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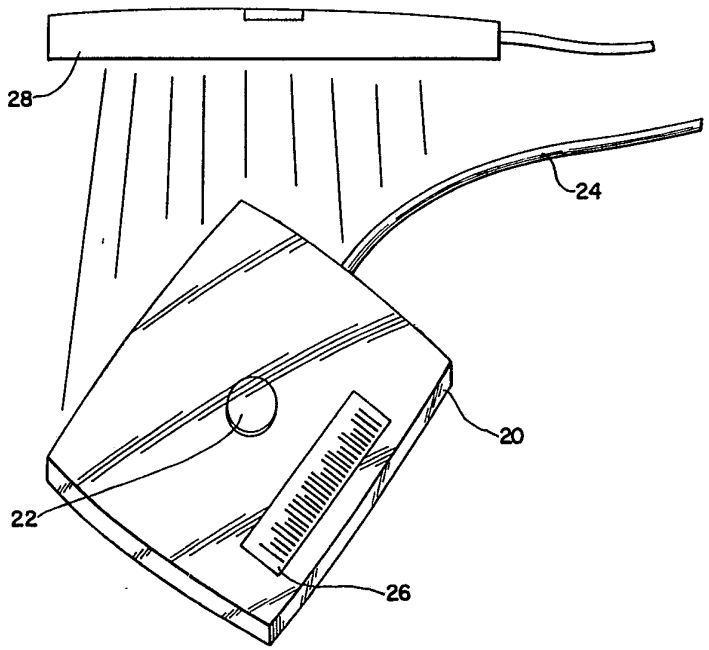

(57) Abstract

The present invention relates to a growth monitoring apparatus for collected transfusable bodily fluids. In particular the apparatus involves a flexible blood collection bag (20) or a sample bag (20) containing microbial growth media. A sensor (22) attached to the inside wall of the bag (20) is used to noninvasively detect microbial contamination within the bag (20). This invention also relates to a method to detect microbial growth in a blood collection bag (20) immediately prior to transfusion.

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METHOD AND APPARATUS TO DETECT BACTERIAL CONTAMINATION OF TRANSFUSABLE BLOOD

This is a continuation-in-part of U.S. Patent Application Serial No. 07/638,481, filed January 4, 1991; which is in turn a continuation-in-part of U.S. Patent Application Serial No. 07/609,278, filed November 5, 1990.

Field of the Invention

This invention relates to a noninvasive method and apparatus to detect the presence or determine the concentration of microorganisms in a container of transfusable blood prior to transfusion.

Background of the Invention

Microorganisms present in bodily fluid can be detected using a culture bottle. Generally, a culture bottle is a flask allowing positive cultures to be detected rapidly. The flask is generally a transparent closed container filled with nutrient that promotes the growth of the organism. In particular, bacteria in blood can be detected in culture. U.S. Patent No. 4,772,559 (Hammann).

Many different qualitative and quantitative detection means are used to monitor the growth of microorganisms in a culture bottle. The microorganisms in a culture bottle have been detected by use of external detectors such as a magnifying lens, U.S. Patent No. 4,543,907 (Freudlich).

Additionally, internal detectors such as liquid level indicators can show bacterial growth as a function of increased pressure in the vessel. Swaine et al., EPA 124,193. Additionally, microorganisms can be detected by measuring changes in pH caused by bacterial growth, Mariel, G.B. Patent No. 1,601,689.

Still another method to detect microorganisms involves the use of a culture media that contains a compound which changes color or appearance according to the growth of microorganisms. The change in the media can be detected with a spectrophotometer. There are many examples of reactions used in Microbiology that rely on a color change. Bascomb, Enzyme Tests in Bacterial Identification, Meth. Microbiol. 19, 105 (1987). For example, a variety of organisms can be classified in large part by their pattern of fermentation, oxidation or assimilation of carbon sources. Fermentation of carbohydrates results in the production of acid which causes a decrease in pH. This drop in pH can be easily detected by including a pH indicator like bromthymol blue or phenol red. With both indicators, acid conditions representing the fermentation of a particular carbohydrate result in a yellow color (changing from blue-green for bromthymol blue or pink/red for phenol red). The same approach can be adopted for a variety of carbohydrates, ranging from monosaccharides like glucose to polysaccharides like inulin. In an analogous fashion, increasing pH can be also be monitored. Assays for detecting the

presence of decarboxylase and urease, and the ability to use malonate are based on an increase in pH, as indicated by a color change in the indicator. Turner, et al. U.S. Patent No. 4,945,060 discloses a device for detecting microorganisms. In this device changes in the indicator medium resulting from pH changes in CO_2 concentration in the medium are detected from outside the vessel.

