

PATENT SPECIFICATION

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(54) PROCESS FOR THE ACTIVATION OF CHOLESTEROL OXIDASE

(71) We, BOEHRINGER MANN-HEIM GMBH, of 112-132, Sandhofer Strasse, Mannheim-Waldhof, Federal Republic of Germany, a Body Corporate organised under the laws of the Federal Republic of Germany, 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:—

The present invention is concerned with a process for the activation of cholesterol oxidase and with a diagnostic agent for carrying out this process.

German Patent Specification No. 2,224,132 describes a process for the determination of cholesterol in which cholesterol is incubated in an aqueous medium with cholesterol oxidase, followed by the determination either of the oxygen consumption or of the amount of hydrogen peroxide or of cholestenone formed. This German Patent Specification also describes a reagent for the determination of cholesterol which comprises cholesterol oxidase and a system for the determination of hydrogen peroxide or a system for the determination of cholestenone.

We have now found that cholesterol oxidase has an insufficient storage stability and is inactivated relatively quickly. In the course of the investigation of this instability, we have found that the inactivation is brought about by small amounts of detergent which are present as an accompanying substance and originate from the process used for the preparation of the enzyme. This preparation of the enzyme can be carried out, for example, by the process described in German Patent Specification No. 2,224,131 in which a micro-organism which metabolises cholesterol is digested by destruction of the cell walls with a non-ionic, surface-active agent present in a buffer solution and then extracted, whereafter the extract is centrifuged, the precipitate obtained is discarded and the supernatant liquid is applied to an anion exchanger, the enzyme then being

eluted with a buffer solution containing the non-ionic surface-active agent and thereafter isolated from the eluate.

We have also found that, upon removal of these residual traces of detergent, a completely satisfactory storage stability of the enzyme can be achieved. However, we have also ascertained that the enzyme freed from detergent has a remarkably reduced activity. If, for example, the enzyme is used for the determination of cholesterol, then it is necessary either to use a comparatively freshly prepared preparation or considerably larger amounts of enzyme must be employed for the test or the time required for carrying out the determination must be considerably increased.

Consequently, it is an object of the present invention to provide a process for the activation of cholesterol oxidase. More particularly, it is an object of the present invention to provide such a process which, on the one hand, permits the use of cholesterol oxidase which is completely free from traces of detergent and, therefore, is sufficiently storage-stable and, on the other hand, provides an activity of the enzyme during use thereof which corresponds to that of the freshly prepared enzyme but which has not been subjected to the removal of small amounts of detergents.

Thus, according to the present invention, there is provided a process for the activation of cholesterol oxidase, wherein at least one surface-active compound with lipophilic and hydrophilic properties is added to the cholesterol oxidase before the use thereof.

As surface-active compounds with lipophilic and hydrophilic properties, there are preferably used non-ionic detergents which contain at least one hydroxyl group in the molecule. Polyoxyethylene derivatives of alkyl, aryl and aralkyl alcohols are preferably employed. Examples of such preferred surface-active compounds include polyoxyethylene alkyl ethers, polyoxyethylene alkyl-carboxylic acid esters, polyoxyethylene-sorbitan alkyl carboxylic acid esters, polyoxyethylene-glycerol alkyl carboxy-

lic acid esters, polyoxyethylene-alkylamines, polyoxyethylene-polyoxypropylene block polymers, polyoxyethylene-alkyl thio-ethers and polyoxyethylene alkyl aryl ethers. Specific examples thereof include hydroxy-polyethoxy-dodecane, ethyleneoxy adducts of alkyl-phenols and polyethoxy-ethylene derivatives of sorbitan anhydrides. Polyoxyethylene derivatives modified with mercaptans are also equally suitable.

According to the present invention, the above-mentioned surface-active compounds are preferably used in an amount of between about 0.005 and 0.1 wt.%, referred to the aqueous enzyme solution.

Considerable numbers of the above-mentioned surface-active materials are commercially available and differ from one another by the number of oxyethylene groups present therein. The most useful ones are those which are water-soluble and which contain, on average, 5 to 20 oxyethylene groups per molecule. Lower oxyethylated types of compounds are usually only dispersible and are, therefore, less useful. Higher ethoxylated compounds (greater than 25) are admittedly water-soluble but are so hydrophilic that their effectiveness is reduced.

Similarly good results are also obtained with physiological surface-active substances which satisfy the above-given definition, for example, desoxycholates.

