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(57) Abstract

Method for rapid determination of biochemical data for microorganisms. Microorganisms in a sample are bound to an adsorbent, which usually is a carrier, provided with antibodies corresponding to the microorganisms that shall be adsorbed. After washing for removing of all non-specifically bound cells a nutritient medium is added in order to start the metabolism of the cell. For this metabolism certain substances must be present, and products are produced simultaneously. This fact is utilized for the determination of the amount of microorganisms, for instance by measurement of the amount of consumed oxygen, produced carbon dioxide, change of pH-value or electrical conductivity in the nutritient medium. Calibration curves are used for the determinations. The method can also be used for the determination of the concentration of biochemical substances, which influence the metabolism of the cell, for instance vitamins and antibiotica. At the determination of the vitamin content, a microorganism that can only metabolize when the vitamin in question is present, isadded to the adsorbent. The degree of metabolism is dependent on the amount of vitamin, which can be determined by comparison with a calibration curve. At the determination of inhibiting substances, like antibiotica, a predetermined amount of a predetermined microorganism is added to the adsorbent. The sample containing antibiotica is added and any change of the products from the metabolism, for instance in the form of a pH-value change is observed. The amount of antibiotica in the sample can be determined by comparison with a calibration curve that has been obtained with known amounts of antibiotica.

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A method for the determination of biochemical data.

The present invention concerns a method to the determination of the content of microorganisms and species of microorganisms in a sample. There are already several different procedures to determine species and amount, but a common drawback with all these is that they require long time, often several days to make one determination. So far, one has used a procedure involving plating, which means spreading on a plate or glass surface of a layer of substrate to which is added the microorganisms to be determined concerning number and species. At first nothing is seen in the medium, but later on the microorganisms divide and eventually form a number of colonies that are observable by the naked eye. Based on the assumption that one cell creates one colony, the number of microorganisms are allowed to divide, the species of the microorganism can be determined. Another procedure to determine amount and kind of microorganism is to use specific sorbents containing antibodies. Such sorbents immobilize a predetermined species of microorganisms against which the antibodies are directed. When the cells in this way have been captured they can be incubated in a growth medium, and when the microorganisms through division have given rise to a larger group of cells that is observable by the naked eye, the amount of microorganisms can be determined by colony counting. The choice of substrate to which the adsorbent with the microorganisms are added gives an indication of the kind of microorganisms that has been adsorbed, since only certain microorganisms are able to grow in a certain substrate, while

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other need another substrate for their growth.

The present invention is aimed at creating a system of procedures by which it will be possible to determine biochemical data concerning microorganisms within a short period of time, in many cases within two hours.

The procedure is based upon the use of an adsorbent directed against a preselected microorganism, where the sample from which the microorganism is bound, is washed after the adsorption together with the sorbent in order to eliminate all non-specifically bound cells. The washed microorganisms bound to the sorbent are exposed to a substrate so that the cell metabolism is started. For this metabolism there is a need for certain substances concomittant with an excretion of products.

The quantity of generated above mentioned products is fully related to the number of adsorbed cells. One or more products may be utilized either directly or indirectly to determine the amount of microorganisms. The species of the microorganisms is determined by the adsorbent due to its specific binding properties.

The adsorbent is as a rule a carrier provided with antibodies directed against the microorganisms that shall be adsorbed. If it is desirable to adsorb all microorganisms then as an alternative either mixtures of antibodies or other binding structures with a broader specificity, eg. lectins are used.

The above mentioned substrate and products may be eg. consumption of oxygen due to metabolic activity. The

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amount of oxygen consumed can be determined with established measuring equipment.

The other parameters may be pH of the substrate-medium. The pH-value can be determined by using a pH-indicator and fotometric equipment.

Still another parameter is reduction equivalents formed during the metabolition. These reduction equivalents are uncoupled from the ordinary metabolism by eg. artificial electron acceptors, the degree of reduction of which can be determined by established technique.

Still another parameter may be the amount of carbon-dioxide generated during the metabolism, as this amount can be determined by using established analytical instruments.

