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(54) PROCESS FOR THE DETERMINATION OF CHOLESTEROL

(71) We, BOEHRINGER MANNHEIM G.M.B.H., 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 determination of total cholesterol or bound cholesterol in biological materials.

Cholesterol is present in biological materials, for example in blood serum, partly in free form and partly in bound form as esters. For the determination of bound cholesterol or of total cholesterol, it is necessary first to liberate the cholesterol present in bound form. Hitherto, this has taken place by saponification under alkaline conditions, for example, with alcoholic potassium hydroxide solution. After the saponification, the liberated cholesterol can then be determined according to one of the known methods, either chemically or enzymatically. Chemical determination can take place, for example, by the Liebermann-Burchard reaction and the enzymatic determination by means of cholesterol oxidase, cholesterol dehydrogenase or cholesterol dehydrase. Since, as is known, the individual cholesterol esters, as well as free cholesterol, have, in the case of chemical methods of determination, differing extinction coefficients, it is necessary to convert the cholesterol esters into free cholesterol by alkaline hydrolysis.

However, the alkaline saponification of bound cholesterol is a disturbing and time-consuming process. Furthermore, the relatively aggressive reagents used can result in decomposition of the cholesterol. In order to prevent such a decomposition and thus a falsification of the analytical results, hydrolysis must be carried out under relatively mild conditions which, in turn, undesirably pro-

long the period of time necessary for the determination.

The alkaline liberation of cholesterol is especially disadvantageous when the determination of the cholesterol is subsequently to be carried out by the preferred enzymatic methods. Since, as is known, the enzymes are inactivated in a strongly alkaline medium, the hydrolysate must be adjusted to a pH of 5 to 8 by the addition of acid before it is possible to commence the enzymatic determination. The result of all this is that the determination of total cholesterol or of bound cholesterol still takes far too long and is very laborious.

It is, therefore, an object of the present invention to provide a process for the determination of total cholesterol or of bound cholesterol which overcomes the above-mentioned disadvantages.

Thus, according to the present invention, there is provided a process for the determination of total cholesterol or of bound cholesterol in biological material, by liberation of the bound cholesterol and subsequent determination of the liberated cholesterol according to known methods, wherein the bound cholesterol is liberated by treating said material with cholesterol esterase.

We have found that a rapid and quantitative saponification of bound cholesterol can be carried out with cholesterol esterase. This process is of quite special advantage when the subsequent determination of the liberated cholesterol also takes place enzymatically, for example, with cholesterol oxidase or with cholesterol dehydrase. In this case, the process according to the present invention permits the completely enzymatic determination of cholesterol and thus a decisive improvement of routine medical diagnosis, as well as a simple adaptation of the process for carrying it out in automatic analysis apparatus.

It was, admittedly, already known that a cholesterol esterase activity is present in the pancreas, liver and in *Nocardia restrictus*. However, it could not be assumed from this

that an enzyme of this kind would be suitable for the rapid and complete saponification of cholesterol esters within the scope of a quantitative analytical process since the ascertained degrees of fission were not quantitative but rather only reached a maximum of 80% (see *Biochimica et Biophysica Acta*, 270, 156—166/1972). Furthermore, bound cholesterol is present in biological material in the form of esters with greatly differing acids. In order to be of use in an analytical process, it is a prerequisite that all the esters present are split quantitatively at approximately the same rate and with the same degree of dependability. On the basis of the known properties of this enzyme, it is surprising that the cholesterol esterases are able quantitatively to split all the cholesterol esters which occur in a very short period of time. This is particularly surprising because it was known that, in the case of the known cholesterol esterases, considerable differences exist with regard to the effectiveness against various cholesterol esters.

Cholesterol esterases from micro-organisms have been found to be especially suitable for the process according to the present invention. They prove to be superior to cholesterol esterases of other origins with regard to the rate of fission of and the degree of effectiveness towards various cholesterol esters and are, therefore, preferably used according to the present invention.

We have also found that various micro-organisms contain especially active cholesterol esterases and can, therefore, be used directly for the process according to the present invention, without separation and purification of the cholesterol esterases. Besides the particularly ready availability, this has also the advantage that, in the case of the omission of any kind of separation and enrichment of the cholesterol esterases, the danger is avoided that from the mixture, which is in practice usually present, of several cholesterol esterases specific for various cholesterol esters, a separation thereof, which would make difficult a quantitative inclusion of all bound cholesterol, is avoided.