In addition, other surface-active substances with lipophilic and hydrophilic properties which contain at least one hydroxyl group, can also be used. Examples of such compounds include ethanol, butyl-diglycol and hexylene-glycol. However, lower mono- and dialcohols of this type must be added in relatively large amounts of 10 to 20 vol.% for the achievement of the desired activation and the suitability thereof must be ascertained experimentally in each case. Thus, for example, no activation is achieved with methanol, ethylene glycol, polyethylene glycol, cyclohexanol, glycerol, lecithin and saponin. Surface-active compounds which have lipophilic and hydrophilic properties but which do not contain a hydroxyl group in the molecule can also be used.

Of the anionic wetting agents, the salts of bile acids, such as cholic acid, taurocholic acid

and desoxycholic acid, are especially preferred, as well as fatty acid salts and fatty acid sarcosides. Of special importance are the sulphuric acid derivatives which, as is known, have the additional property of stabilising the coloured radical oxidation products of some indicators, such as *o*-tolidine and heterocyclic azines. These include, for example, the following classes of compounds: sulposuccinic acid esters, alkyl aryl sulphonates, alkyl sulphates and alkyl polyoxyethylene sulphates.

Of the cationic wetting agents, there can be used, for example, alkyl pyridinium and trimethyl ammonium salts, as well as more complex compounds, for example, benzethonium chloride.

Of the amphoteric wetting agents, there can be used, for example, the imidazolium betaines.

Particular examples of surface-active compounds which can be used according to the present invention include sodium (2-ethylhexyl)-sulphosuccinate, sodium dodecyl sulphate, sodium oleate, benzalkonium chloride and cetylpyridinium chloride.

The ionic or amphoteric detergents are preferably used in admixture with the above-mentioned non-ionic detergents, the appropriate amounts thereof corresponding to those of the non-ionic detergent.

The non-ionic, surface-active detergents containing at least one hydroxyl group in the molecule are preferred because they provide an approximately 5 to 10 times greater activation and thus a corresponding increase of the reaction velocity or shortening of the period of reaction in comparison with the other surface-active substances which can be employed. They are also effective in smaller amounts.

By alkyl radicals, there are here to be understood those containing up to 20 carbon atoms and especially those containing 12 to 18 carbon atoms.

The inactivation of cholesterol oxidase in the presence of traces of detergent is especially marked when the enzyme is present in ammonium sulphate solution. The following Table I shows the storage stability of the enzyme in 1M ammonium sulphate solution at 33°C. in the presence of differing amounts of detergent. The experiments were carried out with an octyl-phenol-ethylene oxide adduct.

TABLE I

Activity of cholesterol oxidase in %, referred to freshly prepared enzyme

storage period in days	detergent				
	without	0.02%	0.05%	0.1%	0.6%
10	89%	60%	10%	7%	3%
22	85%	48%	1%	1%	1%

The effective amount of surface-active compound used in the process according to the present invention depends upon its molecular weight and upon the degree of its hydrophobic and hydrophilic properties. Too small an amount does not bring about an activation but too large an amount leads to the result generally known for surface-active substances of denaturing the enzyme. The appropriate concentration range, as well as the optimum concentration, can be experimentally determined for all appropriate surface-active agents. Such ranges are, for example, 0.15 to 0.6 wt.% for hydroxypolyethoxydodecane, 0.03 to 0.1 wt.% for octylphenol-ethylene oxide adducts, 0.15 to 0.6 wt.% for polyoxyethylene derivatives of sorbitan anhydrides, 0.03 to 0.05 wt.% for sodium desoxycholate and 0.005 to 0.02 wt.% for mercaptan-modified alkyl-phenol ethylene oxide adducts (for example "Sterox" SE). ("Sterox" is a Registered Trade Mark.)

Of the non-ionic surface-active polyoxyethylene compounds, those are especially useful which have a balanced ratio of hydrophobic residues to polyoxyethylene chain and are water-soluble. A measurement value for the ratio of hydrophobic residue and polyoxyethylene chain is the so-called HLB value (cf. W. C. Griffin, J. Soc. Cosmetic Chem., 1, 311/1950 and 5, 249/1954).

Those surface-active materials with HLB values between about 10 and 17 are especially useful. These values are, however, only to be regarded as being optimum standard values since the effectiveness also depends upon the nature of the hydrophobic residue.

The present invention is also concerned with a diagnostic agent for the detection and determination of cholesterol and of cholesterol esters in body fluids which can be used for carrying out the above-described process.