Chemical and enzymatic reactions are used to detect or quantitate the presence of certain substances in microbiological or other assays. Many of these tests rely on the development or change of color or fluorescence to indicate the presence or quantity of the substance of interest.

Another approach to determine if an organism can degrade a particular substrate, is to use a reagent which is capable of reacting with one or more of the intermediates or final products. For example, the detection of the reduction of nitrate to nitrite. If nitrite is formed, then a pink to deep red color will result when sulfanilic acid and alpha-naphthylamine are added to the reaction mixture.

In contrast to the indirect detection of an enzymatic reaction illustrated by the nitrate/nitrite test, it is possible to use a synthetic analog of a natural substrate to directly indicate the presence of an enzyme. For example, methylene blue can be reduced under certain conditions by the action of reductase, resulting in a shift from blue to colorless. In another test, the oxidase assay relies on the interaction of cytochrome oxidase with N, N, N', N'-tetramethyl-*p*-phenylene-diamine producing a blue color.

Another example is the ability of microorganisms to degrade sulfur-containing amino acids as indicated by the production of H_2S. Typically, the organism is incubated with a high concentration of a sulfur-containing substrate (e.g. cysteine, cystine) in an acid environment. The production of H_2S is indicated by the formation of a black precipitate in the presence of ferric ammonium citrate.

Enzymes can usually act on more than one substrate. This allows for the use of synthetic enzyme substrates for the detection of enzyme activities. Synthetic substrates contain a metabolic moiety conjugated with a chromatic or fluorescent moiety. The conjugated molecule usually has a different absorption and/or emission spectrum from the unconjugated form. Moreover, the unconjugated chromatic or fluorescent moiety shows a considerably higher absorption or fluorescence coefficients than those of the conjugated molecule. This allows the measurement of small amounts of products of enzyme activities in the presence of the large amounts of conjugated substrate required for maximal enzyme activity. An example of a synthetic enzyme substrate is *o*-nitro-phenol-β-galactopyranoside used for the detection of activity of the enzyme β-galactosidase. The conjugated substrate is colorless. The β-galactosidase enzyme hydrolyzes the substrate to yield β-galactosidase and *o*-nitro-phenol. *o*-nitro-phenol absorbs strongly at 405nm, and its release can be measured by the increase in absorbance at that wavelength. Bascomb, Enzyme Tests in Bacterial Identification, Meth. Microbiol. 19, 105 (1987), reviewed the synthetic moieties used for

enzyme substrates and the enzymatic activities measurable using this principle.

Presently, the monitoring of color or color end-product in chemical and microbial reactions is usually achieved in either of two ways; 1) the detection of color or color end-product can be achieved by visual observation and estimated qualitatively, or 2) the detection of color end-products or loss of color can be achieved by measuring the intensity of color instrumentally. Spectrophotometers that measure light absorbance are commonly used for this purpose. When measuring the concentration of a number of substances it is advantageous to use one instrument based on one principle of measurement, otherwise cost is increased.

Although the use of colorimetric reactions is widespread there are limitations, especially in the sensitivity of detection. In order to improve sensitivity and, in the case of identification of microorganisms, thereby to decrease the time required to obtain a result, fluorescence-based methods frequently are used. Unfortunately, it may not be possible to develop a fluorescent equivalent to every assay. Additionally, the fluorescent reagents themselves may be highly toxic and therefore difficult to commercialize.

In such cases one might need to measure activities of some enzymes fluorometrically, the others colorimetrically However, most instruments are suited to measure either absorbance or fluorescence, and very few can be used to measure both.

