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(54) SELECTIVE MEASUREMENT OF SOMATIC AND MICROBIAL CELLS

(71) We, SEPPO KOLEHMAINEN, a Belgian citizen, of Ganzenstraat 11, 3540 Zolder, Belgium, and VEIKKO TARKKANEN, a Netherlands citizen of Breulsweg 1, 5119 Wijlre, the Netherlands, 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:—

Introduction

This invention relates to selective measurement of the number or physiological activity of different types of viable cells through the utilization of surface active agents. Method is based on selective releasing of nucleotides from different types of cells and subsequent measuring of nucleotides by chemi- or bioluminescence.

Measurement of viable microbial and somatic (non-microbial) cells is of great importance in medical laboratories, veterinary science, food hygiene, fermentation industry and environmental studies. Bacteria, yeasts and fungi are measured either by colony counting in a growth media or by instruments, such as microscope, turbidometer, refractometer and like. Somatic cells are counted with particle counters, such as Coulter Counter (Coulter Electronics, Ltd.) and laser-based optical and fluorescence apparatuses, or indirect measurements through the quantity of metabolic products, such as nucleotides, or by microscopy. These methods require either complex, expensive equipment or are not accurate due to the interference of non-cellular particles or non-somatic cells. Furthermore, most conventional methods do not make a distinction between dead and viable cells. ("Coulter" is a Registered Trade Mark).

With conventional methods it has been difficult or time consuming to selectively determine different types of cells such as somatic cells and bacteria in a sample containing both types of cells. Furthermore, conventional methods especially in microbiology, are slow as the results are obtained only after one or several days. In clinical tests results should be obtained as soon as possible in order to properly treat the patient, and in food hygiene it is also important to determine bacterial contamination of products as rapidly as possible so that spoilage of raw materials and processed food can be avoided. Therefore, rapid alternative methods to conventional colony counting and somatic cell counting have been looked for.

Firefly bioluminescent measurement of adenosine triphosphate (ATP) (see US Patent No. 3,745,090) is a rapid and sensitive method for determining the number of somatic and bacterial cells in a sample. In this method the quantity of measured ATP is converted to number of cells as the ATP content of a specific type of cell is relatively constant. Similarly, other bioluminescent measurement, such as the photobacterium bioluminescence reaction can be used to determine the number of viable cells with the aid of flavine mononucleotide (FMN) and reduced form of nicotinamine dinucleotide (NADH).

In order to determine the number of bacteria in food stuff and body fluids, such as milk, urine, blood, central spinal fluid, saliva, the ATP, FMN, and NADH, in somatic cells, such as blood, muscle, and epithelial cells has to be eliminated. Likewise, the interference effect of nucleotides from microbial cells has to be avoided in order to accurately measure somatic cells. The utilization of bioluminescence methods for measurement of different types of cells has been limited by the lack of suitable sample preparation methods that enable the measurement of nucleotides somatic cells and microbial cells separately.

Selective measurement of somatic or microbial cells are needed in many fields

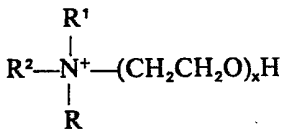
of science, medicine, hygienic control and industrial quality control. In these fields most samples fall within the following measuring conditions:

1. Measurement of somatic cells only in presence of microbial cells.
2. Measurement of total cells in the sample.
3. Measurement of microbial cells only in the presence of other cells.

According to the present invention there is provided a method of measuring adenosine triphosphate (ATP) selectively in somatic (non-microbial) cells and microbial cells which comprises contacting said cells with a surface active agent to release nucleotides, including ATP, through the cell wall and membrane and measuring the concentration of the released ATP by means of the firefly bioluminescence reaction.

In order to release ATP selectively from non-microbial cells there is preferably used a non-ionic surface active agent selected from ethoxylated alkyl phenols having 2 to 30 ethoxyl groups, and fatty acid polyglycol esters having from 2 to 20 ethoxyl groups, in a concentration of 0.02—0.5 percent.

In order to release ATP selectively from microbial cells there is preferably used an ionic surface active agent selected from ethoxylated amines, diamines or amides; polyethylene glycol esters derived from fatty acids having 8 to 18 carbon atoms and 2 to 50 ethoxyl groups, quaternary ammonium salts of the formula



wherein R^1 and R^2 are alkyl, alkyl-aryl-alkyl, ethoxyalkyl or hydroxyalkyl groups, the ethoxylated alkyl radicals having 2 to 15 ethoxyl groups and R is a methyl group or an ethyl group, and y is halogen, sulphite, or phosphate, said surface active agent being present in a concentration of 0.02—0.5 percent.