The determination of cholesterol is of considerable importance in medical diagnosis.

In clinical chemistry, rapid tests are being used more and more for the detection of substances in body fluids. Although they frequently do not provide sufficiently accurate results, nevertheless, they do permit a quicker

and cheaper indication for routine and large-scale investigations. The diagnostic agents employed for rapid tests are either absorbent carriers or water-stable films which contain all of the reagents needed for the reaction. When these diagnostic agents are brought into contact with the body fluids to be tested, then colour reactions are obtained which can be evaluated either on the basis of comparative colours or with the use of simple reflection photometers.

Experiments for the production of agents for cholesterol rapid tests by impregnation of absorbent paper with cholesterol oxidase, peroxidase, an oxidation indicator and a buffer do not give satisfactory results because the test papers thus obtained do not react with cholesterol-containing serum of the usual concentration.

We have now found that useful test papers or test films are obtained, which react in graduated stages with cholesterol-containing serum when the carrier additionally contains at least one of the above-described surface-active agents.

Thus, according to the present invention, there is also provided a diagnostic agent for the detection and determination of cholesterol and of cholesterol esters in body fluids which comprises an absorbent carrier or a synthetic resin film which is impregnated with or has embedded therein cholesterol oxidase, a system for the detection of hydrogen peroxide, a buffer and at least one of the above-described surface-active agents. The surface-active agent is preferably present therein in a concentration of 2 to 30% by weight and more preferably of 10 to 20% by weight, referred to the solid reagents.

A preferred system for the detection of hydrogen peroxide comprises peroxidase and an oxidation indicator, optionally together with a swelling agent and/or a stabiliser.

For the detection of cholesterol in serum, test papers are outstandingly useful which have been obtained by the impregnation of absorbent papers with the necessary reagents. If, however, it is desired to detect cholesterol in

whole blood, then the test papers are preferably rendered hydrophobic, for example, in the manner described in German Patent Specification No. 1,598,048 or are coated with a semi-permeable membrane of cellulose esters. However, for the detection of cholesterol in whole blood, it is especially preferred to use test films; such as are obtainable according to German Patent Specification No. 1,598,153, containing the necessary reagents.

For the preparation of such test films, the reagents, for example, cholesterol oxidase, peroxidase, buffer, indicator and optionally a swelling agent, are stirred, together with the surface-active materials to be used according to the present invention, into an aqueous dispersion of a film-forming polymer. This dispersion is then coated as a thin film and left to dry. When cholesterol-containing blood is dropped on to such a reagent-containing film and then wiped off after about one minute, colorations are also obtained, the colour depth of which depends upon the amount of cholesterol present in the blood. These colorations are particularly suitable for a quantitative evaluation with simple remission photometers.

The diagnostic agent according to the present invention can be used for the detection and determination of free cholesterol. If, however, cholesterol esters are also present, then the cholesterol must first be liberated therefrom. This can be carried out in known manner, for example, by saponification with aqueous alkali. However, it is particularly advantageous to split the ester with cholesterol esterase, which is preferably isolated from micro-organisms, since it is then possible to work under very mild conditions.

The cholesterol esterase can be added to the body fluid, followed by incubation. This process can be carried out, for example, by drawing the body fluid into capillaries, the inner walls of which are coated, in a manner analogous to that described in German Patent Specification No. 2,240,672, with cholesterol esterase and optionally with adjuvant materials. After incubation in the capillaries, the body fluid is then applied to the test strips in the above-described manner. If free and esterified cholesterol were both present in the body fluid, then, of course, the sum of the two is detected. However, the cholesterol esterase can also be incorporated especially advantageously into the test strips to give diagnostic agents with which, in one step, the sum of the free and esterified cholesterol can be detected and determined.

Systems which can be used for the detection of the hydrogen peroxide formed by the oxidation of the cholesterol, for example peroxidase, buffer, oxidation indicator and optionally a swelling agent, are known, for example, from the descriptions of rapid tests for glucose. Examples of components for this preferred system are given in the following:

Of the peroxidases, that from horseradish is especially preferred but lactoperoxidase can also be employed.

As buffers, there can be used those which are conventional, for example, phosphate, citrate and borate buffers. The pH value which they give on the diagnostic agent should be between 4 and 9 and preferably between 5 and 8.

As oxidation indicators, there can be used various classes of compounds, namely, benzidine derivatives, for example *o*-tolidine and *o*-vanillin derivatives according to German Patent Specification No. 1,598,133, or heterocyclic azines according to German Patent Specification No. 1,648,840.