The general principle of fluorescence quenching has been accepted as a way to detect or determine enzymatic or chemical reactions. For

example, Fleminger et al. synthesized intramolecularly quenched fluorogenic substrates for the assay of bacterial aminopeptidase, P. Fleminger et al., Fluorogenic Substrates for Bacterial Aminopeptidase P and its Analogs Detected in Human Serum and Calf Lung, Eur. J. Biochem. 125, 609 (1982). In this case, the fluorescence of the aminobenzoyl group is quenched by the presence of a nitrophenylalanyl group. When the enzyme is present, the nitrophenylalanyl group is cleaved, with a concomitant increase in the sample's fluorescence. A variety of enzymes have been assayed by this type of procedure, including hydrolytic enzymes, other amino- and carboxypeptidases and an endopeptidase. Yaron et al., Intramolecularly Quenched Fluorogenic Substrates for Hydrolytic Enzymes, Anal. Biochem. 95, 229 (1979); Carmel et al., Intramolecularly - Quenched Fluorescent Peptides as Fluorogenic Substrates of Leucine Aminopeptidase and Inhibitors of Clostridial Aminopeptidase, Eur. J. Biochem. 73, 617 (1977); Carmel et al., An Intramolecularly Quenched Fluorescent Tripeptide as a Fluorogenic Substrate of Angiotensin-I-Converting Enzyme and of Bacterial Dipeptidyl Carboxypeptidase, Eur. J. Biochem. 87, 265 (1978); Florentin et al., A Highly Sensitive Fluorometric Assay for "Enkephalinase", a Neutral Metalloendopeptidase that Releases Tyrosine-Glycine-Glycine from Enkephalins, Anal. Biochem. 141, 62 (1984). In each of the previous approaches, a synthetic substrate containing a quenching group and a fluorescing group was generated in order to detect the activity of the enzyme.

An alternative to this approach would involve the synthesis of a resonance energy transfer pair of fluorescing groups on a substrate molecule. In this method, cleavage by the enzyme of one of the groups would result in a decrease in fluorescence, since the critical distance would be exceeded, eliminating the transfer of energy. However, the previously discussed approaches are limited to specifically designed substrates.

Still another approach involves the estimation of a chromophore by fluorescence measurement. See W. Blumberg et al., Hemoglobin Determined in Whole Blood "Front Face" Fluorometry, Clin. Hemo. 26, 409 (1980). Blumberg disclosed an assay based on attenuation of fluorescence of a dye, whose excitation wavelengths overlap with the absorption wavelengths of the chromophore.

Subsequently, M. Shaffer, U.S. Patent No. 4,495,293 (hereinafter Shaffer) filed a patent application disclosing a method to fluorometrically determine a ligand in an assay solution using conventional fluorometric techniques. In Shaffer the intensity of the fluorescence emitted by the assay solution is related to the change in transmissive properties of the assay solution produced by the interaction of the ligand to be determined and a reagent system capable of producing change in the transmissive properties of the assay solution in the presence of the ligand. More particularly, Shaffer discloses a method to monitor absorbance using a fluorophore in solution with the chromophore. In this method the fluorophore may interact with the assay cocktail and produce changes in fluorescence intensity which are unrelated to the change being measured. The

selection of the fluorophores is also restricted, in that pH dependent or environment sensitive fluorophores cannot be utilized. Additionally, when the fluorophore is in solution, less than accurate measure of absorbance may be obtained because light is absorbed exponentially through the chromophore sample.

Similarly, Beggs & Sand, EPA 91,837 disclosed a solution based method for determination of tryptophan-deaminase activity by measuring the reduction in fluorescence in the presence of a chromophore produced by the interaction between indole pyruvic acid and metal ions using a fluorophore "whose fluorescence is capable of being quenched by the indole pyruvate-metal ion complex, the ions of the fluorophore being present throughout the incubation period".

Also, Sands, U.S. Patent No. 4,798,788 discloses a process to detect a nitrate reducing microorganism by measuring reduction of fluorescence in solution by causing the diazotization of the fluorophore. In all these cases a specific fluorophore needs to be chosen for each test to ensure that it will fluoresce under the conditions of the test, e.g. only few fluorophores fluoresce at pH of less than 2.0.

In addition to blood culture tests, a need exists to develop a noninvasive means to determine bacterial contamination of blood in a collection bag immediately prior to transfusion. Although, the previously discussed blood culture test can be used to determine bacterial contamination of transfusable blood, these test may result in errors. First, the transfusion bag must be later matched with a separate blood culture bottle sent to a test center to make a

determination of potential microbial contamination prior to transfusion of the blood. This requirement for subsequent matching could result in errors. Additionally, blood culture bottles are cultured at higher temperature than the temperature that blood is normally stored; as such blood culture bottle tests yield an accelerated picture of bacterial contamination, while a test that simulates actual blood storage conditions may yield more accurate results.