The present invention makes it possible to accomplish these measurements rapidly, simply and accurately. These different alternatives are shown in the accompanying examples.

Principle of Selective Measurement of Somatic and Microbial Cells

A. Sample Preparation

It is a known fact that surface active agents have been used to lyze single cells in order to eliminate ATP from somatic cells (U.S. Patent No. 3,745,090), but now it has been possible to develop a method of selectively releasing nucleotides through the cell wall from different types of cells with specific surface active agents in order to measure the concentration of nucleotides in living somatic or microbial cells, or to determine the number of viable cells in the sample.

In studies on metabolism of cells, in biochemistry, medicine and in studies related to living organisms the concentrations of nucleotides play an important role. Most of the known 2000 enzymes use nucleotides, such as adenosine and other nucleotide phosphates (ATP, ADP, AMP, GTP, ITP, etc.), flavine mononucleotides (FMN, $FMNH_2$), nicotinamide adenine dinucleotides (NAD, NADH, NADPH) as a substrate. Until now it has been difficult to release nucleotides from cells without allowing enzymes of cells to break down them, or to have nucleotides being affected by the treatment. Present methods are both laborious, expensive, slow, inaccurate, or difficult to do in a reproducible manner. Often the separation method dilutes the sample, introduces chemicals that interfere with analysis of nucleotides, or plainly introduce extra sources of errors due to numerous manipulations. Conventional methods include disruption of cells by mechanical or chemical means, and inactivating enzymes by freezing, heating or chemical means; and subsequent separation of nucleotides from sample by precipitation, liquid chromatography, thin layer chromatography, liquid extraction with organic solvents and other methods.

In the present invention the nucleotides are released from single cells or a single layer of cells through the cell walls by making the cell walls permeable by means of surface active agents. Nucleotides having small molecular size can penetrate the cell wall after a treatment with surface active agents, but enzymes having a large molecular size will not penetrate the cell wall and stay inside the cell. This allows the releasing of nucleotides to extracellular liquid without the breakdown of nucleotides by the cell's own enzymes. Red blood cells

(erythrocytes) are disrupted by the surface active agents, but released ATP can still be measured accurately if done within one minute, and preferably within 15 seconds after adding the surface active agent. Now the surface active chemicals can be selected by their specific properties, such as the alkyl chain length of the compound, the nonionic and ionic characters, degree of alkoxylation (degree of lipophility or hydrophility) and presence of special radicals, such as quaternary ammonium salts, thus the releasing of nucleotides can selectively be done from different types of cells. Consequently, nucleotides, such as adenosine phosphates, can be released selectively from somatic cells (non-microbial cells) by means of nonionic surface active agents while ionic surface active agents are used to release nucleotides from all types of cells. This selective release of nucleotides is an advantage over the previous used methods where no selective extraction can be made between different types of cells. Another important advantage of the present invention is that the release of nucleotides from single cells is fast, in less than 10 seconds. Furthermore, only 0.02—0.1% concentration of the surface active agent is needed to make the cell wall permeable and to release nucleotides, thus the sample is not diluted by the addition of the nucleotide releasing reagent. Surface active agents can be selected in such a manner that their presence will not interfere with the analysis of the nucleotides.

B. Measurement

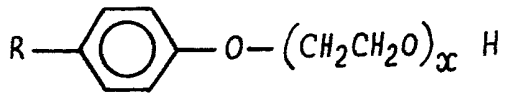
A known application of measuring concentration of nucleotides, such as adenosine triphosphate (ATP) and flavine mononucleotide (FMN) is the utilization of bioluminescence. With bioluminescence, extremely low concentration, such as 10^{-15} M of nucleotides can be measured (U.S. Patent No. 3,745,090). This high sensitivity enables the measurement of concentration of ATP in a single living cell. This analyzing principle has numerous applications in measuring the number of cells in a sample. The bioluminescence measurement is very rapid and sensitive, thus this measuring method provides an attractive way of rapidly measure bacterial, yeast, mold and somatic cells in hygieny, food industry, microbiology, clinical chemistry, virology, tissue culture, veterinary science and all fields of the life science. The present invention enables a rapid, simple, reproducible and complete releasing of nucleotides, including ATP, from all types of cells, thus the use of this invention makes it possible to apply bioluminescence measurements faster, more effectively and selectively than has been possible until now with the conventional extraction methods.