Moreover, for certain types of cells specific metabolites may be excreeted, the concentration of which in the medium can be used to determine the number of cells bound to the sorbent. Analysis is in such a case performed with a dedicated specific analysis developed for the individual metabolites.

Still another physical property of the medium that is changed during the metabolic events is the conductivity. By measuring same and the changes in conductivity over a certain time of incubation, the quantity of metabolizing cells or the degree of metabolic activity in a fixed number of cells can be determined.

The procedure according to the present invention can furthermore also be used to determine the concentra-

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tion of certain biochemical products that in a clear way influences on the metabolism of the microbial cells.

Examples on such substances are vitamins and antibiotics. Analyses of such substances are carried out using predetermined numbers of a specific microorganism which is bound to the sorbent.

When assaying the content of a vitamin in a nutrient, then microorganisms are bound to the sorbents that for their metabolism have an absolute requirement for the actual vitamin. The sample to be analysed is mixed with a full medium for the cells from which the substance to be analysed for has been omitted. Thus, to the washed sorbent with adsorbed microorganism the substrate that is to be analysed is added. The degree of metabolic activity is dependent upon the amount of the vitamin present. By measuring metabolic activity for the cell preparations at known concentrations of vitamins, the metabolic activities registered can be used as reference values when the content in an unknown sample is to be evaluated from the calibration curve.

The procedure according to the invention can also be used to determine the influence by inhibiting substances on cell metabolism, eg. antibiotics. In this case the adsorbent is charged with a certain microorganism in a predetermined amount. Antibiotics are introduced together with a nutrient medium to a washed adsorbent with adsorbed microorganisms. After metabolic activity has been on for a present period of time, the change in concentration of metabolites can be read eg. as a change in pH-value and thus it is possible also to

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determine the amount antibiotics in the sample by comparative studies with a calibration curve which is obtained with known concentrations of antibiotics.

Additional properties valid for the present invention are found in the later presented claims.

In the following the present invention shall be described more in detail reference being made to four figures wherein

Fig. 1 shows a calibration curve for a predetermined microorganism, where the product generated, here read as change in absorbance, is plotted versus the number of microorganisms, N;

Fig. 2 shows a calibration curve valid for a predetermined vitamin, with the concentration of vitamin C plotted against measured change in absorbance after a given period of incubation of a predetermined number of cells;

Fig. 3 shows a calibration curve valid for a predetermined antibioticum and where the absorbance read is plotted versus concentration of antibiotica in the sample A;

Fig. 4 shows a simple unit for practically performing the present invention.

In the present invention it is essential pro primo, to use an adsorbent to which cells are adsorbed, pro secundo to have a washing unit where the adsorbent with bound cells is washed to remove all unspecifically bound material and pro tertio to have a unit where adsorbed cells are supplied with nutrients so that cell metabolism can take place. This three-step system must, in order to be useful, be calibrated and this is done

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by construction calibration curves out from the behaviour of known amounts of known microorganisms. By constructing the curves, one uses the changes in physical parameters that take place as a result of the metabolism of the cells. These parameters may be acidity (read at pH-value), oxygen tension, redox-level, conductivity, concentration of a certain metabolite, carbon dioxide pressure etc.

One of the most useful parameters is the pH-value. To easily determine this a pH-electrode may be used, but in most cases a coloured pH-indicator has been used, the colour of which is related to the pH-value in the solution. Such pH-indicators may be of very different chemical structures. Several different ones have been used in this system. Examples on suitable substances are heptametoxired and phenolred. Usually the indicator is added to the nutrient medium and the used nutrient medium is analysed, usually spectrophotometrically. After the above mentioned three step procedure has been calibrated against known quantities of the species to be analysed, the analytical system can be used to primarily determine amount of a certain microorganism in a sample. The capture of the above mentioned predetermined microorganism is carried out by the use of a carrier-substance to which antibodies are bound and directed against the microorganism that shall be investigated. As carrier is usually used a gel with a low tendency of unspecific adsorption of cells and other biological material. A frequently used carrier in such cases is Sepharose. The carrier must be modified by capting an antibody to it - an antibody with specificity for the microorganism that shall be quantified in the analytical procedure. In certain cases it may also be of interest to collect the whole spectrum

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of microorganisms, and then a sorbent with ability to generally adsorb microorganisms must be used. Such an adsorbent can be based on a binding structure of lectin-type, which is well-known for, in many cases, to have a much broader substrate specificity than what specific antibodies usually have.

