are bound the partners P_2 of a specifically-binding pair is incubated with receptor R_2, the partner P_1 of the specifically-binding pair, a solid phase thereby resulting to which receptors R_2 are bound via P_1. In a second step, this solid phase is then incubated with receptors R_1, R_3 and the sample solution.

Variant 1b shows an embodiment in which, in a first step, a solid phase to which are bound the partners P_2 is incubated with receptor R_2 and the sample solution. In a second step, receptors R_1 and R_3 are added thereto.

Variant 1c shows an embodiment in which a solid phase, to which are bound partners P_2, and conjugates of labelling and partner P_2 are used which, in each case, are independent of the substance to be determined. The receptors R_1, R_2 and R_3' as well as the

sample, are incubated with the solid phase and labelling conjugate.

Figure 2 shows a calibration curve for a T_4 determination with the use of T_4-POD conjugate (reaction scheme 1a). Curve 1:10 nmole/liter T_4-biotin (end concentration in the test).

Figure 3 shows a calibration curve for a T_4 determination with the use of a T_4-POD conjugate (reaction scheme 1b); and

Figure 4 shows a calibration curve for a T_4 determination with the use of a T_4-biotin conjugate and of a streptavidin-POD conjugate (reaction scheme 1c).

For carrying out the process according to the present invention, the sample solution is incubated with three receptors R_1, R_2 and R_3. Receptor R_1 is thereby specifically bindable with the receptors R_2 and R_3, as well as with the substance to be determined, and carries a labelling. Receptor R_2 brings about the binding t the solid phase. Various reaction principles which can be carried out with the process according to the present invention are illustrated in Fig. 1 of the accompanying drawings. A preferred process variant is thereby that indicated with 1a). In this case, into a reaction vessel, to the walls of which are bound a plurality of partners P_2 of a specifically binding pair complementary to P_1, there is added receptor R_2 which is a conjugate of the substance S and the partner P_1 of the specifically binding pair. The conjugate binds via partner P_1 to partner P_2. Subsequently, there is added

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to the reaction vessel receptor R_1 which has at least two binding positions for an epitope of the substance to be determined and R_3 which is a conjugate of the substance S and a labelling. In the solution,

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the part S of R_2, the part S of R_3 and the substance to be determined then compete for the binding to receptor R_1. If R_1 is, for example, a bivalent antibody, then the following complexes are formed:

R_1 to the two paratopes of which is bound R_2 via S

R_1 to the two paratopes of which is bound the substance to be determined

R_1 to the two paratopes of which is bound R_3 via S, as well as mixed complexes, namely:

R_1 to one paratope of which is bound the substance to be determined and to the other paratope of which is bound R_3 or R_2 via S and

R_1 to one paratope of which is bound R_3 via S and to the other paratope of which is bound R_2 via S.

Only the complexes which are bound to R_2 are immobilised and only the complexes in which a receptor R_3 is bound can be evaluated via the determination of the bound labelling. The more of the substance to be determined is contained in the solution, the less R_2 and R_3 are bound to R_1 and the less complexes are thus immobilised or carry a labelling. Thus, the proportion of bound labelling is an indirect measure for the substance to be determined and can be evaluated via a calibration curve.

The process of carrying out by variant b) serves for the detection of substances which are present in the sample solution in very low concentrations. Here,

in the first step, the matrix to which are bound the partners P_2 of the specifically binding pair is incubated with the substance to be determined, as well as receptor R_1. The substance to be determined present in the sample solution can thereby bind with receptor R_1. A binding to the solid phase cannot take place. In a second step, receptors R_2 and R_3 are then added thereto. The added receptors then compete for the binding to receptor R_1 to which the substance to be determined is already partly bound. The more of the substance to be determined is already bound, the less receptors R_2 and R_3 can be bound. Since receptor R_2 also contains partner P_1 which is bindable with partner P_2 of the matrix, it results in the immobilisation of the complexes being formed. The evaluation again takes place after separation of bound phase from liquid phase by evaluation of the labelling, which takes place via a calibration curve.

Variant c) shows a form of carrying out in which the matrix forming the solid phase and the labelling conjugate can remain the same for all determinations. Here again, in a first step, the matrix, which carries the partner P_2 of the specific binding pair, is incubated with the substance to be determined and receptor R_1, whereby the substance to be determined can bind to receptor R_1. In the second step, there are then simultaneously added thereto the conjugate R_2 of the

substance to be determined and partner P_1 and a conjugate R_3' of partner P_2 and a labelling. The conjugate R_2 of substance to be determined and partner P_1, as well as the substance to be determined from the sample solution, then compete for the binding to receptor R_1. Furthermore, the partner P_2 bound to the matrix, as well as the conjugate R_3' of partner P_2 and labelling, compete for the binding to one of the partners P_1. Here, too, there again takes place an evaluation of the bound labelled complexes via a calibration curve.

In the case of this variant, the concentrations of R_1, thus especially of the antibody, and of R_2 are adapted to the sample concentration in the test. The conjugate of partner P_2 and a labelling should thereby be used in an amount which binds not more than about one half of the conjugate R_2 of the substance to be determined and P_1 in order that of the latter conjugate sufficient is available for the binding to the matrix-bound partner P_2, which should be present in excess. The conjugate R_2 containing P_1 is advantageously used in an amount which lies in the order of magnitude of the substance to be determined, thus about 10^{-5} mole/litre (e.g. in the case of theophylline) to 10^{-10} mole/litre (e.g. in the case of digoxin). In the case of variant c), R_2 should have only one binding position for P_2 (on the matrix or in the conjugate) in order to

avoid a disturbance of the cross-linking. This does not apply to variants a) and b).

This embodiment has, on the one hand, the great advantage that only a single specific conjugate - the conjugate of S and P_1 - must be made available and, on the other hand, the immunological reaction takes place homogeneously, which distinctly increases the sensitivity and reproducibility.