Example of Selective Measurement of Somatic and Microbial Cells

1. Measurement of Somatic Cells

The enumeration of living somatic cells is important in many biological fluids, such as blood samples (erythrocytes, leucocytes and platelets) and milk (leucocytes for mastitis determination). This is normally accomplished by means of electrical particle counters, such as instruments based on the change of electrical conductance of medium as cells pass through a small orifice, by direct microscopial counting, or by utilization precipitation of nucleotides or measuring nucleotide absorbance (California Mastitis Test and DNA-absorbancy for leucocytes). These methods are either subjective, require expensive equipment, or have inherent errors due to other particles than cells. Measurement of somatic cells in biological fluids with bioluminescence is both sensitive, fast and accurate. A nonionic surfactant detergent Triton X-100* has been suggested for lyzing somatic cells in order to have the non-bacterial ATP eliminated by an enzyme, called apyrase (U.S. Patent No. 3,745,090). However, Triton X-100 has not been suggested to be used for releasing of ATP in order to measure the content of ATP in somatic cells. Furthermore, Triton X-100 in a concentration of 0.1% lowers the light production of the bioluminescence reaction by about 27% and Triton X-100 does release ATP from some gram-positive bacteria. Now, it has been possible to develop a method to use specific surfactants to quantitatively and selectively release ATP from somatic cells without any inhibition on the bioluminescent reaction in order to measure the content of ATP in somatic cells. Such nonionic surface active agents include chemicals such as ethoxylated alkylphenols, e.g. octylphenols, having the formula:—

*Reg. Trade Mark of Rohm and Haas Co., Philadelphia, Pensylvania, U.S.A.



and fatty acid esters of polyglycol ethers having the formula:—

(fatty acid acyl) O—$(CH_2CH_2O)_xH$

where R is an alkyl group and x is 2—30. Microbial ATP does not interfere with the measurement as nonionic surface active agents can be chosen in such a way that they will not release ATP from microbial cells. The selective release of nucleotides, such as ATP, from somatic cells is performed by adding nonionic surfactant to the sample in a concentration of 0.01—2%, but preferably 0.05—0.2% of the sample volume. This concentration of nonionic surfactant, such as ethoxylated alcohols and phenols, which do not interfere with the bioluminescence reaction, release nucleotides from somatic cells into the extracellular liquid, such as water, buffer or salt solution within then seconds after mixing the surface active agent with the sample. The nucleotides are reasonably stable in the extracellular fluid if the release of enzymes is avoided. The nucleotide concentration can be measured immediately or after a few minutes after the addition of the surfactant, however, as some nucleotides, such as adenosine phosphates, can be autoxidated or broken down by bacteria in the sample, it is better to measure the concentration of the nucleotides within five minutes of the addition of the surfactant. After the cell wall is made permeable with a nonionic surface active agent, the small molecules travel towards the lower concentration gradient, ATP and other substrates diffuse out of the cells until the concentration is equal inside and outside of the cells resulting in a practically complete release of ATP (>99%) when the cell volume is less than 1% of the total volume of the sample. After the small molecules are released, the cell's own enzymes start utilizing the substrates that are left inside the cells. If samples are let stand over five minutes before measurement, the concentration of substrates, such as ATP, start decreasing inside the cells and the substrates being released from the cells start diffusing back into the cells (toward the lower concentration gradient). This may reduce the total concentration in 30 minutes 10—40%. Therefore, the measurement should be made within five minutes after applying the surface active agent. The concentration of cells must not be more than 10 million and preferably less than one million cells per milliliter to ensure complete and instantaneous release of substrates from somatic cells.

Measurement of ATP in Leucocytes in Milk

In order to determine ATP in leucocytes in raw milk the efficiency of different nucleotide extraction methods were compared. The results showed that the utilization of nonionic surface active agent (ethoxylated alkyl phenol) gave better results (Table 1) than conventional methods: perchloric acid extraction (1.0 N) and boiling for 1.5—10 minutes in 20 mM Tris (Tris-Hydroxymethyl — aminomethane) — 2 mM EDTA (ethylene-diamine tetraacetic acid) buffer. The release of ATP was complete and the use of ethoxylated alcohol in 0.1% of total sample volume did not interfere with the bioluminescence reaction. ATP concentration was measured by a luminescence analyzer by means of the firefly luciferin-luciferase reagent.