It is also to be added that the purification of cholesterol esterases, most of which are bound in lipoid membranes, is laborious and, therefore, leads to a preparation which, because of its cost, is less well suited for a routine diagnostic test than a micro-organism preparation which can be used without any kind of enzyme purification.

Especially advantageous results were obtained with the process according to the present invention when using a cholesterol esterase from *Candida rugosa* (also known as *Cylindracea*) ATCC 14830 or WS 90031 and from *Aspergillus spec.* WS 90030. According to the present invention, these two micro-organisms can be used directly as such or

in digested form, for example as an acetone-dried powder. However, it is, of course, equally possible to use an enriched cholesterol esterase preparation obtained from these micro-organisms, a special advantage being that a certain enrichment can here be achieved particularly simply. The above-mentioned *Candida rugosa* is a micro-organism which is produced on a large scale and is commercially available. The conventional commercial form is an acetone-dried powder stabilised with lactose which has proved to be well-suited for the process of the present invention. Similarly good properties have also been found in the case of the following micro-organisms: *Actinomyces aureovorticillum* WS 90002, *Actinomyces cyaneofuscatus* WS 90003, *Actinomyces griseomyces* WS 90004, *Actinomyces longisporus*-fl. WS 90005, *Actinomyces malachiticus* WS 90006, *Actinomyces roseolus* WS 90007, *Actinomyces toxytricini* WS 90008, *Actinomyces variabilis* WS 90010, *Streptomyces autotrophicus* WS 90011, *Streptomyces canescens* WS 90012, *Streptomyces charlensis* WS 90013, *Streptomyces michiganensis* WS 90014, *Streptomyces murinus* WS 90015, *Streptomyces hachijoensis* WS 90016, *Streptomyces caelestes* WS 90017, *Streptomyces tendae* WS 90018, *Nocardia rubra* WS 90019, *Candida mycoderma* WS 90020, *Candida albicans* WS 90021, *Candida albicans* WS 90022, *Candida albicans* WS 90023, *Candida spec.* WS 90024, *Cunninghamella elegans* WS 90025, *Mucor mucedo* WS 90026, *Rhizopus spec.* WS 90027, *Penicillium spec.* WS 90028 and *Aspergillus spec.* WS 90029.

Besides the preferred cholesterol esterases of microbiological origin, there can, however, also be used, in many cases, cholesterol esterases of other origin.

As already mentioned, an especial advantage of the process according to the present invention is that a completely enzymatic total cholesterol determination is possible. It is hereby of importance that, with the preferred cholesterol esterase preparation from micro-organisms, a rapid and quantitative liberation of the cholesterol from its esters is possible. Particularly with the above-mentioned micro-organisms, it is possible, by the direct use thereof in a very small amount and with the maintenance of the pH value and temperature conditions such as are desired in the case of the subsequent enzymatic cholesterol determination, to achieve a quantitative liberation of cholesterol within the course of a few minutes. We have also found that the conventional stabilising agents based on carbohydrates, which are used for such micro-organisms, do not have a disturbing effect on the fully enzymatic process for the determination of cholesterol.

As already mentioned above, for the process according to the present invention,

there can also be used a separated and enriched cholesterol esterase, preferably from micro-organisms. A suitable enrichment can be achieved by starting from an acetone-dried powder of the micro-organism or other biological material and subjecting this to a dialysis against an appropriate aqueous buffer, for example against 0.01M potassium phosphate buffer (pH 6.0), a treatment with a weakly basic anion exchanger and an ammonium sulphate fractionation. In this way, it is easily possible to achieve a 20 to 30 fold enrichment of the cholesterol esterase. Carbohydrate polymers containing diethyl-aminoethanol radicals have proved to be especially suitable as a weakly basic anion exchanger. In the case of the ammonium sulphate fractionation, the fractions obtained at an ammonium sulphate concentration of from 1.8 to 2.4 Mole are preferably recovered. The enzyme fraction so obtained is subsequently preferably chromatographed on the said exchanger material.

Particularly favourable results were obtained, within the scope of the present invention, with micro-organisms which had been cultured on a nutrient medium containing cholesterol esters. In this case, one or more cholesterol esters can be added to the culture as the sole source of carbon or, in addition, other sources of carbon can also be added. It is especially preferred to use micro-organisms which have been obtained in a multistage culture process, in which, in the first step, culturing is carried out on a suitable source of carbon, for example glycerol, and, in the second stage, on a cholesterol ester. A suitable culturing process is described, for example, in German Patent Specifications Nos. 2,224,133 and 2,307,518.