Thus, for the process principle defined according to the present invention, there are many variants of carrying it out. In every case, at least three receptors are necessary.

The substance to be determined can be any substance capable of a specific binding and, especially as defined above, can be a hapten.

As first receptor R_1, there is used a receptor which has at least two binding positions which bind specifically with an epitope of the substance to be determined. This receptor is selected depending upon the particular substance to be determined. There is here preferred a monoclonal or polyclonal antibody or the Fab_2 fragment thereof.

Receptor R_2 is a conjugate of a partner of a specifically binding pair P_1 and a substance S which corresponds to the substance to be determined or is a derivative thereof and has at least one epitope of the substance to be determined. Pairs which are

specifically bindable with one another are known. Appropriate binding pairs (P_1-P_2) are, in particular, biotin-streptavidin, avidin or antibodies against biotin; hapten-antibody; antigen-antibody; concanavalin-antibody; sugar-lectin; hapten-binding protein, for example thyroxine-binding globulin and thyroxine or oligopeptide-antibody.

As binding pair, there is especially preferably used biotin and streptavidin or avidin so that receptor R_2 especially preferably contains biotin as partner P_1. Accordingly, when P_1 is biotin, P_2, being bindable to P_1, may be an antibody against biotin.

The part S of the receptor R_2 can preferably correspond to the unchanged substance to be determined. However, it can also be a derivative of the substance to be determined, for example a protein epitope. The only thing which is important is that the part S is bindable with receptor R_1, whereby it is not absolutely necessary that S and the substance to be determined bind with the same binding strength to R_1.

The preparation of the conjugates takes place in known manner, for example analogously to the description given in Eur. J. Biochem., 131, 333-338/1980.

Receptor R_2 brings about the binding to the solid phase. R_2 can, in one variant, be bound directly or via a spacer to a solid phase via P_1. In another variant, the binding to the solid phase takes place during the immunological reaction by the binding of P_1 to the partner P_2 of the specifically binding pair immobilized on the solid phase.

In a further preferred embodiment of the process, a receptor R_2 is used which is a conjugate of S and P_1. As solid phase, a matrix is used on which is also immobilised a plurality of partners P_1 of the specifically bind pair. After incubation of the components necessary for the immunological reaction, there is then added partner P_2 which has at least two binding positions for P_1. In this way, there then takes place the immobilisation of the receptors R_2.

As solid phase, there are especially preferred reagent glasses or microtitre plates of polystyrene and similar synthetic resins which are adsorptively coated on the inner surface with P_1 or P_2. There are also suitable particulate substances, for example molecular sieve materials, glass pearls, synthetic resin tubes and the like. As solid phase, there are also suitable porous, laminar carriers, for example paper. Also preferred is an embodiment in which the solid phase consists of magnetic particles such as are described, for example, in European Patent Specification No. 0,240,770 and in U.S. Patent Specifications Nos. 4,141,687 and 4,197,337. The magnetic particles preferably consist of chromium dioxide and iron oxide. The binding of the partner P_2 to the particles can take place, for example, in the manner described in European Patent Specification No. 0,240,770.

Receptor R_3 is a complex which contains at least the substance S and a labelling. As labelling, there is thereby used an enzyme or a fluorescing, chemiluminescing or radio-active substance. Processes for labelling are well known, for example from Clin. Chim. Acta, 81, 1-40/1977, and do not here require any further explanation. The labelling can be determined in known manner.

Receptor R_3 can also first be formed during the immunological reaction from a conjugate which contains the substance S and a partner P_1 of a specifically-binding pair and a conjugate of the partner P_2 of the specifically-binding pair and a labelling.

The process can be carried out in one or more steps. The evaluation takes place in known manner. Since each of the receptors and also the substance to be determined can, in each case, only react specifically with the reaction partner intended for it, it is possible to incubate together all receptors and the sample and to carry out the process in one step. This is especially advantageous in the case of carrying out the process in an automatic analyser.

For the detection of substances of very low or very high concentration, multi-step process variants are preferred.

The carrying out of all process variants preferably takes place in a buffered solution. Buffer systems for these processes are known. For this purpose, there

are especially preferred GOOD buffer and phosphate buffer.

According to the present invention, there is provided a process which can be carried out simply and quickly and which is very sensitive also in the case of using polyclonal antibodies.

The present invention also provides a reagent kit for the determination of a specifically bindable substance in a sample comprising at least three receptors R_1, R_2 and R_3 or R_1, R_2 and R_3', and physically separated therefrom a solid phase, wherein: receptor R_1 has at least two specific binding positions for the substance to be determined, and wherein R_1 is specifically bindable with R_2 and R_3, as well as with the substance to be determined; receptor R_2 is a conjugate of a partner P_1 of a specifically binding pair and of a substance S which corresponds to the substance to be determined or is a derivative thereof and has at least one epitope of the substance to be determined and, wherein R_2 brings about the binding to a second partner P_2 of said specifically binding pair, said second partner P_2 being complementary to P_1; receptor R_3 is a complex which contains a label and the substance S; and receptor R_3' is a complex which contains at least one label and the partner P_2.

In a further embodiment, the reagent kit according to the present invention contains a solid phase to which are bound a plurality of partners P_2 of a specifically-binding pair, as well as, physically separated therefrom the receptors R_1, R_2 and R_3 or R_3'.

In a further embodiment, the reagent kit according to the invention contains a solid phase to which are bound the partners P_1, and physically separated therefrom, partners P_2 of the specifically-binding pair and receptors R_1, R_2 and R_3 or R_3'.

In a further preferred embodiment, there is used a multilayer reagent combination which, in a first layer contains the solid phase with partner P_2 and receptor R_3' and physically separated from said first layer receptors R_1 and R_2.

