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[54] **METHOD FOR THE DETERMINATION OF CHOLESTEROL**

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[58] **Field of Search** ..... 195/103.5 R

[56] **References Cited**

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[57] **ABSTRACT**

Total cholesterol or bound cholesterol in a sample is determined by treating the sample with cholesterol esterase, thereby releasing the bound cholesterol, and then determining the resulting total cholesterol by known methods; specifically preferred are cholesterol esterases from Candida rugosa ATCC 14830, Rhizopus spec. WS 90027 and Aspergillus spec. WS 90030.

18 Claims, No Drawings

METHOD FOR THE DETERMINATION OF CHOLESTEROL

The present invention relates to a method of determining cholesterol, and more specifically to a method of determining either total cholesterol or bound cholesterol.

Cholesterol is present in biological matter, such as serum or the like, partially in free form and partially in bound form as ester. For the determination of either bound cholesterol or total cholesterol it is necessary first to release the cholesterol that is present in bound form. This has been done hitherto through saponification under alkaline conditions, using alcoholic potash lye, for example. After the saponification, the released cholesterol can then be determined either chemically or enzymatically by one of the known methods. The chemical determination may be performed, for example, by the Liebermann-Burchard method, and enzymatic determination may be performed by means of cholesterol oxidase, cholesterol dehydrogenase or cholesterol dehydrase. Since the individual cholesterol esters as well as the free cholesterol are known to have different extinction coefficients in the chemical methods of determination, it is necessary to transform the cholesterol esters to free cholesterol by alkaline hydrolysis.

In any case, however, the alkaline saponification of the bound cholesterol is a troublesome and time-consuming step in the procedure. Furthermore, the relatively agressive reagents used may lead to a decomposition of the cholesterol. In order to prevent such decomposition and thus forestall falsification of the results of the analysis, a hydrolysis must be performed under relatively mild conditions, and this in turn undesirably increases the length of time required for the determination.

The alkaline liberation of the cholesterol is especially disadvantageous when the determination of the cholesterol is afterwards to be performed by the preferred enzymatic methods. Since the enzymes are inactivated, as it is known, in the strongly alkaline medium, the hydrolyzate must be acidified by the addition of acid to pH 5 to 8 before the enzymatic determination can be started. All this results in the fact that the determination of the total cholesterol or of the bound cholesterol still takes an undesirably long time and requires too much work.

The present invention provides a process for the determination of total cholesterol or bound cholesterol which substantially obviates the above-mentioned disadvantages.

Essentially, the process of the invention comprises the determination of total cholesterol or of bound cholesterol by releasing the bound cholesterol using cholesterol esterase, followed by determination of the released cholesterol by known methods.

It has been found that, using cholesterol esterase, a rapid and quantitative saponification of bound cholesterol can be performed. This process is especially advantageous whenever the subsequent determination of the released cholesterol is performed enzymatically, using cholesterol oxidase or cholesterol dehydrase, for example. In this case, the process of the invention makes possible the all-enzymatic determination of cholesterol and therefore a decided improvement of routine medical diagnosis plus an easy adaptation of the process for performance in automatic analysis apparatus.

It is already known that in the pancreas and liver a cholesterol esterase is present. It could not be concluded from this knowledge, however, that such an enzyme would be suitable for the rapid, complete saponification of cholesterol esters in the framework of a quantitative analysis process, because the cleavage rates determined were not quantitative, amounting to only 80% maximum (Biochimica et Biophysica Acta 270, (1972), 156–166). Furthermore, bound cholesterol is present in biological matter in the form of esters of widely different acid. For an enzymatic process to be useful in the framework of a process of analysis, it is required that all of the esters that may occur be cleaved quantitatively with approximately the same speed and with the same reliability. On account of the known properties of these enzymes it is surprising that the cholesterol esterases are capable of cleaving quantitatively, within a very short time, all of the cholesterol esters that occur. This is especially surprising also because in the known cholesterol esterases there are considerable differences with regard to their activity against various cholesterol esters.

Cholesterol esterases from microorganisms have proven to be especially suited for the process of the invention. They have proven superior to cholesterol esterases of other origin in regard to speed of cleavage and effectiveness and they are therefore preferred within the scope of the invention.

It has also been found that a number of microorganisms contain particularly active cholesterol esterases and can therefore be used directly within the scope of the invention without separation and purification of the cholesterol esterases. This is advantageous in avoiding the separation of certain esterases from the normally used mixture of a plurality of cholesterol esterases specific for various cholesterol esters. Such separation would greatly complicate the quantitative determination of all of the bound cholesterol.