TABLE 1

The intensity of bioluminescent light emitted by ATP and firefly luciferin-luciferase reaction in 1 ml samples of raw milk containing living leucocytes.
A. With 0.1 ml M perchloric acid and neutralized with 0.1 ml 1 M KOH.
B. With 9 ml of boiling 0.02 M Tris — 0.002 M EDTA buffer pH 7.4 and,
C. With 1 ml 0.2% ethoxylated alkyl phenol.
In each case three 0.1 ml aliquotes of samples were measured.

	A Perchloric acid extraction	B Tris-EDTA extraction	C Nonionic surfactant (ethoxylated alkylphenol)
Relative Light Units	1480	36,200	55,300

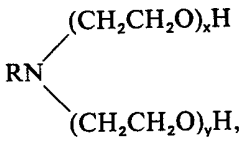
In a study (Tarkkanen, P. R. Driesch and H. Greiling. A rapid enzymatic micro-method for the determination of intracellular and extracellular ATP and its clinical-chemical applications. Analytische Chemie 290(2): 180, 1978) on different extraction methods of ATP from blood cells the nonionic surfactant (NRS*, Nucleotide Releasing Reagent for Somatic Cells) was compared to boiling tris method and to enthanol-EDTA (0.1 M) 9:1 mixture. Extraction of ATP with NRS was rapid, quantitative (96.8—102.2%) and gave ATP values that correlated well with conventional methods (r=0.92—0.93). NRS was simpler, more rapid and reproducible.

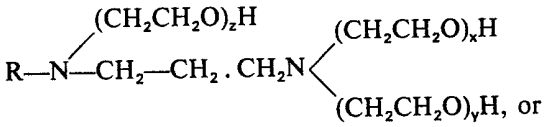
One great advantage in the utilization of nonionic surfactant is that some of them do not interfere with the enzymatic activity of the bioluminescence catalyzing enzyme, luciferase, thus these nonionic surfactants can be mixed together with the bioluminescence reagents. It is thus possible to combine all necessary chemicals required for releasing ATP from cells (nonionic surfactants) and for producing the bioluminescence light reaction (luciferase, substrates, enzyme activators and stabilizators, Mg-salt, and buffer all in one single reagent). In the present invention the sample preparation and measurement are extremely simple and lead easily for automation. Somatic cells can be measured with a manual instrument in less than 30 seconds including pipetting of sample, placing the sample in the photometer, dispensing the reagent and obtaining the relative light reading.

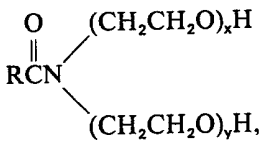
2. Measurement of Samples Having a Majority of Microbial Cells

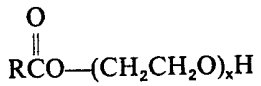
Samples having primarily microbial cells, e.g. activated sludge and microbial cultures, can be measured without prior elimination of ATP from non-microbial cells. The cell wall of bacteria has a polymer, called peptidoglycan that makes the cell wall very resistant towards outside chemical and physical interference. Therefore, it is more difficult to make bacterial cell wall permeable than that of somatic cells. The present invention makes it possible to affect the cell wall of bacteria in such a way that it becomes permeable for substances having a relatively small molecular size, such as nucleotides. This permeability is obtained by treating bacteria with ionic surface active agents, the best types being those that contain quaternary ammonium salts and fatty acid esters of polyglycol ethers, the fatty acids having preferably a chain length of 12 carbon atoms, but any chain length of carbon atoms between 4 and 22 can be used.

Ethoxylated amines, ethoxylated diamines, ethoxylated amides and polyethylene glycol esters of fatty acids having a chemical structure of





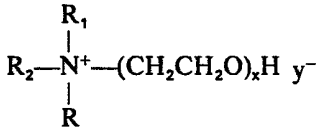




where

*Registered Trade Mark of Lumac Systems A. G. Basel.

is a fatty acid acyl group derived from fatty acids having 8—18 carbon atoms and x, y and z range from 2 to 50, and quaternary salts of ethoxylated amines may be represented by the formula:



where R_1 and R_2 are alkyl, alkyl aryl, ethoxylated amino, alkyl aryl alkyl, ethoxyalkyl or hydroxyalkyl group and R a methyl or ethyl group, x is 2 to 15 and y is, e.g. halogen, sulphate, sulfite or phosphate.

Such surface active agent can also be one of fluorinated hydrocarbons or hyamine chloride.