In a further preferred embodiment, there is used a reagent combination which, in a first reagent, contains a suspension of the magnetic particles coated with partner P_2 and a solution of the receptor R_3' (reaction scheme 1c), and physically separated therefrom, the receptors R_1 and R_2. As specifically-binding pair for P_1/P_2, there is preferably used biotin/avidin or streptavidin.

This reagent kit is suitable for the determination of a plurality of parameters in body fluids and tissue extracts.

In a preferred embodiment, the reagent kit additionally contains buffer substances. Especially preferably, it contains phosphate buffer or GOOD buffer.

In another embodiment, there is also provided a reagent kit for the determination of a specifically bindable substance, wherein it contains receptors R_1, R_2 and R_3 or R_3', wherein, receptor R_1 has at least two specific binding positions for the substance to be

determined and wherein R_1 is specifically bindable with R_2 and R_3, as well as with the substance to be determined; receptor R_2 is a conjugate of a partner P_1 of a specifically binding pair and of a substance S which corresponds to the substance to be determined or is a derivative thereof and has at least one epitope of the substance to be determined and, wherein R_2 brings about the binding to P_2; receptor R_3 is a complex which contains a label and the substance S; receptor R_3' is a complex which contains at least one label and a partner P_2; and P_2 is the partner of the specifically binding pair complementary to P_1.

The present invention will now be described in more detail with reference to the following Examples and the accompanying drawings, in which:

Fig. 1 shows three reaction schemes for preferred embodiments of the process according to the present invention;

Fig. 2 shows a calibration curve for a T4 determination with the use of T4-POD conjugate (reaction scheme 1a). Curve 1: 10 nmole/litre T4-biotin; curve 2: 1 nmole/litre T4-biotin (end concentration in the test).

Fig. 3 shows a calibration curve for a T4 determination with the use of a T4-POD conjugate (reaction scheme 1b); and

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Fig. 4 shows a calibration curve for a T_4 determination with the use of a T_4-biotin conjugate and of a streptavidin-POD conjugate (reaction scheme 1c).

In Fig. 1 are shown schemes for the reaction principles of various preferred embodiments of the process according to the present invention. I_n all of the variants shown, receptor R_1 is an antibody.

Fig. 1a shows a variant in which first a solid phase to which are bound the partners P_2 of a specifically-binding pair is incubated with receptor R_2, the partner P_1 of the specifically-binding pair, a solid phase thereby resulting to which receptors R_2 are bound via P_1. In a second step, this solid phase is then incubated with receptors R_1, R_3 and the sample solution.

Variant 1b shows an embodiment in which, in a first step, a solid phase to which are bound the partners P_2 is incubated with receptor R_2 and the sample solution. In a second step, receptors R_1 and R_3 are added thereto.

Variant 1c shows an embodiment in which a solid phase, to which are bound partners P_2, and conjugates of labelling and partner P_2 are used which, in each case, are independent of the substance to be determined. The receptors R_1, R_2 and R_3', as well as the sample, are incubated with the solid phase and labelling conjugate.

Example 1.

Determination of T_4 according to reaction scheme 1a).

Buffer A:

120 mmole/litre barbiturate

18.2 mmole/litre phosphate buffer (pH 8.6)

1.27 mmole/litre 8-anilino-1-naphthalenesulphonic acid

0.2% by weight bovine serum albumin

980 µl. of buffer A and 20 µl. of a solution of a conjugate of T_4 and biotin (preparation according to Example 4 (end concentration 1 or 10 mmole/ml.)) are introduced into a streptavidin-coated polystyrene vessel (produced according to European Patent Specification No. 0,269,092) and incubated for 30 minutes at 25°C. Subsequently, the vessel is washed and 50 µl. of sample (human serum made up with T_4), 980 µl. of buffer A, 20 µl. of a solution of 0.1 mg./ml. polyclonal antibody against T_4 and 2.5 U/ml. T_4-peroxidase conjugate (5 mU/test) added thereto, incubated for 30 minutes at 25°C., washed and 1 ml. of a solution of 9.1 mmole/litre ABTS® (2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid diammonium salt) added thereto. After a further incubation for 30 minutes at 25°C., the optical density is determined at 422 nm as a measure for the T_4 content. The results are given in Fig. 2 of the accompanying drawings.

Example 2.

Determination of T_4 according to reaction scheme 1b).

50 µl. of sample (human serum made up with T_4) are incubated with 480 µl. of buffer A and 20 µl. of a solution of a polyclonal antibody against T_4 (0.1 mg./ml.)

at 25°C. for 30 minutes in a polystyrene vessel coated with streptavidin. Subsequently, 480 µl. of buffer A and 20 µl. of a solution of T_4-biotin conjugate (5 x 10^{-8} mole/litre, corresponding to 1 nmole/litre end concentration in the test) and T_4-POD conjugated (2.5 U/ml., 50 mU/test) are added thereto and incubated for 30 minutes at 25°C. The vessel is washed and subsequently 1 ml. ABTS ® solution (9.1 mmole/litre) added thereto, incubated for 30 minutes at 25°C. and subsequently the optical density is determined at 422 nm as a measure for the T_4 content. The results are given in Fig. 3 of the accompanying drawings.

Example 3.

Determination of T_4 according to reaction scheme 1c).

50 µl. of sample (human serum made up with T_4) are incubated with 480 µl. of buffer A and 20 µl. of a solution of a polyclonal antibody against T_4 (0.1 mg./ml.) for 30 minutes at 25°C. in a polystyrene vessel coated with streptavidin. Subsequently, 480 µl. of buffer A and 20 µl. of a solution of a conjugate of T_4-biotin (5 x 10^{-7} mole/litre, corresponding to 10 nmole/litre end concentration in the test) and 2.5 U/ml. streptavidin-POD conjugate (50 mU/test) are added thereto and incubated for 30 minutes at 25°C. The vessel is washed and 1 ml. ABTS ® solution (9.1 mmole/litre) added thereto, incubated at 25°C.

for 30 minutes and the optical density determined at 422 nm as a measure of the T_4 content. The results are given in Fig. 4 of the accompanying drawings.