The formulations for the diagnostic agents and especially those for test films, can also contain a conventional swelling agent, such as sodium alginate or carboxy-methyl-cellulose, as well as a stabilising agent for the enzyme, for example dithioerythritol.

Substrate materials for test films are synthetic resin dispersions of, for example, polyvinyl propionate or acetate. Into these are stirred all the necessary reagents, preferably in dissolved form, whereafter the mixture obtained is then coated into thin films and dried.

Test papers can be produced by dissolving the reagents in water or in a mixture of water and organic solvents with which filter papers are impregnated and then dried. However, the paper can first be impregnated with the water-soluble reagents and then impregnated with, for example, the indicators in organic solution.

The following Examples are given for the purpose of illustrating the present invention:—

Example 1.

0.05 ml. of serum are added to 10 ml. 0.5M potassium phosphate buffer of pH 7.5 which contains 0.4% hydroxypolyethoxydodecane. The extinction (E_1) is read off at 240 nm in a suitable spectrophotometer and the reaction started with 0.02 ml. (0.1 U) of storage-stable cholesterol oxidase, freed from traces of detergent, in 1M aqueous ammonium sulphate solution. After 3 minutes, the extinction (E_2) is read off. The concentration of the cholesterol formed and thus of the cholesterol is given by the difference between the first and second readings, having regard to the molar extinction coefficients for cholesterol at 240 nm. The measurement of a typical sample gave 62 mg.% free cholesterol and 167 mg.% total cholesterol (after saponification).

A comparative determination but without the addition of the surface-active agent required a reaction time of 15 minutes.

The separation of traces of detergent for the improvement of the storage stability of the enzyme is preferably carried out with the use of hydrophobic adsorption resins. Especially preferred for this purpose are, for example, the material which is commercially available as "Bio-Beads" from Biorad, as well as the

products obtainable from Rohm & Haas under the designation XAD-resins.

Example 2.

0.05 ml. serum are added to 10 ml. 0.5M potassium phosphate buffer of pH 7.5 which contains 0.02% hydroxypolyethoxy-dodecane and 0.03% sodium desoxycholate. The extinction (E_1) is read off at 240 nm in a suitable spectrophotometer and the reaction is started with 0.02 ml. (= 0.1 U) storage-stable cholesterol oxidase, freed from traces of detergent, in 1M aqueous ammonium sulphate solution.

After 3 minutes, the extinction (E_2) is again read off. The concentration of the cholestenone formed and thus of the cholesterol is given from the difference between the first and second readings, having regard to the molar extinction coefficients for cholestenone at 240 nm ($\epsilon = 15.5 \text{ cm}^2/\mu\text{mol}$).

The measurement of a typical sample gave 65 mg.% free cholesterol and 170 mg.% total cholesterol (after saponification).

Example 3.

0.05 ml. serum are added to 10 ml 0.5M potassium phosphate buffer of pH 7.5 which contains 0.02% hydroxypolyethoxy-dodecane and 0.1% secondary alkyl sulphate. The extinction (E_1) is measured at 240 nm in a suitable spectrophotometer and the reaction is started with 0.02 ml. (= 0.1 U) storage-stable cholesterol oxidase, freed from traces of detergent, in 1M ammonium sulphate solution.

After 3 minutes, the extinction (E_2) is again read off. The concentration of the cholestenone formed and thus of the cholesterol is given by the difference between the first and second readings, having regard to the molar extinction coefficients of cholestenone at 240 nm.

The measurement of a typical sample gave 62 mg.% free cholesterol and 167 mg.% total cholesterol (after saponification).

Example 4.

Filter paper (Schleicher & Schüll No. 597 NF Ind.) is impregnated with a solution of the following composition and dried at 40°C.:

1 M citrate buffer, pH 5.25	20 ml.
cholesterol oxidase (60 U/mg.)	0.1 g.
peroxidase (70 U/mg.)	0.05 g.
distilled water	ad 100 ml.

This paper is then impregnated with solutions of 0.2 g. *o*-tolidine in 100 ml. methylene chloride which, in addition, each contain 1 g. of the following surface-active materials:

- a) polyoxyethylene tributyl-phenol ether
- b) polyoxyethylene sorbitan monolaurate
- c) polyoxyethylene-nonyl-phenol ether
- d) polyoxyethylene lauryl ether

- e) polyoxyethylene cetyl ether
- f) polyoxyethylene stearate
- g) polyoxyethylene dodecyl thioether.