A quaternary ammonium salt is not necessary as cationic ethoxylated amines also release nucleotides from bacterial cells, but quaternary ammonium salts of ethoxylated amines having the carbon chain length of 12 work faster and are less affected by buffers, pH and other agents possibly encountered in the microbial samples. A mixture of 5 to 95% of cationic ethoxylated amine and 5 to 95% of quaternary polyoxyethylated amine can be used to obtain a rapid nucleotide release from cells without interfering with or inactivating luciferase enzymes used in the bioluminescent systems.

The rate of luminescence light reaction using firefly luciferin-luciferase reagents can be altered by changing the ratio between ethoxylated amine with chain length of 12 carbons and quaternary ethoxylated amine with a chain length of 12 carbons. The release of ATP and the bioluminescence reaction with ethoxylated amine alone is slow. Using a mixture of quaternary ethoxylated amine and ethoxylated amine the release is moderately slow or moderately fast depending on the proportion of the two surface active agents in the mixture. This effect is illustrated in the accompanying drawing in which the graph plots the magnitude of the light signal as a function of time after adding ionic surface active agent in bacterial sample. Numbers refer to the percentage of ethoxylated amine and quaternary ethoxylated amine, respectively in a total concentration of 0.1% in the sample. Both had a chain length of 12 carbons. *A* refers to the time of adding the surface active agent and *B* to the time of adding the firefly reagent. The more quaternary ethoxylated amine is present the faster is the release, enzyme kinetics and light reaction. A mixture of 70% ethoxylated amine and 30% quaternary ethoxylated amine gives a moderate rate of reaction and 1.2—2.0 times elevated relative light reading in the firefly bioluminescence reaction as compared to the same concentration of ATP in water without the surfactant mixture when measured with 10-second integration. It appears that this mixture increases the turnover rate of the luciferase enzyme possibly affecting the hydrophilic site of the enzyme. More than 50% of quaternary salt of ethoxylated amine will reduce the activity of luciferase enzyme, thus its proportion in relation to ethoxylated amine has to be controlled.

Measurement of Microbial Cells

If a sample, such as pure bacterial culture, or mixed bacterial culture contain only bacteria or 1000 times more bacteria than other cells, the measurement of bacterical nucleotides is made by simply adding 0.01—2%, but preferably 0.05—0.2% of ionic surfactants, such as ethoxylated amines, and preferably having a quaternary ammonium salt as part of it, mixing this with the sample to release nucleotides through the cell walls. Then the sample can be placed in an instrument to have the nucleotide concentration measured with, for example, ATP with the firefly bioluminescence reaction. The light intensity produced by the reaction in the sample is directly related to the concentration of ATP or number of viable bacteria in the sample as shown in Table 2.

TABLE 2

Relative light intensity produced by ATP, released from different number of bacterial cells by the ethoxylated amine — quaternary ethoxylated amine in 0.1% concentration. Bacteria were suspended in 0.5 ml of physiological NaCl solution (0.9%) and measured with firefly bioluminescent method.

Number of bacteria in sample	Relative light units measured by a photometer
150,000	1480
500,000	4800
1,000,000	9580
2,000,000	18900

The extraction efficiency of the mixture of ethoxylated amine and quaternary salt of ethoxylated amine in 70:30 ratio in concentration of 0.1% of the volume of sample was compared to Tris (0.02 M) — EDTA (0.002 M) pH 7.75 boiling method and to perchloric acid extraction similarly to Table 1. Results of three replicate tests are given below:

	0.1% Ethoxylated amine and quaternary salt of ethoxylated amine in 70:30 ratio, respect.	Perchloric acid 0.1 M, neutralized with KOH	Tris-EDTA boiling buffer
Whole blood	100%	7%	80%
Escherichia coli Suspension 10^6 cells	100%	3%	95%
Chlorella sp. alga	100%	—	100%

The release of ATP from yeast cells and fungi with the mixture of ethoxylated amine and its quaternary salt is slower than the release of ATP from bacteria. From bacteria the release is complete in less than 15 seconds while from yeast and fungi cells quantitative release takes place in 30—60 seconds. For quantitative release the volume of cells must not be over 1% of total volume of sample. Release is not complete from thick sections of tissues and thick flockulates of microbes (over 1 mm^3) as the reagent does not rupture microbial cells but releases small molecules through the cell wall.