Example 4.

5 Preparation of T_4-biotin conjugate.

N-tert.-butoxycarbonyltetraiodothyronine is coupled via pentamethylenediamine with biotin in the manner described in Eur. J. Biochem., 131, 333-338/1980.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. Process for the determination of a specifically bindable substance comprising:

a) an incubation of a sample solution with at least three receptors R_1, R_2 and R_3 or R_1, R_2 and R_3', wherein;

R_1 is specifically bindable with R2 and R3, as well as with the substance to be determined;

R_2 brings about the binding to a solid phase or to R_3'; and

R_3 or R_3' carries a labelling;

b) a separation of bound labelling from unbound labelling; and

c) a measurement of the labelling in one of the two phases, wherein;

as receptor R_1 a receptor is used which has at least two binding positions which bind specifically with an epitope of the substance to be determined;

as R_2 a conjugate of a partner P_1 of a specifically binding pair and of a substance S which corresponds to the substance to be determined or is a derivative thereof and has at least one epitope of the substance to be determined, the partner P_1 thereby being bound to a second partner P_2 of said specifically binding pair;

as R_3 a complex is used which contains at least the substance S and a labelling; or

as R_3' a complex is used which contains at least one labelling and the partner P_2.

2. Process according to claim 1, wherein, as receptor R_1, there is used an antibody specifically bindable with the substance to be determined or the Fab_2 fragment thereof.

3. Process according to claim 1, wherein, as receptor R_2, there is used a solid phase to which the substance S is bound via the partner P_1.

4. Process according to claim 2, wherein, as receptor R_2, there is used a solid phase to which the substance S is bound via the partner P_1.

5. Process according to claim 1, 2, 3 or 4, wherein, as receptor R_2, there is used a conjugate of the substance S and a partner P_1 of a specifically binding pair and, as solid phase, there is used a matrix to which are bound many partners P_2 of the specifically binding pair complementary to P_1.

6. Process according to claim 1, 2, 3 or 4, wherein, as receptor R_2, there is used a conjugate of the substance S and a partner P_1 and, as solid phase, there is used a matrix to which are bound a plurality of partners P_1, the immobilization taking place after incubation by the addition of a component P_2 which has at least two specific binding positions for P_1.

7. Process according to claim 5, wherein as receptor R_2, there is used a conjugate of the substance S and a partner P_1 and, as solid phase, there is used a matrix to which are bound a plurality

of partners P_1, the immobilization taking place after incubation by the addition of a component P_2 which has at least two specific binding positions for P_1.

8. Process according to claim 6, wherein, as partner P_1, there is used biotin and, as component P_2, streptavidin, avidin or antibodies against biotin.

9. Process according to claim 7, wherein, as partner P_1, there is used biotin and, as component P_2, streptavidin, avidin or antibodies against biotin.

10. Process according to claim 1, 2, 3, 4, 7, 8 or 9, wherein, as receptor R_3, there is used a conjugate of the substance S and a labelling.

11. Process according to claim 5, wherein, as receptor R_3, there is used a conjugate of the substance S and a labelling.

12. Process according to claim 6, wherein, as receptor R_3, there is used a conjugate of the substance S and a labelling.

13. Process according to claim 10, wherein, as labelling, there is used a radio-active, chemiluminescing or fluorescing substance or an enzyme.

14. Process according to claim 11 or 12, wherein, as labelling, there is used a radio-active, chemiluminescing or fluorescing substance or an enzyme.

15. Process according to claim 1, 2, 3, 4, 7, 8, 9, 11, 12 or 13, wherein, as receptor R_3, there is used a conjugate of the substance S and the partner P_1 of a specifically binding pair and a conjugate of the partner P_2 of the specifically binding pair complementary to P_1 and a labelling.

16. Process according to claim 5, wherein, as receptor R_3, there is used a conjugate of the substance S and the partner P_1 of a specifically binding pair and a conjugate of the partner P_2 of the specifically binding pair complementary to P_1 and a labelling.

17. Process according to claim 6, wherein, as receptor R_3, there is used a conjugate of the substance S and the partner P_1 of a specifically binding pair and a conjugate of the partner P_2 of the specifically binding pair complementary to P_1 and a labelling.

18. Process according to claim 10, wherein, as receptor R_3, there is used a conjugate of the substance S and the partner P_1 of a specifically binding pair and a conjugate of the partner P_2 of the specifically binding pair complementary to P_1 and a labelling.

19. Process according to claim 14, wherein, as receptor R_3, there is used a conjugate of the substance S and the partner P_1 of a specifically binding pair and a conjugate of the partner P_2 of the specifically binding pair complementary to P_1 and a labelling.

20. Process according to claim 1, 2, 3, 4, 7, 8, 9, 11, 12, 13, 16, 17, 18 or 19, wherein, as binding pair P_1-P_2, there is used biotin-streptavidin or avidin, biotin-biotin antibody, antigen-antibody, hapten-binding protein or oligopeptide-antibody.

21. Process according to claim 5, wherein, as binding pair P_1-P_2, there is used biotin-streptavidin or avidin, biotin-biotin antibody, antigen-antibody, hapten-binding protein or oligopeptide-antibody.

22. Process according to claim 6, wherein, as binding pair P_1-P_2, there is used biotin-streptavidin or avidin, biotin-biotin antibody, antigen-antibody, hapten-binding protein or oligopeptide-antibody.