If in a milieu, the amount of a certain microorganism is to be quantified, then an adsorbent is produced suitable for binding the specific microorganism. The adsorbent with its binding structures, either they are lectins or antibodies, is allowed to be in contact with the milieu where the microorganisms are present. When the adsorbent has been exposed for microorganisms for a given period of time, the sorbent is washed to remove unspecifically bound cells.

The thus washed sorbent with microorganisms is supplied with nutrient, which can be a sugar solution with suitable buffering substances and eg. a pH-indicator, or some other liquid medium, in which the cells that shall be quantified can metabolize.

When the adsorbent with the captured microorganisms is exposed to nutrient medium, metabolism starts and is allowed to proceed for a fixed number of minutes, usually 30-120. After incubation in nutrient medium analysis is performed of the medium, usually by a spectrophotometer. Based on the value read in that analysis, it is then possible, by consulting a calibration curve (Fig. 1), to determine the number of microorganisms of the selected species in relation to the numbers used as references when setting up the calibration curve for a selected organism. In the case here described has, as a parameter, been used changes in pH-value as a result of metabolic activity. In meta-

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bolism there is in many cases a demand for externally added oxygen. The amount of oxygen consumed can be determined and from this value it is possible to determine the number of cells by using a calibration curve similar to that in Fig. 1. In a similar way the number of redox-equivalents as well as the amount of carbondioxide generated by the metabolic activity can be determined. These values are directly related to the number of microorganisms.

The way in which the number of microorganisms discussed so far are quantitated is generally by using laboratory equipment often well-known to the chemists. However, it is also possible to use the present invention in a more primite way. One prerequisite however is that a calibration curve of the type in Fig. 1 is available and that it is possible, by the use of charts with different colours or maybe spectrophotometry, to quantitate the change in colour that appears in the nutrient medium as a result of metabolism by the investigated microorganisms. The simplified method for quantitation is illustrated in Fig. 4 with the subfigures 4a - 4f.

In these figures is shown a disposable plastic syringe 5, with a piston 6, and a piston handle 7. Just in front of the tip of the syringe is placed a net 8, eg. of nylon with holes of 25 µm. On top of the net is placed the adsorbent equipped with antibodies or other binding structures directed against the microorganism to be quantified. The suringe 5 is dipped into a vessel 9, containing a fluid with microorganisms to be quantified. The fluid with the microorganisms is sucked into the syringe 5, where the adsorbent captures that amount of microorganisms that it is capable of

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under the conditions used. Then the liquid is removed together with non-bound microorganisms and other impurities. This is done according to a washing procedure as illustrated in Fig. 4c. In 4d a nutrient medium is sucked into the syringe and mixed with the sorbent containing captured microorganisms. The nutrient medium may consist of a buffered glucose solution with suitable indicator-molecules or other substances.

After a sufficient amount of nutrient medium is drawn into the syringe 5, it is removed from the vessel 11. The nutrient medium is kept in the syringe 5, and is allowed to react for a certain time, usually between 30 and 120 minutes, sometimes shorter or longer. After this time period the syringe 5 is emptied of nutrient medium. The nutrient medium is added to a vessel 12 that preferentially is transparent. By measuring the colour of the nutrient medium the amount of microorganisms in the sample 9 can be determined.

The procedure according to the present invention can also be used to determine concentration of vitamin in a substrate. Hereby, the procedure involves the selection of a microorganism that needs external supply of the vitamin of interest in order to be capable of maintaining metabolism. The adsorbent with such microorganisms is washed in the usual way and is then exposed to a nutrient medium that is a complete medium with the exception of the vitamin to be quantified, mixed with the sample to be analysed. The degree of metabolic activity is related to the content of vitamins in the sample. Also in this case changes in pH-value can be utilized for determining metabolic activity and thereby via a calibration curve also the vitamincontent. To simplify the procedure a calibration

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curve must be constructed according to Fig. 2 which can be used to evaluate the vitamin content by the measured colour-intensity.