In addition, the purification of the bound cholesterol esterases in lipoid membranes is difficult, and therefore results in a preparation which is less suitable, on account of its price, for use in routine diagnosis than a microorganism preparation which can be used without any enzyme purification.

Particularly advantageous results have been obtained in the process of the invention by the use of a cholesterol esterase derived from *Candida rugosa* (also referred to as Cylindracea) ATCC 14830 and WS 90031, respectively, and from Aspergillus spec. WS 90030. These two microorganisms may be used directly as such or in processed form, e.g., in the form of an acetone dry powder, within the scope of the invention. It is also, of course, possible to use a concentrated cholesterol esterase preparation made from these microorganisms, there being a special advantage in the fact that a certain concentration may in this case be achieved very simply. *Candida rugosa* is a microorganism that is produced on a large technical scale and is available commercially. The customary commercial form is an acetone dry powder stabilized with lactose, which has proven to be outstandingly suited for the invention. Similarly attractive properties have been found in:

Actinomyces aureoverticillium	WS 90002
Actinomyces cyaneofuscatus	WS 90003
Actinomyces griseomycini	WS 90004
Actinomyces longisporus-fl.	WS 90005
Actinomyces malachiticus	WS 90006
Actinomyces roseolus	WS 90007

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Actinomyces toxytricini	WS 90008
Actinomyces variabilis	WS 90009
Streptomyces spec.	WS 90010
Streptomyces autotrophicus	WS 90011
Streptomyces canescens	WS 90012
Streptomyces chartreusis	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
Aspergillus spec.	WS 90029

In addition to the preferred cholesterol esterases of microbiological origin, however, cholesterol esterases of other origin may also be used in many cases.

As previously mentioned, an especially important advantage of the process of the invention consists in the fact that it makes possible an all-enzymatic determination of total cholesterol. It is important in this case that, with the preferred cholesterol esterase preparations made from microorganisms, a rapid and quantitative release of the cholesterol from its esters is possible. Especially with the preferred microorganisms mentioned above, it is possible by the direct addition of same in a very small quantity, with the maintenance of the pH values and temperatures which are desirable in the subsequent enzymatic determination of cholesterol, to achieve within a few minutes a quantitative release of the cholesterol, it having been found that the common carbohydrate-based stabilizing agents which are used for such microorganisms do not interfere with the cholesterol determination performed within the framework of the allenzymatic process.

As mentioned, a separated and concentrated cholesterol esterase, preferably one obtained from microorganism, may also be used for the process of the invention. A suitable concentration may be achieved by setting out from an acetone dry powder of the microorganism or other biological material and subjecting it to a dialysis, a treatment with weakly basic anion exchanger and to an ammonium sulfate fractionation. In this manner, it is easy to achieve a 20-fold to 30-fold concentration of the cholesterol esterase. A preparation on a carbohydrate basis, modified with diethylaminoethanol groups, has proven to be an especially suitable weakly basic anion exchanger. In the ammonium sulfate fractionation, the fraction between 1.8 and 2.4 moles of ammonium sulfate is preferably obtained. The enzyme fraction thus obtained is then chromatographed, preferably on the above-named exchanger material.

Particularly good results are obtained within the scope of the invention with microorganisms which have been cultivated in a nutrient medium containing cholesterol ester. In this case, the cholesterol ester or a mixture of cholesterol esters may be added during the cultivation as the sole source of carbon, or may be used together with another carbon source. Especially preferred is the use of microorganisms which are obtained in a multi-stage cultivating process, in which they are cultivated in the first stage on a suitable carbon supplier, such as glycerin, and in the second stage on a cholesterol ester. A suitable cultivation process is described, for example, in German Published Specifications ("Offenlegungsschriften") Nos. 2,224,133 and 2,307,518.

The cholesterol esterase from *Candida rugosa* ATCC 14830 used preferentially in accordance with the invention has very good stability in the weakly acid region between pH 5 and 6.5. The optimum pH for the enzyme is 7.5. One peculiarity of the enzymes is that the catalytic reaction takes place especially well when the salt content of the reaction medium is relatively high. Preferably, therefore, the process is performed in an 0.2 to 0.8 molar buffer solution. The pH may range between 4.5 and 7.5, and will preferably range, as stated above, between pH 5 and 6.5. The effectiveness of the cholesterol esterase is preferably increased by the addition of surface active substances. Especially preferred is the addition of hydroxypolyethoxydodecane.

As previously mentioned, it is especially preferred that the process of the invention be performed allenzymatically, i.e., the cholesterol determination that follows is also performed enzymatically, preferably with the use of cholesterol oxidase. However, cholesterol dehydrase or dehydrogenase may also be used.