23. Process according to claim 10, wherein, as binding pair P_1-P_2, there is used biotin-streptavidin or avidin, biotin-biotin antibody, antigen-antibody, hapten-binding protein or oligopeptide-antibody.

24. Process according to claim 14, wherein, as binding pair P_1-P_2, there is used biotin-streptavidin or avidin, biotin-biotin antibody, antigen-antibody, hapten-binding protein or oligopeptide-antibody.

25. Process according to claim 15, wherein, as binding pair P_1-P_2, there is used biotin-streptavidin or avidin, biotin-biotin antibody, antigen-antibody, hapten-binding protein or oligopeptide-antibody.

26. Process according to claim 5, wherein, as solid phase, there is used a matrix which consists of a synthetic resin and on which a plurality of binding partners P_2 is bound directly or via a spacer.

27. Process according to claim 1, 2, 3, 4, 7, 8, 9, 11, 12, 13, 16, 17, 18, 19, 21, 22, 23, 24, 25 or 26, wherein, as partner P_1, there is used biotin and, as partner P_2, streptavidin or avidin.

28. Process according to claim 5, wherein, as partner P_1, there is used biotin and, as partner P_2, streptavidin or avidin.

29. Process according to claim 6, wherein, as partner P_1, there is used biotin and, as partner P_2, streptavidin or avidin.

30. Process according to claim 10, wherein, as partner P_1, there is used biotin and, as partner P_2, streptavidin or avidin.

31. Process according to claim 14, wherein, as partner P_1, there is used biotin and, as partner P_2, streptavidin or avidin.

32. Process according to claim 15, wherein, as partner P_1, there is used biotin and, as partner P_2, streptavidin or avidin.

33. Process according to claim 20, wherein, as partner P_1, there is used biotin and, as partner P_2, streptavidin or avidin.

34. Reagent kit for the determination of a specifically bindable substance in a sample comprising at least three receptors R_1, R_2 and R_3 or R_1, R_2 and R_3', and physically separated therefrom a solid phase wherein:

receptor R_1 has at least two specific binding positions for the substance to be determined, and wherein R_1 is specifically bindable with R_2 and R_3, as well as with the substance to be determined;

receptor R_2 is a conjugate of a partner P_1 of a specifically binding pair and of a substance S which corresponds to the substance to be determined or is a derivative thereof and has at least one epitope of the substance to be determined and, wherein R_2 brings about the binding to a second partner P_2 of said specifically binding pair, said second partner P_2 being complementary to P_1;

receptor R_3 is a complex which contains a labelling and the substance S; and

receptor R_3' is a complex which contains at least one labeling and the partner P_2.

35. Reagent kit of claim 34, wherein, as receptor R_1, there is used an antibody specifically bindable with the substance to be determined or the Fab_2 fragment thereof.

36. Reagent kit of claim 34 or 35, wherein, as receptor R_2, there is used a conjugate of the substance S and a partner P_1 of a specifically binding pair and, as solid phase, there is used a matrix to which are bound many partners P_2 of the specifically binding pair complementary to P_1.

37. Reagent kit of claim 34 or 35, wherein, as receptor R_2, there is used a conjugate of the substance S and a partner P_1 and, as solid phase, there is used a matrix to which are bound a plurality of partners P_1,

the immobilization taking place after incubation by the addition of a component P_2 which has at least two specific binding positions for P_1.

38. Reagent kit of claim 36, wherein, as partner P_1, there is used biotin and, as component P_2, streptavidin, avidin or antibodies against biotin.

39. Reagent kit of claim 37, wherein, as partner P_1, there is used biotin and, as component P_2, streptavidin, avidin or antibodies against biotin.

40. Reagent kit of claim 34, 35, 38 or 39, wherein, as the labelling, there is used a radio-active, chemiluminescing or fluorescing substance or an enzyme.

41. Reagent kit of claim 36, wherein, as the labelling, there is used a radio-active, chemiluminescing or fluorescing substance or an enzyme.

42. Reagent kit of claim 37, wherein, as the labelling, there is used a radio-active, chemiluminescing or fluorescing substance or an enzyme.

43. Reagent kit of claim 36, wherein, as solid phase, there is used a matrix which consists of a synthetic resin and on which a plurality of binding partners P_2 is bound directly or via a spacer.

44. Reagent kit of claim 34, 35, 38, 39, 41, 42, or 43, wherein, as a binding pair P_1-P_2, there is used biotin-streptavidin or avidin, biotin-biotin antibody, antigen-antibody, hapten-binding protein or oligopeptide-antibody.

45. Reagent kit of claim 36, wherein, as a binding pair P_1-P_2, there is used biotin-streptavidin or

avidin, biotin-biotin antibody, antigen-antibody, hapten-binding protein or oligopeptide-antibody.

46. Reagent kit of claim 37, wherein, as a binding pair P_1-P_2, there is used biotin-streptavidin or avidin, biotin-biotin antibody, antigen-antibody, hapten-binding protein or oligopeptide-antibody.

47. Reagent kit of claim 40, wherein, as a binding pair P_1-P_2, there is used biotin-streptavidin or avidin, biotin-biotin antibody, antigen-antibody, hapten-binding protein or oligopeptide-antibody.

48. Reagent kit of claim 34, 35, 38, 39, 41, 42, 43, 45, 46 or 47, wherein, as partner P_1, there is used biotin and, as partner P_2, streptavidin or avidin.