The assay unit according to the present invention can also be used to determine the concentration of a known antibioticum by studying the influence by various samples containing antibioticum on the metabolism of various microorganisms. In this procedure, the adsorbent is exposed to, and thus can adsorb a predetermined number of microorganisms. After washing of the exposed adsorbent to remove all cells not properly bound it is treated with a nutrient medium in combination with an antibioticum. Metabolism starts operating, but it will be inhibited due to the fact that the antibioticum to a certain extent inhibits the activity of the microorganisms. By measuring after a certain time of exposure, which usually is between 30 and 120 minutes, the colour intensity of the nutrient medium can be read and thereby the influence of the antibioticum can be evaluated. In Fig. 3 is shown a calibration curve obtained from using a certain number of cells incubated in media containing varying concentrations of the antibioticum that shall be quantified. Translation of values obtained on unknown samples can be done by using a calibration curve. As a further information from such a test, the pattern of resistance for the microorganism can be determined.

All cells contain ATP. This fact can be used to determine the number of cells in a sample. By using the above mentioned adsorption technique the cells are bound to a specific adsorbent. The bound cells are then treated so that ATP is released to the medium surrounding the sorbent. The amount of released ATP

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has empirically been shown to be correlated to the number of cells. By addition of the enzyme-substrate system luciferas --- luciferin a light signal can be obtained that can be related to the amount of ATP in the sample and thus to the number of cells in the primary sample.

This latest described application concerns still another important area of application of general interest, namely concentration of cells out of a sample to a small volume in order to obtain a sensitive and accurate analysis.

Example 1 (counting of yeast cells)

A disposable plastic syringe (2 ml) equipped with a 25 µm-net in front of the outlet is supplied with 1 ml Concanavalin A-Sephadex when 1/2 ml of sedimented Concanavalin A Sepharose (either prepared by ourselves according to established procedures or bought from Pharmacia Fine Chemicals AB, Uppsala, Sweden) is suspended in 2 ml buffer, pH 7.4 (0.004 mM KH_2PO_4, 0.054 mM Tris, 0.41 mM $NaHCO_3$, 0.95 mM $CaCl_2$, 0.80 mM $MgSO_4$, 5.36 mM KCl, 137 mM NaCl) and poured into the syringe. The piston is then introduced and the air is emptied before the excess of liquid is forced out. The sample to be analysed is sucked into the syringe. A total of 2 ml of sample is sucked in. Incubation for 10 minutes to allow the cells to bind to the gel. During incubation the content in the syringe is mixed gently by turning the syringe end-over-end with time intervals of 1-3 minutes. After this incubation, the solution is forced out of the syringe and 2 ml of buffer is sucked in. The solution is forced out again. This washing procedure is repeated 4 times

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before 2 ml of substrate is sucked in after which the syringe is shaken so that a homogenous suspension is obtained. The excess of substrate is emptied so that 0.8 ml (total volume = substrate + gel) remains in the syringe.

The substrate solution consists of buffer according to the composition given above supplied with glucose to a final concentration of 50 mM and neutral red to a final concentration of 0.2 mg/ml. By the addition of indicator to the medium pH must be adjusted to the initial value.

Incubation for 2 hrs in a water bath at 26° C. During this time period mixing is achieved by intermittent shakings of the syringe.

After the incubation period, the substrate solution was forced out of the syringe and collected in collecting vessel. The absorbance at 528 nm was read.

cell number	change absorbance
$4\text{-}10^4$	0.054
$7\text{-}10^4$	0.057
$1.1\text{-}10^5$	0.078
$1.5\text{-}10^5$	0.085
$2.2\text{-}10^5$	0.088
$2.5\text{-}10^5$	0.095
$7.5\text{-}10^5$	0.107
$1.0\text{-}10^5$	0.119

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Example 2

Concentration and counting of yeast cells in various diluted solutions

In this example a 2 ml disposable plastic syringe was used in which 0.5 ml Concanavalin-S-Sepharose had been packed according to the procedure described in the previous example. This small column was used in this example of application as an adsorbent through which the solution to be analysed was drawn with a constant speed. Standard solutions with varying dilutions of yeast cell suspensions were prepared by diluting a yeast cell suspension with the buffer described in example 1.