The cholesterol esterase from *Candida rugosa* (ATCC 14830), which is preferably used according to the present invention, has a very good stability in the weakly acid range between pH 5 and 6.5. The pH optimum of the enzyme is at 7.5. A characteristic of the enzyme is that the catalytic reaction takes place especially well at a relatively high salt content of the reaction medium. Preferably, therefore, the process is carried out in a 0.2 to 0.8M and particularly a 0.4 to 0.6M, buffer solution. The pH value can be within the range of between pH 4.5 to 7.5 and preferably in the above-mentioned range of pH 5 to 6.5. The effectiveness of the cholesterol esterase can be increased by the addition of a surface-active agent, an addition of hydroxypolyethoxydodecane being especially preferred.

As already mentioned, the process according to the present invention is preferably carried out completely enzymatically, i.e. the subsequent cholesterol determination also takes place enzymatically, preferably with the use of cholesterol oxidase. However,

cholesterol dehydrogenase or cholesterol hydrogenase can also be employed.

The determination with cholesterol oxidase is described, for example, in German Patent Specification No. 2,224,132. The process therein described can advantageously be combined with the process according to the present invention. In principle, the utilisation of oxygen, the formation of hydrogen peroxide or the formation of cholestenone can be measured.

The determination of the oxygen utilisation can take place, for example, gas chromatographically, polarometrically or by the polarisation process. These methods of determination are known. Hydrogen peroxide formed can be determined titrimetrically, potentiometrically, polarographically or colorimetrically, as well as enzymatically. The enzymatic determination is preferred, with the use of catalase or peroxidase, especially the determination by means of catalase in the presence of β - diketones, for example acetyl - acetone, lower alcohols and ammonium ion-containing buffers or the determination by means of peroxidase in the presence of a chromogen, for example 2,2' - aminobenzthiazoline - sulphonic acid. Cholestenone can be determined with the help of keto reagents, for example, 2,4 - dinitrophenyl - hydrazine, or also photometrically at 240 nm.

If the completely enzymatic determination of the total or bound cholesterol is to take place with cholesterol oxidase, there is preferably used a cholesterol oxidase derived from *Nocardia erythropolis* (ATCC 17895), *Nocardia erythropolis* (ATCC 4277), *Nocardia formica* (ATCC 14811) or *Proactinomyces erythropolis* (NCIB 9158).

The present invention also provides a test pack for the determination of cholesterol, comprising cholesterol esterase and a reagent or reagent mixture for the determination of free cholesterol. A test pack of this kind preferably comprises a cholesterol esterase of microbiological origin, cholesterol oxidase and a reagent or reagent mixture for the determination of hydrogen peroxide or a reagent or reagent mixture for the determination of cholestenone. Very particularly preferred is a test pack in which, as cholesterol esterase, there is used one of the above-mentioned micro-organisms, especially in the form of an acetone-dried powder, or a protein fraction with cholesterol esterase activity obtained therefrom.

In a special and preferred embodiment of the present invention, such a test pack comprises cholesterol oxidase, a cholesterol esterase preparation from one of the above-mentioned micro-organisms, catalase, acetyl - acetone, methanol and ammonium ion-containing buffer, the individual components of the test pack being separate from one another or in the form of mixtures of compatible

components, i.e. components which do not react under storage conditions. In another preferred embodiment of the present invention, the test pack comprises cholesterol oxidase, a preparation of one of the above-mentioned micro-organisms with cholesterol esterase activity, peroxidase, a chromogen and a buffer, there again, the individual components of the test pack can be separate from one another or can be in the form of mixtures of compatible components. The chromogen is preferably 2,2' - aminobenzthiazoline - sulphonic acid.

According to yet another preferred embodiment of the present invention, the test pack comprises cholesterol oxidase, a cholesterol esterase preparation of one of the above-mentioned micro-organisms, a hydrazine derivative which can react with keto groups with the formation of hydrazone and possibly also a buffer. The hydrazine derivative is preferably 2,4 - dinitrophenyl - hydrazine.

The above-mentioned preferred test packs can, in addition to the mentioned obligatory components, also contain conventional solvents, stabilisers for preventing the components of the test pack being attacked by micro-organisms and/or surface-active agents. All these additives are known and are conventional in detection systems for hydrogen peroxide or cholestenone.