49. Reagent kit of claim 36, wherein, as partner P_1, there is used biotin and, as partner P_2, streptavidin or avidin.

50. Reagent kit of claim 37, wherein, as partner P_1, there is used biotin and, as partner P_2, streptavidin or avidin.

51. Reagent kit of claim 40, wherein, as partner P_1, there is used biotin and, as partner P_2, streptavidin or avidin.

52. Reagent kit of claim 44, wherein, as partner P_1, there is used biotin and, as partner P_2, streptavidin or avidin.

53. Reagent kit for the determination of a specifically bindable substance, wherein it contains receptors R_1, R_2 and R_3 or R_3', wherein,

receptor R_1 has at least two specific binding positions for the substance to be determined and

wherein R_1 is specifically bindable with R_2 and R_3, as well as with the substance to be determined;

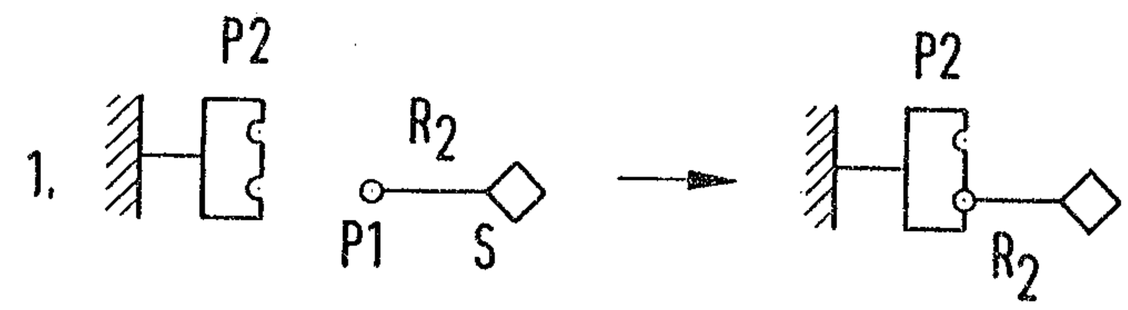
receptor R_2 is a conjugate of a partner P_1 of a specifically binding pair and of a substance S which corresponds to the substance to be determined or is a derivative thereof and has at least one epitope of the substance to be determined and, wherein R_2 brings about the binding to a second partner P_2 of said specifically binding pair, said second partner P_2 being complementary to P_1;

receptor R_3 is a complex which contains a labelling and the substance S; and

receptor R_3' is a complex which contains at least one labeling and the partner P_2.

Fig.1

a)



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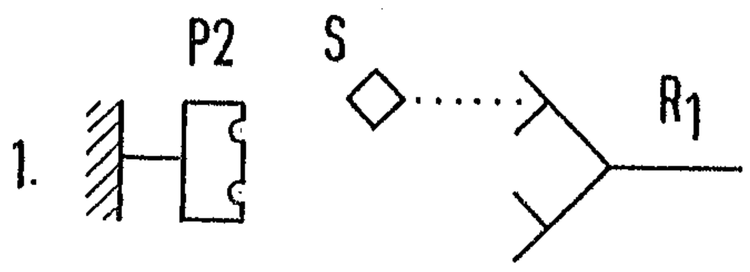