The volume of the sample sucked into the sorbent was adjusted with respect to the cell number, so that in total an equally large number of cells were introduced in the Concanavalin A-Sepharose column. When the cell suspension had been drawn through the column, incubation was allowed to proceed for 10 minutes thereby giving possibilities for the creation of strong so called multi-point attachment bonds. The experimental procedure followed that given in example 1,

In the experiment, except for the blank experiment, 6 milj cells were sucked into the sorbent.

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No of cells per ml	Absorbance at 528 nm
0	0.020
0.1 milj	0.080
0.2	0.116
0.6	0.115
1.2	0.120
6	0.122

Example 3

Quantitation of thiamin by yeast cells

The practical details in essence follow that given in example 1, however, with another buffer, substrate and indicator. Furthermore, the condition of the cells in the starting moment is quite different. They are grown on Difcos special medium for thiamine assay. (The cells were grown for two days according to Difcos manual).

Buffer: 0.1 M sodium acetate/acetic acid pH 4.5 containing also 0.95 mM $CaCl_2$, 0.81 mM $MgSO_4$, 5.36 mM KCl, 137 mM NaCl and 1.0 mM $MnCl_2$.

Indicator: Bromophenylblue dissolved in the same buffer, 4 mg/l.

Thiamine-solution: After capturing the cells by the Concanavalin A-Sepharose, the syringe was filled (2 ml/syringe) for 30 minutes with a thiamine solution or a sample solution where the content of thiamin was to be determined. Incubation at 37° C with regular mixing. The thiamin solution was afterwards washed out through 2 repeated washings with buffer.

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To each syringe was then added substrate, 50 mM glucose in buffer with indicator 4 mg/L. Incubation for 2 hrs at 37° C under gentle mixing. The substrate solution is emptied and A_{585} is read.

Thiamine µg/ml	A_{585}
0	0
0.1	0.0253
0.05	0.040
0.10	0.047
0.50	0.0585
1.0	0.062

Example 4

Quantitation of E. coli cell

The basic experimental procedure was the same as given in example 1.

Buffer used: $CaCl_2$ 0.95 mM, KCl 5.35 mM, NaCl 137 mM, $MgSO_4$ 0.80 mM, KH_2PO_4 0.004 mM $NaHCO_3$ 0.04a mM Tris 0.053 mM, pH 7.4.

Indicator: Neutralred 0.2 mg/ml substrate: 50 mM glucose in the above buffer.

In a 2 ml disposable syringe was added 0.5 ml Sepharose 4B to which was coupled antibodies with binding specificites against Hemophilus influencae, but also a certain activity against E. coli. The coupling technique used is known (C. Borrebaeck, J.Börjesson and B. Mattiasson, Clin. Chim. Acta 86, (1978) 267-278). Incubation for 20 minutes was used. After wash-

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ing with buffer incubation with substrate and indicator took place for 2 hrs at 37° C. Then the absorbance was read at 528 nm.

Number of cells	A_{528}
$1\ 10^6$	0.030
$2\ 10^6$	0.058
$3\ 10^6$	0.080
$4\ 10^6$	0.103
$5\ 10^6$	0.110

Example 5

Quantitation of amphotericin

Quantitation of amphotericin is done by measuring the inhibiting effect of amphotericin on metabolic response from immobilized cells.

The procedure follows example 1, with the exception that 6 ml cells were used and exposed to varying concentrations of amphotericin for 20 min prior to incubation in substrate.

Amphotericin µg/ml	A
0	0.067
2	0.055
4	0.051
8	0.042
16	0.038
32	0.041

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Claims

1. A method for the determination of biochemical date, c h a r a c t e r i z e d in that a carrier, provided for instance with antibodies and or lectins with a binding capacity for a certain microorganism or other unicellular structure or group of organisms is utilized for immobilization of microorganisms, in that microorganisms are added to said carrier, whereupon carrier with biospecifically binding structures with bound microorganisms is washed, whereupon washed carrier with biospecifically bound microorganisms is fed with a nutrient medium, the metabolism being started at this addition of nutritient medium, products being formed as a result of this metabolism, the products being enriched in the medium, giving rise to a change of the physical-chemical properties of the medium, which change is utilized for obtaining chemical data.