The above-mentioned test packs preferably contain the essential components in the following ratios:

1. 13 to 150 U cholesterol oxidase, 0.05 to 0.5 mg. micro-organism cholesterol esterase, 2×10^4 to 5×10^5 U catalase, 0.05 to 0.2 ml. acetyl - acetone and 2 to 10 ml. methanol in 100 ml. ammonium ion-containing buffer of pH 5 to 7, as well as possibly also 0.02 to 0.3 ml. of a surface-active agent, preferably hydroxypolyethoxydodecane.

2. 3 to 40 U cholesterol oxidase, 0.05 to 0.5 mg. micro-organism cholesterol esterase, 2×10^3 to 1×10^4 U peroxidase and 50 to 200 mg. 2,2' - aminobenzthiazolinesulphonic acid, as well as possibly also 0.05 to 0.5 ml. surface-active agent, preferably hydroxypolyethoxydodecane, in 100 ml. buffer of pH 6 to 8.

3. 0.1 to 1 U cholesterol oxidase, 0.05 to 0.5 mg. micro-organism cholesterol esterase and 1 to 5 ml. of a 1 mM solution of 2,4 - dinitrophenyl - hydrazine, as well as possibly also 0.005 to 0.1 ml. of surface-active agent, preferably hydroxypolyethoxydodecane, in 10 ml. buffer of pH 6 to 8.

4. 2 to 100 U cholesterol oxidase and 0.05 to 0.5 mg. micro-organism cholesterol esterase, as well as possibly also 0.1 to 2.0 ml. of surface-active agent, preferably hydroxypolyethoxydodecane, in 50 ml. buffer of pH 5 to 9, preferably in 0.5M sodium phosphate buffer of pH 7.5.

With the process and test pack according

to the present invention, an extraordinarily quick and complete saponification of bound cholesterol can be carried out. For example, under the conditions of the cholesterol determination with cholesterol oxidase according to the present invention, there is achieved a quantitative splitting of bound cholesterol within the course of 1 to 3 minutes when using *Candida rugosa* (ATCC 14830) or *Aspergillus* sp. (WS 90030) acetone-dried powder in an amount of between 0.1 and 0.3 mg.

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

Example 1

With the use of the process described in Example 1 of our British Patent Specification No. 23082/73 (Serial No. 1429525), the content of free cholesterol in serum is determined as being 63 mg.% (63 mg. in 100 ml. serum). For the determination of bound cholesterol, a comparison sample of the serum is treated for 30 minutes with alcoholic potassium hydroxide solution at 70°C. After neutralisation and a second measurement of the cholesterol present, there is given a total content of 181 mg.% cholesterol. From this, it follows that 118 mg. cholesterol/100 ml. was present in bound form.

The same determination was made on untreated serum but 0.3 mg. (referred to the protein) *Candida rugosa* (ATCC 14830) acetone-dried powder in commercially-available form was added to the serum sample. After 3 minutes, polarographic determination gave a content of 183 mg.% total cholesterol.

Example 2

For the enrichment of the cholesterol esterase activity, a commercially-available acetone-dried powder of *Candida rugosa* (ATCC 14830) was dissolved in potassium phosphate buffer of pH 6.0 and dialysed against the same buffer. After removal of the lactose present therein as stabilising agent, there was obtained, in the dialysed solution, a specific cholesterol esterase activity of 0.3 U/mg. protein.

The solution thus obtained was stirred with an ion exchanger (dextran containing diethylaminoethanol radicals), whereafter the exchanger was separated and eluted with 0.2M phosphate buffer of pH 6.0. In the eluate there was found a specific cholesterol esterase activity of 1.2 U/mg.

The solution thus obtained was subjected to an ammonium sulphate fractionation. The protein fraction which precipitated between 1.8 and 2.4M ammonium sulphate was separated off. It had a specific cholesterol esterase activity of 2.5 U/mg.

The product obtained was again dissolved in a phosphate buffer of pH 6.0, dialysed

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against the same buffer until free from salt and then chromatographed over a column which was filled with the same anion exchanger as used above. Elution was again carried out with 0.2M phosphate buffer of pH 6.0. In the fractions with cholesterol esterase activity, there was found a specific activity of 7 U/mg. protein.