Determination with cholesterol oxidase is described, for example, in German Offenlegungsschrift No. 2,224,132. The process therein described may advantageously be combined with the process of the invention. In this case, it is possible in principle to measure the oxygen consumption, the H_2O_2 formation or the formation of cholestenone. The determination of the oxygen consumption may be performed, for example, by gas chromatography or polarometry, or by the polarization method. These methods of determination are in the prior art. The hydrogen peroxide that forms may be determined titrimetrically, potentiometrically, and colorimetrically, as well as enzymatically. Enzymatic determination is preferred, with the use of catalase or peroxidase, especially determination by catalase in the presence of beta diketones such as acetylacetone, low alcohols and buffer containing ammonium ions, or determination by peroxidase in the presence of a chromogen such as 2,2'-aminobenzothiazolinesulfonic acid. Cholestenone is determined by means of keto reagents such as 2,4-dinitrophenylhydrazine, or by photometry at 240 nm.

If the all-enzymatic determination of the total or bound cholesterol is performed with cholesterol oxidase, a cholesterol oxidase obtained from *Nocardia erythropolis* ATCC 17895, *Nocardia erythropolis* ATCC 4277, *Nocardia formica* ATCC 14811 or *Proactinomyces erythropolis* NCIB 9158 is preferably used.

Additional subject matter of the invention is a reagent for the determination of cholesterol which consists of cholesterol esterase and a reagent for the determination of free cholesterol. Preferably, a reagent of this sort consists of a cholesterol esterase of microbiological origin, cholesterol, oxidase, a system for the determination of hydrogen peroxide, or a system for the determination of cholestenone. Quite especially preferred in this case is a reagent in which the cholesterol esterase is one of the microorganisms mentioned further above, especially in the form of an acetone dry powder or a protein fraction obtained therefrom having cholesterol esterase activity.

In a special and preferred embodiment, such a reagent consists of cholesterol oxidase, a cholesterol esterase preparation made from one of the above-mentioned microorganisms, catalase, acetyl acetone, methanol and aluminum ion-containing buffer, individually or mixed. In still another preferred embodiment, the reagent consists of cholesterol oxidase, a preparation made of the above-mentioned microorganisms having cholesterol esterase activity, peroxidase, chromogen and buffer, individually or mixed. 2,2'-aminobenzothiazolinesulfonic acid is preferred as the chromogen.

In still another preferred embodiment, the reagent of the invention consists of cholesterol oxidase, a cholesterol esterase preparation made from one of the named microorganisms, and a hydrazine derivative reacting with keto groups with the formation of hydrazone, and in some cases a buffer. 2,4-dinitrophenylhydrazine is preferred as the hydrazine derivative.

The above-mentioned preferred reagent combinations may contain, in addition to the specified essential components, commonly used solvents, stabilizers and/or suface active substances. All these additive substances are known to persons skilled in the art and commonly used in detection systems for hydrogen peroxide and cholestenone.

Preferably, the above-mentioned reagent combinations will contain the essential components in the following rations:

1. 13 to 150 U of cholesterol oxidase, 0.05 to 0.5 mg of microorganism cholesterol esterase, 2×10^4 to 5×10^5 units of catalase, 0.05 to 0.2 ml of acetyl acetone and 2 to 10 ml of methanol in 100 ml of a pH 5 to 7 buffer containing ammonium ions, plus, if desired, 0.02 to 0.3 ml of a surface active agent, preferably hydroxypolyethoxydodecane.
2. 3 to 40 U of cholesterol oxidase, 0.05 to 0.5 mg of microorganism cholesterol esterase, and $2 \times$ [illegible] to 1×10^4 U of peroxidase, 50 to 200 mg. of 2,2'amino-benzothiasolinesulfonic acid, and, if desired, 0.05 to 0.5 ml of surface active agent, preferably hydroxypolyethoxydodecane, in 100 ml of pH 6 to 8 buffer.
3. 0.1 to 1 U of cholesterol oxidase, 0.05 to 0.5 mg of microorganism cholesterol esterase, 1 to 5 ml of a 1 mM solution of 2,4-dinitrophenylhydrazine, and, if desired, 0.005 to 0.1 ml of surface active agent in 10 ml of pH 6 to 8 buffer.
4. 2 to 100 U of cholesterol oxidase, 0.05 to 0.5 mg of microorganism cholesterol esterase, and, if desired, 0.1 to 2.0 ml of surface active agent (preferably hydroxypolethoxydodecane), in 50 ml of pH 5 to 9 buffer, preferably 0.5 m of sodium phosphate pH 7.5 buffer.