3. Measurement of Microbial Cells in Presence of Large Number of Somatic Cells

When the bacteria sample, from which released ATP is to be measured, contains non-microbial cells, the nucleotides of the non-bacterial cells have to be eliminated before the bacterial nucleotides can be measured. In earlier firefly bioluminescence methods this is done by lyzing the non-bacterial cells by a nonionic surfactant Triton X-100, and adding an ATP-ase enzyme, such as apyrase, to the sample to break down the non-bacterial ATP. Later the bacterial ATP is measured by extracting ATP and inactivating apyrase by boiling Tris-EDTA buffer, organic solvents, bases, acids and other conventional methods, (see U.S. Patent No. 3,745,090).

The above mentioned method has some drawbacks: Triton X-100 does not quantitatively extract ATP from all types of non-bacterial cells. Furthermore, Triton X-100 releases ATP from gram-positive bacteria and it reduces the bioluminescent light reaction. The above mentioned method can lead to inaccuracies in measuring bacteria in body fluids and foods. When the sample is treated with boiling Tris-EDTA buffer to inactivate apyrase and to release ATP from bacterial cells (see U.S. Patent No. 3,745,090), it is greatly diluted lowering the sensitivity of the method.

Four improved methods of eliminating non-bacterial ATP from bacteria samples will now be described. The first method is as following: Non-bacterial nucleotides, such as ATP, are released by a nonionic surfactant, or other surface active agent that does not affect the permeability of bacterial cell wall and the sample 50 μl—100 ml, but preferably 50—1000 μl, is filtered through a membrane filter 0.1—2 μm, but preferably 0.2—0.45 μm of pore size. Non-bacterial ATP goes through the filter and is so eliminated while the intact bacteria are left on the filter. The filter is subsequently rinsed with buffer, physiological salt solution or distilled water, and placed in a transparent measuring vial, and ionic surfactant, such as the mixture of ethoxylated amine and quaternary ethoxylated amine in concentration of 0.05—2% is added to release the bacterial ATP and subsequently measured with the firefly bioluminescence reaction. In this method manipulations are simple and fast, and possible interfering substances besides the non-bacterial ATP, such as salts, coloured substances, enzyme inhibitors and like, are also removed. Furthermore, the sample does not get diluted, but actually concentrated, thus the sensitivity of the analyses is enhanced. When a flat bottom measuring vial, and a

filter, smaller in diameter than the inside diameter of the vial, is used, the filter membrane can be placed on the bottom of the vial, and as a result the filter will not intercept light between the liquid sample and the light detector.

The second method is as following:

The sample 0.1—100 ml, but preferably 0.5—10 ml is filtered through a 0.1—0.45 μm membrane filter until a thin film of liquid is left on the top of the filter. Filter should not go dry as this causes stress on microbes and reduces their ATP level from normal. Nonionic surface active agent, such as 0.01—1%, but preferably 0.1—0.2% solution of ethoxylated alkyl phenols is added in 0.01—10 ml, but preferably 0.1—0.5 ml volume on the filter. After 10—60 seconds the liquid is filtered to waste but without having the filter to get dry. The filter is rinsed one to three times with 0.1—10 ml of sterile water, physiological salt solution or suitable buffer to remove all non-bacterial ATP from the filter, cell debris, and walls of the filterholder, but during the filtering of the rinsing solution the filter must not get dry. After rinsing the filter is covered with 0.05—10 ml but preferably 0.1—1 ml of 0.01—1%, but preferably 0.05—0.2% solution of ionic surfactants, preferably with a mixture of an ethoxylated amine and quaternary ammonium salt of ethoxylated amine in a 95:5—5:95, but preferably in a 70:30 ratio. The chain length of 12 carbons is preferred in the ethoxylated amine. This reagent is allowed to affect the microbial cells on the filter for 10—30 seconds and the solution containing ATP released from microbial cells is filtered to a test tube or directly into a measuring cuvette. The filter and filterholder is rinsed with 0.05—10 ml, but preferably with 0.1—0.5 ml of distilled water into the same container as the solution of ionic surfactant with ATP. Now the sample is ready for measurement.

Another method of eliminating non-bacterial nucleotides is centrifugation. Nucleotides of somatic cells are released by a nonionic surface active agent, such as ethoxylated alkyl phenols and the bacteria are sedimented by centrifugation for 1—20 minutes of 3000—20,000 g. The supernatant with released non-bacterial nucleotides is discarded and the bacteria are resuspended with small volume of water, saline or buffer, and bacterial nucleotides released by addition of ionic surface active agents, such as ethoxylated amines in concentration of 0.05—2% of total volume. Immediately afterwards the concentration of bacterial nucleotides can be measured with a bioluminescent method. High blank values (non-bacterial ATP) can be lowered by an additional washing with water, buffer or saline solution and subsequent centrifugation before the addition of ionic surface active agent and measurement of bacterial nucleotides.