Summary of the Invention

This invention relates to a multi-layer body fluid culture sensor comprised of a pH sensitive absorbance based dye spectrally coupled to a pH insensitive, or pH sensitive dye that is highly buffered, fluorescence based dye. The pH sensitive absorbance based dye is encapsulated or isolated in a polymeric layer that is permeable to CO_2 and water, but impermeable to protons. The pH insensitive fluorophore is encapsulated or isolated in the second polymeric layer that may or may not be permeable to CO_2 and water. This type of sensor may be used to detect or determine the concentration of microorganisms in bodily fluid. The spectral criterion required to make this determination are such that the absorption spectrum of the chromophore must overlap the excitation and/or emission spectrum of the fluorophore, thereby allowing the change in fluorescence to be related to the change in the reaction and consequently related to the presence or quantity of the substance of interest.

Further, this sensor is used to monitor microbial growth in collected transfusable blood. In

particular, this sensor can be used to monitor bacterial growth in a collection bag of bodily fluid that is to be transfused into a patient. As bacteria grow they generate CO_2. The CO_2 generated by the bacteria diffuses into the polymeric layer that is in direct contact with a hydrated pH sensitive absorbance based dye. The CO_2 reacts with the aqueous environment to form carbonic acid (H_2CO_3), which lowers the pH of the absorbance dye environment. This results in a concomitant change in the pH sensitive spectrum of the dye. Typically, as the absorbance of an absorbance based dye decreases more light reaches the fluorophore for excitation which results in a larger amount of emitted fluorescence.

The sensor is attached to a blood collection bag or separate sampler test bag. If a separate sampler bag is used this bag may contain microbial growth media or an inert substance such as a saline. With this system microbial contamination of transfusable blood in a collection bag can be determined immediately prior to transfusion. In one embodiment of this invention a detector, such as a handheld fluorescence detector, is used to monitor the emitted fluorescence.

Brief Description of the Figures

Fig. 1 shows a schematic diagram of a multi-layer blood culture sensor.

Fig. 2 shows a blood culture growth curve detected by a xylenol blue-rhodamine 101 sensor.

Fig. 3 shows a blood culture growth curve detected by xylenol blue in silicone-rhodamine B in acrylic sensor.

Fig. 4 shows a blood culture growth curve for a xylenol blue in silicone-6213 acrylic sensor.

Fig. 5 shows a blood culture growth curve for a bromthymol blue in silicone-rhodamine 101 in silicone sensor.

Fig. 6 shows a sample test blood collection bag and fluorescent detector for monitoring growth of microorganisms in blood.

Fig. 7 shows the percent change of fluorescent intensity versus time for two blood samples.

Detailed Description - Best Mode

In this approach, fluorescence from a fluorophore embedded in an inert light-transparent matrix, is modulated by a pH sensitive absorbance dye embedded in a polymeric gas permeable, but proton impermeable matrix. The assay is carried out in a blood collection bag or sampler test blood collection bag.

In a fluorometric based colorimetric assay the fluorescence intensity is regulated by changes in absorbance of an interfering chromophore. As a pH change occurs the chromophoric material alters the amount of emitted light reaching the fluorophore and/or the amount of emitted light reaching the detector. Spectrally compatible fluorescent and colorimetric indicators are selected so that as the pH changes due to the production of CO_2 by microorganisms present in the blood, the colorimetric indicators regulate the amount of light reaching the fluorophore and/or photodetector and, thus cause a change in the excitation and/or emission of the

fluorescent dye. This change is detected with a fluorescent reader and can be correlated with the presence or concentration of microorganisms in the blood.

A bodily fluid culture sensor is comprised of a pH sensitive absorbance based dye in or isolated by a polymeric gas permeable, but proton impermeable matrix, and a fluorescent dye in a second polymeric matrix.

Spectrally compatible fluorescent and colorimetric indicators are selected so that when an organism is present in blood, the colorimetric indicator will regulate the amount of light reaching the fluorophore thereby causing a change in the emission intensity from the fluorescence dye reaching the photodetector. The change, indicating the presence of bacteria, is detected with a fluorometric reader.