With the process and reagent of the invention, an extremely rapid and complete saponification of bound cholesterol determination with cholesterol oxidase in accordance with the invention, a quantitative cleavage of bound cholesterol is accomplished within one to three minutes with the addition of *Candida rugosa* ATCC 14830 or Aspergillus sp. WS 90030 acetone dry powder in a quantity between 0.1 to 0.3 mg. The following examples are illustrative.

EXAMPLE 1

Using the method described in Example 1 of German Offenlegungsschrift No. 2,224,132, the content of free cholesterol in serum was found to be 63 mg% (63 mg in 100 ml). For the determination of bound cholesterol, a specimen of the same serum was treated for 30 minutes with alcoholic potash lye at 70°C. After neutralization and measurement of the cholesterol present, a total content of 181 mg% of cholesterol was found. From this appears that 118 mg of cholesterol were present in bound form for every 100 ml.

The process was repeated with untreated serum, but at the beginning of the determination 0.3 mg% (with reference to the protein) of an acetone dry powder of *Candida rugosa* ATCC 14830 in commercial form was added. After 3 minutes, polarographic determination showed a content of 183 mg% total cholesterol.

EXAMPLE 2

To concentrate the cholesterol esterase activity, commercial acetone dry powder of *Candida rugosa* ATCC 14830 was dissolved in potassium phosphate buffer pH 6.0 and dialyzed against the same buffer. After removal of the lactose contained in the solution as stabilizer, the specific cholesterol esterase activity was 0.3 U per mg of protein in the dialyzed solution.

The solution thus obtained was stirred together with an ion exchanger on a dextran basis modified with diethylaminoethanol groups; the exchanger was separated and eluted with 0.2 M of pH 6.0 phosphate buffer. A specific cholesterol esterase activity of 1.2 U/mg was found in the eluate.

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

The product obtained was again dissolved in pH 6.0 phosphate buffer, dialyzed against the same buffer until salt-free, and then chromatographed on a column filled with the same anion exchanger as above. Elution was again performed with 0.2 M of pH 6.0 phosphate buffer. A specific activity of 7 U per mg of protein was found in the fraction having cholesterol esterase activity.

The concentrated cholesterol esterase preparation thus obtained was used in the cholesterol determination as described in Example 1, except that the amount used was only 0.001 mg with reference to protein. The results were the same as in Example 1.

The cholesterol esterase from *Candida rugosa* can be further purified by the conventional methods of enzyme refinement. Instead of the concentration procedures cited above, other conventional biochemical refinement procedures may be used, such as precipitation or fractionation with polyethyleneimine, organic solvents or salts, by chromatography through molecular sieve materials or weak anion exchanger with functional groups other than diethylaminoethanol groups, by protamine sulfate precipitation and the like.

EXAMPLE 3

To 0.5 ml of serum in the one case and cholesterol standard in the other, 1.0 ml of 0.5 M potassium phosphate pH 7.5 buffer containing 0.4% hydroxypolethoxydodecane, and 2.5 U of cholesterol esterase from Example 2 were added. This reaction mixture was incubated for 40 minutes at 37°C. Then 0.25 ml of this solution was added to 3 ml of cholesterol reagent containing two parts acetic acid, three parts acetic acid anhydride and one part sulfuric acid (Liebermann-Burchardt reagent).

By using a standard as a reference magnitude, 170 mg% total cholesterol was found in a typical specimen. Comparative determination after saponification of the cholesterol ester with alcoholic potash lye gave 165 mg%.

EXAMPLE 4

10 ml of 0.5 M potassium phosphate buffer containing 0.4% hydroxypolyethoxydodecane, and 0.2 U of the cholesterol esterase of Example 2, were added to 0.02 ml of serum. The reaction solution was incubated for 60 minutes 37°C. Then the extinction (E_1) at 240 nm was read in a suitable spectral photometer and the reaction was started with 0.1 U of sterol dehydrase obtained from *Brevibacterium sterolicum*. After fifteen minutes, the extinction (E_2) was again read. The concentration of the Δ^4 cholestenone and hence of the cholesterol was found from the difference between the first and second reading on the basis of the molar extinction coefficient for Δ^4 cholestenone at 240 nm. Measurement of a typical specimen gave 183 mg% total cholesterol.