2. A method according to claim 1 for the determination of the amount of microorganisms or other cells in a milieu, c h a r a c t e r i z e d in that immobilized lectins are used as adsorbent.

3. A method according to claim 1 for the determination of a predetermined microorganism or other cell, c h a r a c t e r i z e d in that immobilized antibodies, corresponding to the predetermined type of cell are used as adsorbent.

4. A method according to claim 1, wherein the concentration of vitamin or other chemical substance, essential for the metabolism of a certain type of cell, in a predetermined sample shall be determined, c h a r a c t e r i z e d in that a microorganism or other

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type of cell is chosen that demands the addition of the chemical substance to be assayed for its metabolism that the adsorbent utilized is formed by immobilized antibodies to the organism chosen, or other binding structures which can be utilized to bind cells biospecifically, that the nutritient medium in excess of containing the sample to be assayed for the concentration of a certain chemical substance, also contains all the other substances that are essential for the full metabolism of the cell, and that the metabolic activity of the cell during exposition to this medium can be related to the concentration of chemical substance in the medium and thus make possible the determination of the concentration of the specific chemical substance of the nutritient medium.

5. A method according to claim 1, wherein the effect on microorganism of antibiotica or other substance, acting inhibiting on cell metabolism/cell growth can be utilized for the determination of the concentration of the inhibiting substance, c h a r a c t e r i z e d in that a predetermined amount of the also predetermined microorganism or other type of cell is fed to the carrier, provided with antibodies directed toward the chosen organism, that the immobilized cell preparation is exposed to a predetermined antibioticum or other inhibiting substance, either simultaneously with the addition of the nutritient medium or in a separate step preceding this one, and that the cells metabolize at the addition of the nutritient medium.

6. A method according to any of the preceding claims, c h a r a c t e r i z e d in that the change of pH-value in the medium, as a result of metabolic activity, is determined, preferably by aid of an indicator.

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7. A method according to any of the preceding claims, c h a r a c t e r i z e d in that oxygen, needed for the metabolism is utilized and that the reduction in oxygen pressure in the medium can be determined.

8. A method according to any of the preceding claims, c h a r a c t e r i z e d in that reduction equivalents, formed during the metabolism are conveyed to special red-ox-indicators in the medium and that the change in red-ox-status on these can be utilized for the determination of metabolic activity and thus also the number of immobilized cells.

9. A method according to any of the preceding claims, c h a r a c t e r i z e d in that the amount of carbon dioxide, formed at the metabolism of the nutrient medium, is utilized for the determination of the amount of microorganisms on the adsorbent and thus also in the original sample.

10. A method according to any of the preceding claims, c h a r a c t e r i z e d in that the metabolism of the immobilized cells cause an accumulation of metabolites, which in turn change the physical-chemical properties of the medium, and that these changed properties can be utilized for the determination of the degree of metabolism and from this either the number of cells or in the case of identical numbers, the degree of metabolic activity of the different cell preparations, and from this the degree of stimulating or inhibiting effect, which is caused by special chemical substances, present in the medium.

11. A method according to any of the preceding claims, c h a r a c t e r i z e d in that products formed at

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the metabolism have electrical charge and that the accumulation of these products in the sample cause a change of the electrical conductivity of the medium, and that this change is related to the degree of metabolic activity and thus also to the number of cells or to the degree of metabolism at a constant number of cells.

12. A method according to any of the preceding claims, c h a r a c t e r i z e d in that at the metabolism of the medium one or a plurality of specific product is/are formed, which are emitted from the cell and which are enriched in the medium, where the concentration of these products indicate the degree of metabolic activity and thus the number of the cells or the degree of the metabolism in the case of constant cell number.

13. A method according to any of the preceding claims, c h a r a c t e r i z e d in that an adsorbent is utilized for enriching cells to a small volume of adsorbent before these cells are treated to emit a specific or some specific substance/s to the medium, which substance/s thereupon can be quantified and thus give information about the number of cells that have been enriched to the adsorbent.