The enriched cholesterol esterase thus obtained was used, as described in Example 1, for the determination of cholesterol. The amount used was, however, only 0.001 mg., calculated as protein. The result corresponded to that of Example 1.

The cholesterol esterase from *Candida rugosa* can be further purified by the conventional methods used for the purification of enzymes. Instead of the above-mentioned enrichment steps, there can also be employed other conventional biochemical enrichment steps, for example, precipitation or fractionation with polyethyleneimine, organic solvents or salts, chromatography over molecular sieve materials or weak anionic exchangers with functional groups other than diethylamino-ethanol groups or protamine sulphate precipitation.

Example 3

1.0 ml. 0.5M potassium phosphate buffer of pH 7.5, which contains 0.4% hydroxypolyethoxydodecane, is added to 0.5 ml. serum or cholesterol standard and 2.5 U cholesterol esterase according to Example 2 added thereto. This reaction mixture is incubated for 40 minutes at 37°C. Thereafter, 0.25 ml. of this solution are added to 3 ml. of cholesterol reagent which contains 2 parts acetic acid, 3 parts acetic anhydride and 1 part sulphuric acid (Liebermann-Burchardt reagent).

With the use of a standard as reference, for a typical sample there was found 170 mg.% total cholesterol. A comparative determination with saponification of the cholesterol esters with alcoholic potassium hydroxide solution gave 165 mg.% total cholesterol.

Example 4

0.02 ml. serum are mixed with 10 ml. 0.5M potassium phosphate buffer, which contains 0.4% hydroxypolyethoxydodecane, and 0.2 U cholesterol esterase according to Example 2. The reaction solution is incubated for 60 minutes at 37°C. Thereafter, the extinction (E₁) is read off at 240 nm in a suitable spectrophotometer and the reaction initiated with 0.1 U sterol dehydrase from *Brevibacterium sterolicum*. After 15 minutes, the extinction (E₂) is again read off. The concentration of the Δ⁴ - cholestenone formed and thus of the cholesterol follows from the difference between the first and second reading, having regard to the molar extinction coefficients for Δ⁴ - cholesterol at 240 nm.

The measurement of a typical sample gives 183 mg.% total cholesterol.

A comparison determination with a cholesterol oxidase from *Nocardia erythropolis* instead of with sterol dehydrase gave 181 mg.% total cholesterol.

Example 5

10 g. diammonium hydrogen phosphate are dissolved in 100 ml. water and adjusted to pH 7.0 with 85% phosphoric acid. 10⁵ U catalase are then added thereto. The solution thus obtained is used to make up to 100 ml. a mixture of 0.2 ml. acetyl - acetone; 10 ml. methanol and 0.1 g. hydroxypolyethoxydodecane. 2.5 U cholesterol esterase from *Rhizopus spec.* (WS 90027) are added to this solution.

5.0 ml. of the solution thus obtained are mixed with 0.02 ml. serum or 0.02 ml. of a cholesterol standard solution with a content of 200 mg.% cholesterol. Aliquots of the serum-containing sample, as well as of the cholesterol standard sample are each mixed with 0.1 U cholesterol oxidase and incubated for 60 minutes at 37°C. The coloured material formed is then measured photometrically at 405 nm, having regard to the blank reading.

The cholesterol content of the serum-containing sample was 154 mg.%, with the use of a standard as reference. A control determination with cholesterol esterase from *Candida rugosa* (ATCC 14830), instead of with cholesterol esterase from *Rhizopus spec.* (WS 90027), gave the same value.

WHAT WE CLAIM IS:—

1. Process for the determination of total cholesterol or of bound cholesterol in biological materials by liberation of the bound cholesterol and subsequent determination of the liberated cholesterol according to known methods, wherein the bound cholesterol is liberated by treating said materials with cholesterol esterase.

2. Process according to claim 1, wherein a cholesterol esterase derived from a micro-organism is used.

3. Process according to claim 2, wherein a cholesterol esterase derived from *Candida rugosa* ATCC 14830, *Rhizopus spec.* WS 90027 or *Aspergillus spec.* WS 90030 is used.