More particularly, spectrally compatible fluorescence and absorbance dyes are selected dyes are selected so that as carbonic acid is produced (CO_2 and H_2O), the absorbance of the dye will change thereby regulating the amount of light reaching the fluorophore and/or photodetector, thus producing a change in the measured fluorescence. This change is detected with a fluorescence reader. Spectrally compatible dyes are rhodamine B and xylenol blue. Additionally, bromthymol blue and rhodamine 101 are also spectrally compatible.

For example this can be illustrated by inoculating a bag containing the appropriate growth media with Yersinia enterocolitica. As the organism grows, it produces CO_2 gas. The silicone is permeable to the CO_2. The CO_2 diffuses to the

absorbance layer and reacts with water to produce carbonic acid (H_2CO_3). The carbonic acid causes a drop in the pH in the absorbance dye environment resulting in a change in measured absorbance. For example, as the pH drops in an absorbance layer containing the dye xylenol blue, the absorbance of xylenol blue decreases, thereby allowing more light to reach the fluorophore to excite it and thus increase the amount of fluorescence emitted at 590nm. A positive culture using xylenol blue as the absorbance dye is detected by increase the amount of fluorescence emitted at 590nm. A positive culture using xylenol blue as the absorbance dye is detected by a measured increase in fluorescence as the xylenol blue decreases in absorbance See Fig. 7.

The pH sensitive absorbance based dye is encapsulated in or isolated by a polymeric matrix that is gas permeable, but proton impermeable. The polymeric matrix must be optically transparent in the visible region, permeable to gas, autoclavable, stable for at least six months, and proton impermeable. In particular, silicone may function as the polymeric matrix used to encapsulate or isolate the absorbance based dye. Silicones found to meet these criteria were Dow, Rhone Poulenc, G.E. and Wacker.

Similarly, the fluorescence based dyes can also be encapsulated in a polymeric matrix. The polymeric matrix used for the fluorophore does not have to meet all of the above requirements listed for the matrix used to encapsulate or isolate the absorbance dye. The similar features that it must possess are that it must be optically transparent in the visible region, autoclavable and stable for at least six months.

The polymeric matrix containing or isolating the absorbance based dye must be coupled to the polymeric matrix containing the fluorescent dye. It should be noted that the polymeric matrices must be in close proximity so that light that has been regulated by the absorbance layer will have an effect on the emission intensity of the fluorophore as received by the photodetector. This can be accomplished by applying the same polymeric material to one side of each polymeric matrix and curing these matrices. Once the matrices containing the dyes have been adhered together they must be rehydrated. The clarity of the sensor upon rehydration is also a factor in matrix selection.

In particular, in the present invention, a microorganism growth monitoring system for collected transfusable blood is shown in Fig. 6. The monitoring system shown in Fig. 6 is comprised of a sampler test blood collection bag 20. A bar code 28 can be attached to the bag to record data for later inspection. Blood from the blood collection bag to be transfused is expressed through tube 24 to sampler test blood collection bag 20. The wall of the blood collection bag contains a multi-layer sensor 22 comprising a pH sensitive dye in a light transmissive, gas permeable, proton impermeable matrix and a pH insensitive fluorescence dye in inert light transparent matrix, said first and second matrices being spectrally coupled. The blood collection or blood storage bag can contain whole blood, plasma, serum, erythrocytes, red blood corpuscles, leukocytes, white blood corpuscles, thrombocytes and blood platelets, collectively referred to as blood storage.

In the present invention the sensor can be located on the interior wall of the blood collection bag or a separate sampler test blood collection bag to which blood can be shunted for assessment. These various types of bags are heretofore collectively referred to as blood collection bag.

If a separate blood collection bag is employed the bag may contain a growth media or an inert substance such as saline.

In the present invention the two-layer sensor is mounted inside a blood collection bag such that one layer, of the sensor is positioned facing outside the bag. The second layer which is fluorescent is positioned facing the interior of the bag. The sensor may be formed integrally with the wall of the bag. The invention, then, is comprised of the two-layer sensor outlined above, mounted inside a blood collection container in such a way that by utilizing a fluorometer to excite the fluorescent sensor and detect the emitted fluorescent light, a determination can be made as to the presence of a threshold level of microorganisms contained within the blood collection container.