After drying, test papers are obtained which react with cholesterol-containing sera with a green colour. If the sera also contain cholesterol esters, then stronger green colorations are obtained if the sera have previously been mixed with a drop of cholesterol esterase solution. A test paper which does not contain one of the above-mentioned surface-active materials does not react with the sera.

Example 5.

Filter paper (Schleicher & Schüll No. 597 NF Ind.) is impregnated with a solution of the following composition and dried at 40°C.:

1 M citrate buffer, pH 7	20 ml.
cholesterol oxidase (60 U/mg.)	0.1 g.
cholesterol esterase (18 U/mg.)	0.25 g.
peroxidase (70 U/mg.)	0.05 g.
distilled water	100 ml.

This paper is impregnated with solutions of 0.2 g. *o*-tolidine in 100 ml. methylene chloride which, in addition, each contain 0.5 g. of one of the following surface-active materials:

- a) polyoxyethylene cocoate
- b) polyoxyethylene oleate
- c) polyoxyethylene polypropylene glycol
- d) polyoxyethylene stearylamine
- e) polyoxyethylene glycerol monolaurate.

After drying, test papers are obtained which react with a green colour with those sera which contain cholesterol and/or cholesterol esters. Test papers without surface-active agent show no reaction.

Practically the same behaviour is shown by test papers which contain the same amount of citrate buffer with a pH of 5.25 or 6.

Example 6.

Paper pre-impregnated in the manner described in Example 4 is subsequently impregnated with solutions of 0.3 g. *o*-tolidine in 100 ml. acetone which contain 1.5 g. of one of the following surface-active materials:

- a) dioctyl sodium sulphosuccinate
- b) sodium dodecyl-benzene-sulphonate
- c) sodium lauryl polyglycol ether sulphate
- d) sodium lauryl sarcosinate
- e) sodium laurate
- f) cholic acid
- g) desoxycholic acid
- h) sodium taurocholate

These test papers possess practically the same properties as the test papers produced according to Example 4 but the reaction colours are stable for a longer period of time.

Test papers with a phosphate buffer of pH 7 possess similar properties.

Example 7.

Paper pre-impregnated in the manner described in Example 5 is impregnated with solutions which contain 0.4 g. *o*-toluidine and 0.5 g. of one of the following surface-active materials:

- lauryl pyridinium chloride
- benzethonium chloride
- cetyl trimethyl ammonium chloride
- 1 - hydroxyethyl - 1 - carboxymethyl - 2-alkyl-imidazolium betaine.

The properties of these test papers correspond to those of Example 5.

Example 8.

Filter paper (Schleicher & Schüll No. 597 NF Ind.) is impregnated with a solution of the following composition and dried at 40°C.:

IM phosphate buffer, pH 6	25 ml.
cholesterol oxidase (60 U/mg.)	0.1 g.
peroxidase (70 U/mg.)	0.05 g.
cholic acid	1.0 g.
acetone	10 ml.
distilled water	ad 100 ml.

This paper is subsequently impregnated with solutions of oxidation indicators in 100 ml. acetone.

The amount of indicator used, the chemical name thereof and the colour reaction with cholesterol-containing sera are summarised in the following Table II:

TABLE II

amount	indicator	colour reaction
0.3 g.	<i>o</i> -vanillylidene- <i>p</i> -vanilloyl-hydrazone	violet
0.2 g.	azino-bis-(<i>N</i> -ethyl-benzthiazolone-2-sulphonic acid-5) diammonium salt	green
0.1 g.	azino-bis-(<i>N</i> -alkylquinolone-2-sulphonic acid-6) diammonium salt	blue-violet
0.1 g.	bis-(<i>N</i> -alkyl-quinolone-2)-azine	violet
0.1 g.	(<i>N</i> -methyl-benzthiazolone-2)- <i>N</i> -ethyl-quinolone-(2)-azine	blue-green
0.3 g.	(<i>N</i> -methyl-benzthiazolone-2)-1-phenyl-3,4-dimethyl-triazolone-(5)-azine	blue

Example 9.

A mixture is prepared from the following components:

polyvinyl propionate dispersion	45 g.
sodium alginate, 1.85% in 0.5M phosphate buffer, pH 5.5	35 g.
cholesterol oxidase (60 U/mg.)	0.5 g.
peroxidase (70 U/mg.)	0.25 g.
dioctyl sodium sulphosuccinate	1g.
<i>o</i> -toluidine, dissolved in 6 ml. acetone	0.6 g.
water	50 ml.