The fourth method applies an ATP-ase enzyme, such as apyrase in immobilized form. The method is as following:

A sample of 0.05—10 ml, but preferably 0.1—1 ml, or a filter membrane, containing both somatic and microbial cells is treated with 0.05—10 ml, but preferably with 0.1—1 ml of 0.01—2%, but preferably with 0.05—0.2% nonionic surface active agent, such as ethoxylated alkyl phenols that will not interfere with the firefly bioluminescent reaction. This treatment releases the ATP from somatic cell in a few seconds. Immobilized ATP 0.1—10 units, but preferably 1 to 5 units is added in form of glass beads, a plastic stick, cellulose or other fibers, metal beads, latex or other macromolecule beads, or apyrase could be immobilized on the walls of the sample tube. Sample is incubated for 0.5—30 minutes, but preferably between 1 to 10 minutes at temperatures from 20—37°C with or without agitation to let apyrase to break down somatic ATP. After incubation an aliquot of 0.01—10 ml, but preferably 0.05—1 ml of sample is treated with 0.1—10 ml, but preferably with 0.05—1 ml of 0.01—1%, but preferably from 0.05—0.2% of ionic surface active agent, such as ethoxylated amine and preferably a mixture of ethoxylated amine and ethoxylated amine with a quaternary ammonium salt in ratio of 5:95% to 95:5%, but preferably in a ratio of 70:30% respectively. This releases microbial ATP in a few seconds, from bacteria in 10—15 seconds and from yeasts and fungi in 20—60 seconds quantitatively when the number of cells is less than 10^8 for bacteria and less than 10^6 per milliliter for yeasts and fungi. The concentration of microbial ATP can then be measured with the firefly bioluminescent reagent. The concentration of ATP is converted to number of cells of bacteria using determined values of ATP per cell for known species, or 0.3—2 femtograms ($0.4 \cdot 10^{-15}$) per cell for unknown mixed population of bacteria 130—170 femtograms per cell for brewers yeast as reported in the literature (D'Eustachio, A. J., D. R. Johnson and G. V. Levin. Rapid assay of bacteria populations. Bacteriol. Proc. 21:23-, 1968, and Thore, A., S. Ansehn, A. Lundin and S. Bergmann. Detection of bacteriuria by luciferase assay of

adenosine triphosphate, J. Clin. Microbiol. 1:1—8, 1975, and U.S. Patent No. 3,745,090).

This last alternative method leads easily to automation with already existing sample changer and dispensing units.

The use of apyrase enzyme is reported in the literature and the U.S. Patent No. 3,745,090, but it is not known to have it applied in immobilized form before. The use of immobilized apyrase eliminates the need to inactive apyrase by heating or chemical means and makes it possible to use ionic active agents, such as the ethoxylated amines to release ATP from bacteria. This procedure allows an easy automation both with flowthrough methods and discrete sample tube systems. The immobilized apyrase can be used for several weeks over and over again when washed with buffer and stored in a buffer at +4°C.

General Comments

The method of the invention, as disclosed herein, results in a saving of time when compared to conventional methods of extracting nucleotides, such as ATP for biochemical assays, specifically for the bioluminescent assay of ATP with the firefly lucifern-luciferase system. Furthermore, the measurement of ATP can be made selectively for somatic or microbial cells. Sample preparation is fast, effective and reproducible and the surface active agents can be selected in such a way that they will not interfere with the luciferin-luciferase bioluminescent reaction nor affect the stability of mucleotides such as ATP.

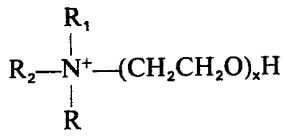
The principles, preferred embodiments, and modes of operation of the present invention are described in the foregoing specification. The examples of surface active agents, modes of operations and application areas described herein, are not to be construed as limited to the particular forms disclosed since they are to be regarded as illustrative rather than restrictive examples.

WHAT WE CLAIM IS:—

1. A method of measuring adenosine triphosphate (ATP) selectively in somatic (non-microbial) cells and microbial cells which comprises contacting said cells with a surface active agent to release nucleotides, including ATP, through the cell wall and membrane and measuring the concentration of the released ATP by means of the firefly bioluminescence reaction.