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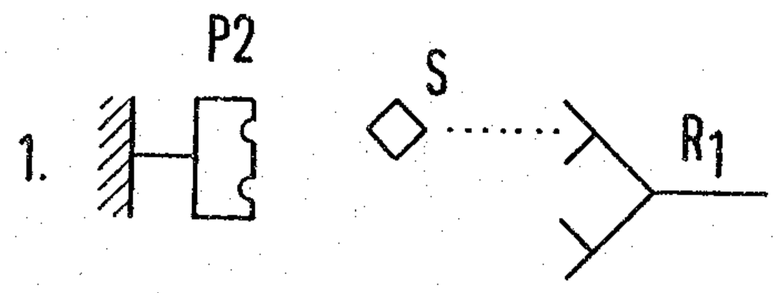
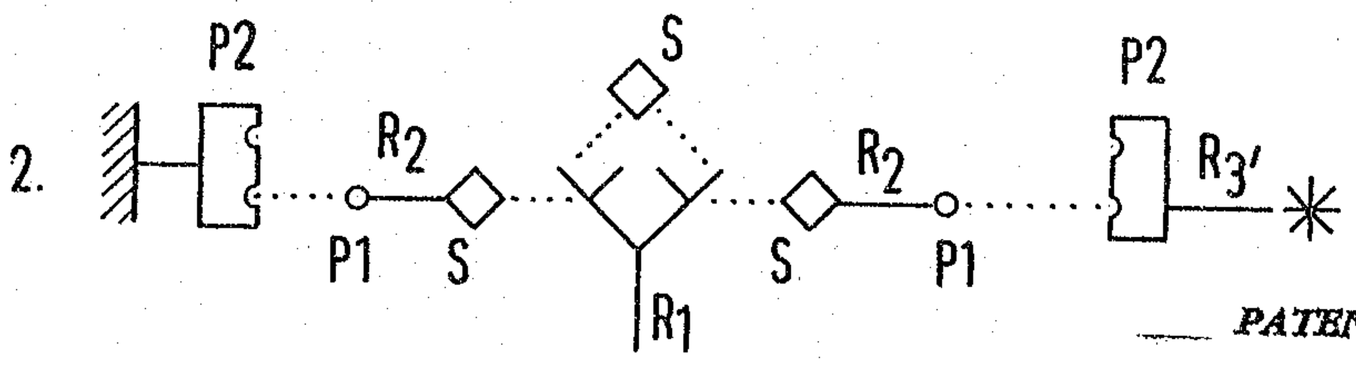
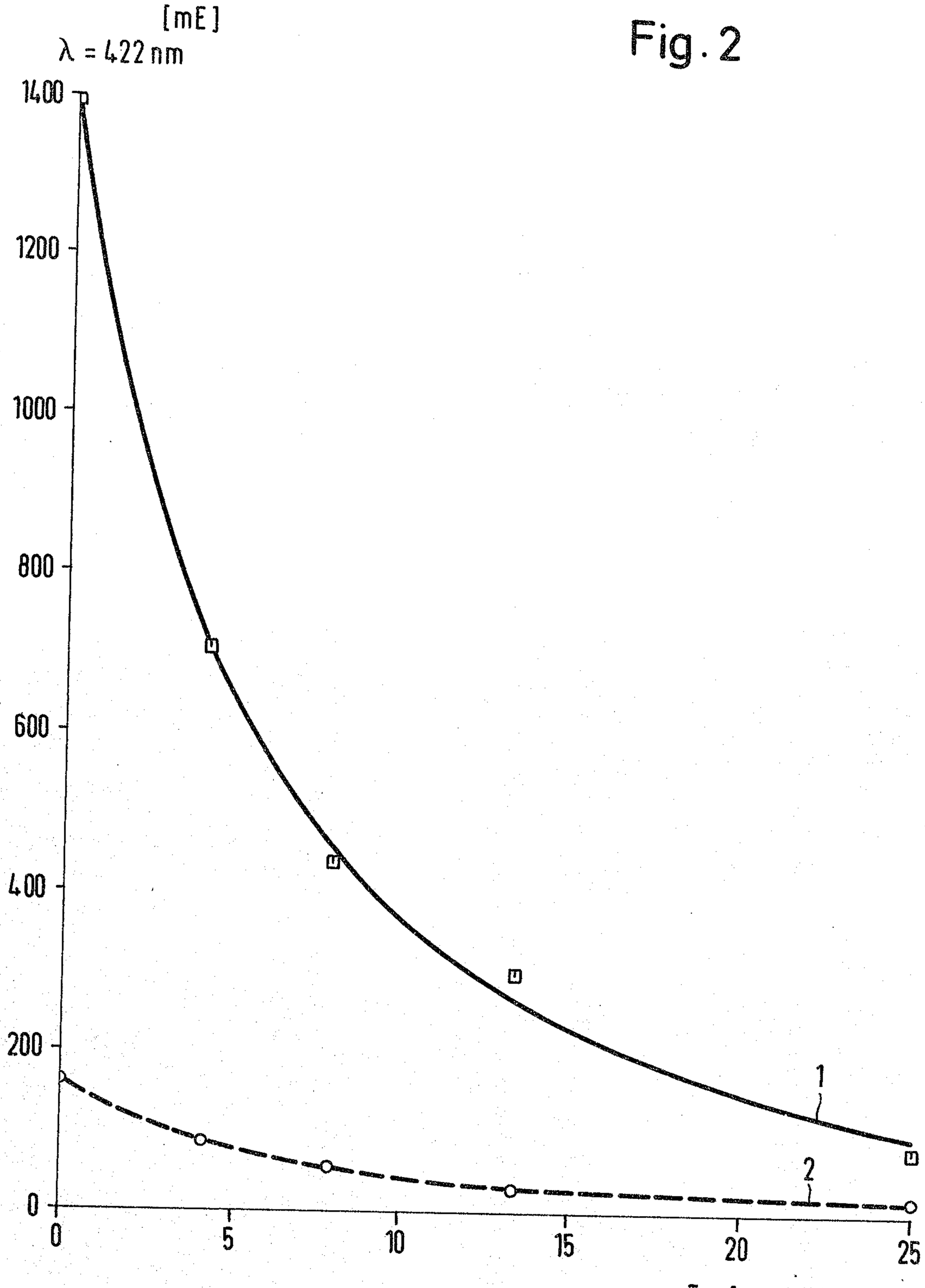