This mixture is either spread out to give a film with a wet film thickness of about 300 nm or is painted on to a solid carrier and dried at 35°C. When blood containing cholesterol is dropped on to the film and the blood wiped off after one minute, then, depending upon the cholesterol concentration, green colorations of varying intensity are obtained.

If, in addition, 1.0 g cholesterol esterase is added to the above-described formulation, then films are obtained with which it is also pos-

sible to determine abnormally high cholesterol ester contents.

WHAT WE CLAIM IS:—

5 1. A process for the activation of cholesterol oxidase, wherein at least one surface-active compound with lipophilic and hydrophilic properties is added to the cholesterol oxidase before use.

10 2. A process according to claim 1, wherein the surface-active compound used is non-ionic and contains at least one hydroxyl group.

3. A process according to claim 2, wherein the non-ionic compound is a polyoxyethylene derivative of an alkyl, aryl or aralkyl alcohol.

15 4. A process according to any of the preceding claims, wherein the surface-active compound is used in an amount of between 0.005 and 0.1 wt.%, referred to the aqueous enzyme solution.

20 5. A process according to claim 1 for the activation of cholesterol oxidase, substantially as hereinbefore described and exemplified.

25 6. Diagnostic agent for the detection and determination of cholesterol and of cholesterol esters in body fluids by oxidation of the cholesterol with oxygen in the presence of cholesterol oxidase to give cholestenone and hydrogen peroxide and detection of the hydrogen peroxide by peroxidase-catalysed oxidation of

an oxidation indicator, which comprises an absorbent carrier or a synthetic resin film impregnated with or having embedded therein cholesterol oxidase, a system for the detection of hydrogen peroxide, a buffer and at least one surface-active compound according to claim 1. 30 35

7. Diagnostic agent according to claim 6, wherein the surface-active agent is present therein in a concentration of 2 to 30% by weight referred to the solid reagents.

8. Diagnostic agent according to claim 7, wherein the surface-active agent is present therein in a concentration of 10 to 20% by weight, referred to the solid reagents. 40

9. Diagnostic agent according to any of claims 6 to 8, wherein cholesterol esterase is also present. 45

10. Diagnostic agent according to any of claims 6 to 9, wherein a swelling agent and/or a stabilising agent is also present.

11. Diagnostic agent according to claim 6, substantially as hereinbefore described and exemplified. 50

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WHAT WE CLAIM IS:—

- 5 1. A process for the activation of cholesterol oxidase, wherein at least one surface-active compound with lipophilic and hydrophilic properties is added to the cholesterol oxidase before use.
- 10 2. A process according to claim 1, wherein the surface-active compound used is non-ionic and contains at least one hydroxyl group.
- 15 3. A process according to claim 2, wherein the non-ionic compound is a polyoxyethylene derivative of an alkyl, aryl or aralkyl alcohol.
- 20 4. A process according to any of the preceding claims, wherein the surface-active compound is used in an amount of between 0.005 and 0.1 wt.%, referred to the aqueous enzyme solution.
- 25 5. A process according to claim 1 for the activation of cholesterol oxidase, substantially as hereinbefore described and exemplified.
6. Diagnostic agent for the detection and determination of cholesterol and of cholesterol esters in body fluids by oxidation of the cholesterol with oxygen in the presence of cholesterol oxidase to give cholestenone and hydrogen peroxide and detection of the hydrogen peroxide by peroxidase-catalysed oxidation of

an oxidation indicator, which comprises an absorbent carrier or a synthetic resin film impregnated with or having embedded therein cholesterol oxidase, a system for the detection of hydrogen peroxide, a buffer and at least one surface-active compound according to claim 1. 30

7. Diagnostic agent according to claim 6, wherein the surface-active agent is present therein in a concentration of 2 to 30% by weight referred to the solid reagents. 35

8. Diagnostic agent according to claim 7, wherein the surface-active agent is present therein in a concentration of 10 to 20% by weight, referred to the solid reagents. 40

9. Diagnostic agent according to any of claims 6 to 8, wherein cholesterol esterase is also present. 45

10. Diagnostic agent according to any of claims 6 to 9, wherein a swelling agent and/or a stabilising agent is also present.

11. Diagnostic agent according to claim 6, substantially as hereinbefore described and exemplified. 50

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