2. The method of Claim 1, wherein the release of ATP is selectively accomplished from non-microbial cells by means of nonionic surface active agents selected from ethoxylated alkyl phenols having 2 to 30 ethoxyl groups, and fatty acid polyglycol esters having from 2 to 20 ethoxyl groups, in a concentration of 0.02—0.5 percent and the concentration of ATP is measured by reacting the ATP with the firefly luciferase reagents in a photometer.

3. The method of Claim 1, wherein the release of ATP from microbial cells is accomplished by means of surface active agents selected from ethoxylated amines, diamines or amides; polyethylene glycol esters derived from fatty acids having 8 to 18 carbon atoms and 2 to 50 ethoxyl groups; and quaternary ammonium salts of the formula



wherein R_1 and R_2 are alkyl, alkyl-aryl-alkyl, ethoxyalkyl or hydroxyalkyl groups, the ethoxylated alkyl radicals having 2 to 15 ethoxyl groups and R is a methyl or ethyl group and y is halogen, sulphite, or phosphate, said surface acting agent being present in a concentration of 0.02—0.5 percent.

4. The method of Claim 1, wherein the release of ATP is accomplished from microbial cells in the presence of non-microbial cells by means of:

A. Treating the sample with nonionic surface active agents selected from ethoxylated alkyl phenols, and fatty acid polyglycol esters in a concentration of 0.02—0.5 percent to selectively release non-microbial ATP,

B. Adding 1—5 units/ml of an ATP-ase enzyme in immobilized form to the sample, and

C. Incubating the sample for 1—10 minutes at 20—37°C during which time ATP-ase hydrolyses non-microbial ATP, and

D. Removing the immobilized ATP-ase from the sample, or by taking an aliquot of the sample, and

E. Measuring the ATP level by reacting the ATP with the firefly luciferin-luciferase reagents in a photometer.

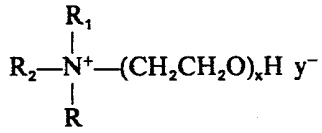
5. The method of Claim 1, wherein the release of ATP is accomplished from microbial cells in the presence of non-microbial cells by means of:

A. Treating the sample with a nonionic surface active agent selected from ethoxylated alkyl phenols, and fatty acid polyglycol esters in a concentration of 0.02—0.5 percent to selectively release non-microbial ATP, and

B. Filtering the sample after 10—60 seconds waiting time to eliminate the released non-microbial ATP, and

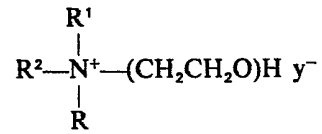
C. Washing the sample and filter unit with sterile water, buffer or saline to remove traces of non-bacterial ATP, and either

D1. Placing the filter membrane on the bottom of a measuring cuvette and adding surface active agent selected from ethoxylated amines, diamines or amides; polyethylene glycol esters derived from fatty acids having 8 to 18 carbon atoms and 2 to 50 ethoxyl groups; and quaternary ammonium salts of the formula



wherein R^1 and R^2 are an alkyl, alkyl-aryl-alkyl, ethoxyalkyl or hydroxyalkyl groups, the ethoxylated alkyl radicals having 2 to 15 ethoxyl groups, and R is a methyl or ethyl group atoms, and y is halogen, sulphite, or phosphate, to release ATP from microbial cells.

D2. Or alternatively pipetting onto the filter membrane, after washing the non-microbial ATP, with a surface active agent selected from ethoxylated amines, diamines or amides; polyethylene glycol esters derived from fatty acids having 8 to 18 carbon atoms and 2 to 50 ethoxyl groups; and a quaternary ammonium salt of the formula



wherein R^1 and R^2 are alkyl, alkyl-aryl-alkyl, ethoxyalkyl or hydroxyalkyl groups, the ethoxylated alkyl radicals having 2 to 15 ethoxyl groups, and R is methyl or ethyl carbon atoms, and y is halogen, sulphite, or phosphate in a concentration of 0.02—0.5 percent to release the microbial ATP, and after 10—60 seconds waiting time the solution of filter is filtered into the measuring cuvette or another container from which it can be pipetted into a cuvette,

E. Whereafter the ATP level of the sample treated according to D1 or D2 is measured by reacting the ATP with the firefly luciferin-luciferase reagents in a photometer.

6. A method of measuring ATP according to Claim 1 and substantially as hereinbefore described.

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