4. Process according to claim 2, wherein a cholesterol esterase is used which is derived from *Actinomyces aureovorticillium* WS 90002, *Actinomyces cyaneofuscatus* WS 90003, *Actinomyces griseomyces* WS 90004, *Actinomyces longisporus-fl.* WS 90005, *Actinomyces malachiticus* WS 90006, *Actinomyces roseolus* WS 90007, *Actinomyces toxicitricini* WS 90008, *Actinomyces variabilis* WS 90009, *Streptomyces spec.* WS 90010, *Streptomyces autotrophicus* WS 90011, *Streptomyces canescens* WS 90012, *Strepto-*



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against the same buffer until free from salt and then chromatographed over a column which was filled with the same anion exchanger as used above. Elution was again carried out with 0.2M phosphate buffer of pH 6.0. In the fractions with cholesterol esterase activity, there was found a specific activity of 7 U/mg. protein.

The enriched cholesterol esterase thus obtained was used, as described in Example 1, for the determination of cholesterol. The amount used was, however, only 0.001 mg., calculated as protein. The result corresponded to that of Example 1.

The cholesterol esterase from *Candida rugosa* can be further purified by the conventional methods used for the purification of enzymes. Instead of the above-mentioned enrichment steps, there can also be employed other conventional biochemical enrichment steps, for example, precipitation or fractionation with polyethyleneimine, organic solvents or salts, chromatography over molecular sieve materials or weak anionic exchangers with functional groups other than diethylamino-ethanol groups or protamine sulphate precipitation.

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1.0 ml. 0.5M potassium phosphate buffer of pH 7.5, which contains 0.4% hydroxypolyethoxydodecane, is added to 0.5 ml. serum or cholesterol standard and 2.5 U cholesterol esterase according to Example 2 added thereto. This reaction mixture is incubated for 40 minutes at 37°C. Thereafter, 0.25 ml. of this solution are added to 3 ml. of cholesterol reagent which contains 2 parts acetic acid, 3 parts acetic anhydride and 1 part sulphuric acid (Liebermann-Burchardt reagent).

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The measurement of a typical sample gives 183 mg.% total cholesterol.

A comparison determination with a cholesterol oxidase from *Nocardia erythropolis* instead of with sterol dehydrase gave 181 mg.% total cholesterol.

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5.0 ml. of the solution thus obtained are mixed with 0.02 ml. serum or 0.02 ml. of a cholesterol standard solution with a content of 200 mg.% cholesterol. Aliquots of the serum-containing sample, as well as of the cholesterol standard sample are each mixed with 0.1 U cholesterol oxidase and incubated for 60 minutes at 37°C. The coloured material formed is then measured photometrically at 405 nm, having regard to the blank reading.

The cholesterol content of the serum-containing sample was 154 mg.%, with the use of a standard as reference. A control determination with cholesterol esterase from *Candida rugosa* (ATCC 14830), instead of with cholesterol esterase from *Rhizopus spec.* (WS 90027), gave the same value.

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1. Process for the determination of total cholesterol or of bound cholesterol in biological materials by liberation of the bound cholesterol and subsequent determination of the liberated cholesterol according to known methods, wherein the bound cholesterol is liberated by treating said materials with cholesterol esterase.

2. Process according to claim 1, wherein a cholesterol esterase derived from a micro-organism is used.

3. Process according to claim 2, wherein a cholesterol esterase derived from *Candida rugosa* ATCC 14830, *Rhizopus spec.* WS 90027 or *Aspergillus spec.* WS 90030 is used.

4. Process according to claim 2, wherein a cholesterol esterase is used which is derived from *Actinomyces aureovercillium* WS 90002, *Actinomyces cyaneofuscatus* WS 90003, *Actinomyces griseomycini* WS 90004, *Actinomyces longisporus-fl.* WS 90005, *Actinomyces malachiticus* WS 90006, *Actinomyces roseolus* WS 90007, *Actinomyces toxicicini* WS 90008, *Actinomyces variabilis* WS 90009, *Streptomyces spec.* WS 90010, *Streptomyces autotrophicus* WS 90011, *Streptomyces canescens* WS 90012, *Strepto-*