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AMENDED CLAIMS
(received by the International Bureau on 15 October 1982 (15.10.82))

(new) 1. A method for determination of biochemical data for certain species of microorganisms or a certain microorganism or unicellular organism, an adsorbent in the form of a carrier provided with lectins and/or antibodies with biospecific binding power structures with the ability to bind said organisms being brought to bind said organisms from a mixture of microorganisms and possibly unicellular organisms, whereupon the adsorbent and bound organisms are separated from non-bound organisms and is then brought into contact with a substrate in order that the metabolism of the organisms is started, c h a r a c t e r i z e d in that the chemical or physical properties of the products, being produced by the metabolism, are determined.

(new) 2. A method according to claim 1, c h a r a c t e r i z e d in that a carrier provided with immobilized lectins is utilized as adsorbent.

(new) 3. A method according to claim 1, c h a r a c t e r i z e d in that a carrier provided with immobilized antibodies is utilized as adsorbent.

(new) 4. A method according to claim 3, utilized with the object to determine the concentration of a certain substance in a sample, c h a r a c t e r i z e d in that a microorganism or unicellular organism is

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chosen, the metabolism of which is influenced, positively or negatively by this substance, the sample being added to the utilized substrate, which preferably does not contain any amount of said substance, in excess of what is added by the sample, whereby the determination of the chemical or physical properties of the products produced by the metabolism will indirectly determine the concentration of said chemical substance.

(new) 5. A method according to any of claims 1 - 4, c h a r a c t e r i z e d in that the pH-change, caused by the metabolism, is determined.

(new) 6. A method according to any of claims 1 - 4, c h a r a c t e r i z e d in that the oxygen consumption, caused by the metabolism, is determined.

(new) 7. A method according to any of claims 1 - 4, c h a r a c t e r i z e d in that the red-ox status is determined for the reduction-equivalents that are produced by the metabolism.

(new) 8. A method according to any of claims 1 - 4, c h a r a c t e r i z e d in that the CO_2 produced by the metabolism is determined.

(new) 9. A method according to any of claims 1 - 4, c h a r a c t e r i z e d in that the change in electrical conductivity, caused by the metabolism, is determined.

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INTERNATIONAL SEARCH REPORT

International Application No PCT/SE82/00193

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC 3

C 12 Q 1/00

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
IPC 3	C 12 Q 1/00,02,04,18,24, G 01 N 33/48,50,54, 27/26,00
US Cl	195:103.5; 204:1E, 195B; 435:29,30,32,34,4

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
	DE, A, 2 826 416 (BOEHRINGER MANNHEIM GmbH) 20 December 1979	1-13
	Methods in Enzymol., Vol 34, pp 220-25, in particular p 224, 2nd paragraph, published 1974 (Ed. JAKOBY W B, WILCHEK M)	1-13
	US, A, 3 843 324 (RESEARCH CORPORATION N.Y.) 22 October 1974	1-3
A	Proc. Nat. Acad. Sci. U.S.A., Vol 68, pp 2153-7, published 1971 (EDELMAN G M et al), "Cell Fractionation and Arrangement on Fibers, Beads, and Surfaces".	1-3
	US, A, 3 403 081 (TRW Inc) 24 September 1968	1, 4-12
A	US, A, 4 072 576 (AB KABI) 7 February 1978 & SE, B, 407 115	1

.../...

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understanc the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
1982-08-16	1982 -09- 19
International Searching Authority [1]	**Signature of Authorized Officer [20]**
Swedish Patent Office	Carl Olof Gustafsson

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
A	Chem. & Eng. News pp 29-35, published 27 January 1975 (RECHNITZ G A), "Membrane bioprobe electrodes".	1-13
A	WO, A1, 80/02849 (JOHNSTON LABORATORIES Inc) 24 December 1980	1,4,5,7
A	Biomed. Eng., Vol 9, pp 18-20, published January 1974 (UR A, BROWN D F J), "Rapid detection of bacterial activity using impedance measurements"	1, 4, 5, 11