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

EXAMPLE 5

10 g of diammonium hydrogen phosphate was dissolved in 100 ml of water and adjusted to pH 7.0 with 85% phosphoric acid. Then 10^5 units of catalase were added. The solution thus obtained was added to a mixture of 0.2 ml of acetyl acetone, 10 ml of methanol and 0.1 g of hydroxypolyethoxydodecane to produce a volume of 100 ml. To this solution, 2.5 units of cholesterol esterase from Rhizopus spec. (WS 90027) were added. 5.0 ml of the solution thus obtained was mixed with 0.02 ml of serum in the one case and 0.02 ml of a cholesterol standard solution containing 200 mg% cholesterol in the other. To aliquots of the serum-containing specimen and of the cholesterol standard-containing specimen 0.1 unit of cholesterol oxidase was added and the mixtures were incubated for 60 minutes at 37°C. Then the dye that was formed was measured photometrically at 405 nm on the basis of the specimen zero value.

Using a standard as a reference magnitude, the cholesterol content of the serum-containing specimen amounted to 154 mg% total cholesterol. The control determination performed with cholesterol esterase from *Candida rugosa* ATCC 14830 instead of cholesterol esterase from Rhizopus spec. (WS 90027) gave the same value.

It will be understood that the specification and examples are illustrative but not limitative of the present invention and that other embodiments within the spirit and scope of the invention will suggest themselves to those skilled in the art.

What is claimed is:

1. Method of determining total cholesterol or bound cholesterol in a sample, which method comprises treating said sample with cholesterol esterase obtained from a micro-organism, thereby releasing the bound cholesterol, and then determining the resulting cholesterol content of said sample using a standard determinatiion.

2. Method as claimed in claim **1** wherein said sample contains only bound cholesterol and the said determination determines the amount of said bound cholesterol.

3. Method as claimed in claim **1** wherein said sample contains free cholesterol and bound cholesterol and the said determination determines total cholesterol.

4. Method as claimed in claim **1** wherein said microorganism is *Candida rugosa* ATCC 14830.

5. Method as claimed in claim **1** wherein said microorganism is Rhizopus spec. WS 90027.

6. Method as claimed in claim **1** wherein said microorganism is Aspergillus spec. WS 90030.

7. Method as claimed in claim **1** wherein said microorganism is

Actinomyces aureoverticillium	WS 90002
Actinomyces cyaneofuscatus	WS 90003
Actinomyces griseomycini	WS 90004
Actinomyces longispurus-fl.	WS 90005
Actinomyces malachiticus	WS 90006
Actinomyces roseolus	WS 90007
Actinomyces toxytricini	WS 90008
Actinomyces variabilis	WS 90009
Streptomyces spec.	WS 90010
Streptomyces autotrophicus	WS 90011
Streptomyces canescens	WS 90012
Streptomyces chartreusis	WS 90013
Streptomyces michiganensis	WS 90014
Streptomyces murinus	WS 90015
Streptomyces hachijoensis	WS 90016
Streptomyces caelestis	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
Penicillium spec.	WS 90028 or
Aspergillus spec.	WS 90029.

8. Method as claimed in claim **1** wherein said determination is an enzymatic determination.

9. Method as claimed in claim **8** wherein the enzyme used for said enzymatic determination is cholesterol oxidase.

10. Method as claimed in claim **9** wherein said cholesterol oxidase is from *Nocardia erythropolis* ATCC 17895, *Nocardia erythropolis* ATCC 4277, *Nocardia formica* 14811 or *Proactinomyces erythropolis* NCIB 9158.

11. Method as claimed in claim **1** wherein said microorganism has been cultivated on a cholesterol ester containing nutrient medium.

12. Reagent composition for the determination of cholesterol in a sample, with composition comprises cholesterol esterase obtained from a microorganism and a system for the determination of free cholesterol.

13. Reagent composition as claimed in claim **12** wherein said composition comprises

a cholesterol esterase from a microorganism,

cholesterol oxidase,

a system for the determination of hydrogen peroxide.

14. Reagent composition as claimed in claim **13** wherein said microorganism is *Candida rugoas* ATCC 14830, Rhizopus spec. WS 90027 or Aspergillus spec. WS 90030.

15. Reagent composition as claimed in claim **12** comprising

cholesterol esterase,

cholesterol oxidase,

catalase,

acetyl acetone,

methanol,

and a buffer containing ammonium ions.

16. Reagent composition as claimed in claim 14 containing
peroxidase,
a chromogen,
a buffer,
a system for the determination of H_2Oh_2.

17. Reagent composition as claimed in claim 12 wherein said composition comprises
a cholesterol esterase from a microorganism,
cholesterol oxidase,
a system for the determination of cholestenone.

18. Reagent composition as claimed in claim 17 wherein said system for the determination of cholestenone is a hydrazine derivative reacting with keto groups, to result in the formation of hydrazone.

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