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5. Process according to any of the preceding claims, wherein the subsequent determination of liberated cholesterol is carried out enzymatically.
6. Process according to claim 5, wherein cholesterol esterase and an enzyme for the determination of free cholesterol are added simultaneously to the biological material.
7. Process according to claim 5 or 6, wherein liberated cholesterol is determined with cholesterol oxidase.
8. Process according to claim 7, wherein there is used a cholesterol oxidase from *Nocardia erythropolis* ATCC 17895, *Nocardia erythropolis* ATCC 4277, *Nocardia formica* ATCC 14811 or *Proactinomyces erythropolis* NCIB 9158.
9. Process according to any of claims 2 to 8, wherein a cholesterol esterase is used from a micro-organism which has been cultured on a nutrient medium containing cholesterol ester.
10. Process according to any of the preceding claims, wherein the reaction with cholesterol esterase is carried out at a pH of between 4.5 and 7.5.
11. Process according to claim 10, wherein the reaction with cholesterol esterase is carried out at a pH of between 5 and 6.5.
12. Process according to any of the preceding claims, wherein the reaction with cholesterol esterase is carried out in a 0.2 to 0.8M buffer solution.
13. Process according to claim 12, wherein the reaction with cholesterol esterase is carried out in a 0.4 to 0.6M buffer solution.
14. Process according to claim 1 for the determination of total cholesterol or of bound cholesterol, substantially as hereinbefore described and exemplified.
15. A test pack for the determination of cholesterol, comprising cholesterol esterase and a reagent or reagent mixture for the determination of free cholesterol.
16. A test pack according to claim 15, comprising a cholesterol esterase of micro-biological origin, cholesterol oxidase, a reagent or reagent mixture for the determination of hydrogen peroxide or a reagent or reagent mixture for the determination of cholestenone.
17. A test pack according to claim 16, wherein the reagent mixture for the determination of hydrogen peroxide comprises peroxidase, a chromogen and a buffer.
18. A test pack according to claim 17, wherein the chromogen is 2,2' - aminobenzthiazoline - sulphonic acid.
19. A test pack according to claim 16, wherein the reagent mixture for the determination of cholestenone comprises a hydrazine derivative which reacts with keto groups to form hydrazone groups.
20. A test pack according to claim 19, wherein the hydrazine derivative is 2,4 - dinitrophenyl - hydrazine.
21. A test pack according to any of claims 15 to 20, wherein the cholesterol esterase used is one obtained from a micro-organism according to claim 3 or 4.
22. A test pack for the determination of cholesterol, comprising cholesterol oxidase, a cholesterol esterase from a micro-organism according to claim 3 or 4, catalase, acetyl - acetone, methanol and an ammonium ion-containing buffer.
23. A test pack according to any of claims 15 to 22, wherein there is additionally present at least one solvent, a stabiliser for preventing the components of the test pack being attacked by micro-organisms and/or a surface-active agent.
24. A test pack for the determination of cholesterol, comprising 13 to 150 U cholesterol oxidase, 0.05 to 0.5 mg. micro-organism cholesterol esterase, 2×10^4 to 5×10^5 U catalase, 0.05 to 0.2 ml. acetyl - acetone and 2 to 10 ml. methanol in 100 ml. ammonium-ion containing buffer of pH 5 to 7.
25. A test pack according to claim 24, wherein 0.02 to 0.3 ml. of a surface-active agent is also present.
26. A test pack for the determination of cholesterol, comprising 3 to 40 U cholesterol oxidase, 0.05 to 0.5 mg. micro-organism cholesterol esterase, 2×10^3 to 1×10^4 U peroxidase and 50 to 200 mg. 2,2' - aminobenzthiazoline - sulphonic acid in 100 ml. of buffer of pH 6 to 8.
27. A test pack according to claim 26, wherein 0.05 to 0.5 ml. of a surface-active agent is also present.
28. A test pack for the determination of cholesterol, comprising 0.1 to 1 U cholesterol oxidase, 0.05 to 0.5 mg. micro-organism cholesterol oxidase and 1 to 5 ml. of a 1 mM solution of 2,4 - dinitrophenyl - hydrazine in 10 ml. of buffer of pH 6 to 8.
29. A test pack according to claim 28, wherein 0.005 to 0.1 ml. of a surface-active agent is also present.
30. A test pack for the determination of cholesterol, comprising 2 to 100 U cholesterol oxidase and 0.05 to 0.5 mg. micro-organism cholesterol esterase in 50 ml. of buffer of pH 5 to 9.



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31. A test pack according to claim 30, wherein 0.1 to 2.0 ml. of a surface-active agent is also present. the determination of cholesterol, substantially as hereinbefore described and exemplified.

32. A test pack according to claim 30 or 31, wherein the buffer is a 0.5M sodium phosphate buffer of pH 7.5.

33. A test pack according to any of claims 23, 25, 27, 29 and 31, wherein the surface-active agent is hydroxypolyethoxydodecane.

34. A test pack according to claim 15 for

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