PATENT AGENTS

Aubrey Ogilvy Renault

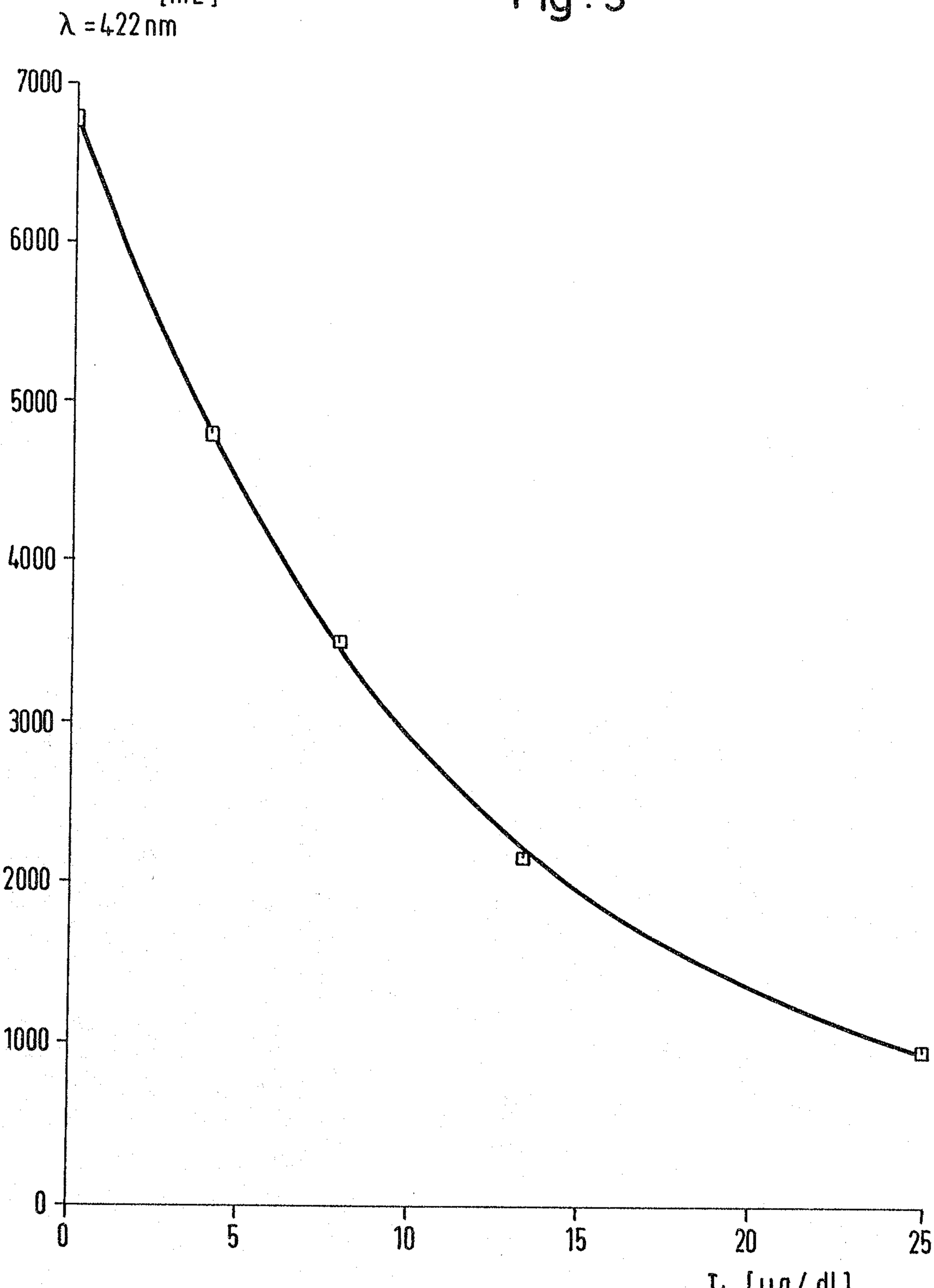
Fig. 2



PATENT AGENTS

Dubrey Gilroy Renault

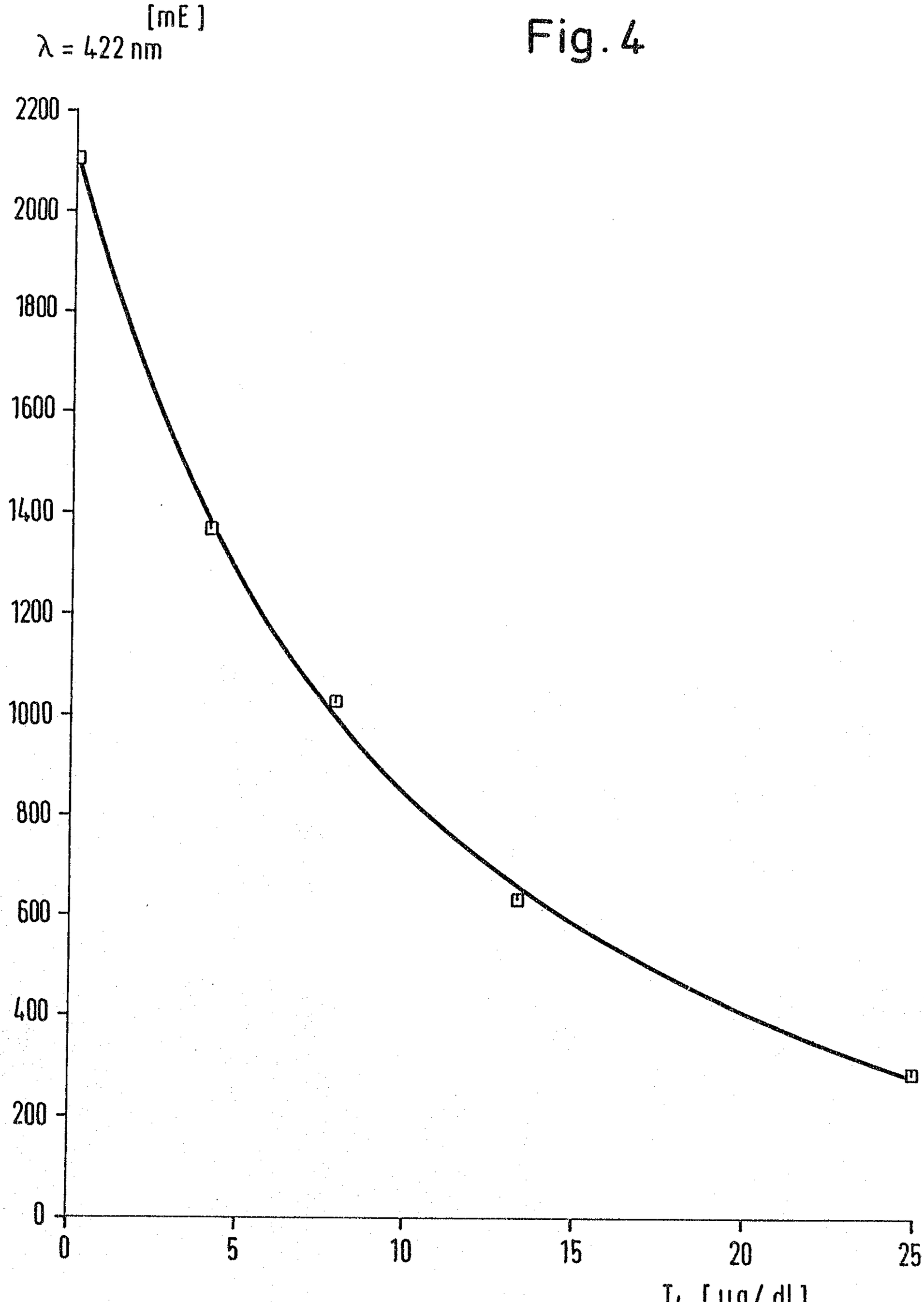
Fig . 3



PATENT AGENTS

Dunlop Ojilvy Renault

Fig. 4



PATENT AGENTS

Dudley Ojilvy Kenault