An additional feature of this invention is that the bag is stored at normal blood storage temperatures, i.e. 4°C. In this environment certain microorganisms are not affected by the cold: Yersinia enterocolitica and Enterobacter agglomerans. Additionally, the system allows for the determination of other bacteria that would not normally grow rapidly in the cold but might be present in such high concentrations over time that it would be unsafe to transfuse into a patient: Citrobacter freundii; Pseudomonas aeruginosa; Staphylococcus aureus; and Staphylococcus epidermidis.

An alternative approach would follow the same design but would consist solely of an absorbance sensor. In this case the increase is CO_2 produced by the growing microorganisms and subsequent drop in pH would result in a visible color change in the sensor. This change in color visible to the blood handler, or detected by a colorimeter would signal that the blood contained a threshold level of microorganisms.

Another feature of this invention involves covering the sensor with a gas permeable membrane to prevent naturally fluorescing substances in the blood from interacting with the fluorescent measurement.

In and alternative embodiment shown in Fig. 1, a bodily fluid culture sensor, is comprised of a pH sensitive absorbance based dye encapsulated in or isolated by a polymeric gas permeable, but proton impermeable matrix 4 and a fluorescent dye in a second polymeric matrix 2. Reflective surface 6 can be included to facilitate the transmission of light to the detecting element 12. In Fig. 1 interrogation light enters the sensor and is regulated by pH sensitive matrix 2 which in turn causes a change in the fluorescence emission 10 of the fluorophore in matrix 4. This sensor offers the advantage of maximal surface area.

A measurement is taken by first reading reference light intensity. Next the reading from the sensor disk is measured. The data is plotted by taking the ratio of reference, excitation light, to sample. In particular, as CO_2 levels increase in the blood collection bag, the absorbance of the absorbance dye changes, thereby changing the amount of light reaching the fluorescence layer and/or photodetector. This causes a change in emitted fluorescence that is detected.

The following examples serve to illustrate the method of the present invention. The concentration of reagents and other variable parameters are only shown to exemplify the methods of the present invention and are not to be considered limitation thereof.

Example 1

At the time a unit of blood is drawn, an additional amount (10ml) is collected in such a manner as to be subsequently sectioned off from the unit to be transfused. This additional blood is then expressed through a tube into a flexible bag, attached to the blood collection bag. This side bag is supplied containing a microorganism growth media and an attached multi-layer sensor. The sensor is capable of detecting microorganism growth by measuring an increase in CO_2 production through a change in fluorescence emitted from the sensor.

After adding the additional blood to the bag containing media and the sensor, and waiting a predetermined amount of time (2 - 6 hours) for the sensor to equilibrate, an initial reading of the sensor is made using a portable handheld fluorometer to produce a baseline fluorescence level. This level can be manually recorded for latter comparison or a bar code can be provided and attached to the bag. The blood and additional monitoring bag are then stored in a normal manner (4°C).

At the time the blood is to be used for transfusion a second reading of the sensor is made and compared to the first reading. This is compared to the original reading. If a bar code was produced, the bar code and sensor are read. The instrument will

compare the initial and final fluorescent values and based upon an established threshold level of change will signal negative or positive for growth. In this particular embodiment, the instrument will signal green for no growth or red for growth based on differences in original and final sensor readings.

Example 2 Xylenol Blue - Rhodamine 101 Sensor

Wacker silicone elastomer 3601 part A is thoroughly mixed with Wacker 3601 catalyst part B in a 9:1 ratio, as recommended by the manufacturer. Next 5% w/w of a 50mM xylenol blue, dissolved in 5mM borate buffer pH 11 containing 1% Tween 80, is added to the silicone and homogenized to ensure a uniform distribution of the dye. The absorbance layer mixture is then poured into an aluminum square mold to a thickness of 30/1000 of an inch and cured at 55°C for 2 hours.

Wacker silicone is prepared, as described above. Next 2% w/w of 7.5mM Rhodamine 101, in 50mM Tris-HCl buffer pH 8.5 in 95% ethylene glycol, is added to the silicone. The mixture is poured over the previously cured xylenol blue layer in the mold, described above, and cured at 55°C overnight. This cured, dehydrated, double layer sensor consists of two distinct layers, each 30/1000 of an inch thick. Disks may now be punched out of the mold and adhered onto the base of bottles using more silicone, ensuring that the absorbance layer is face down. Finally, the bottles are cured at 55°C for 15 minutes, rehydrated with normal saline and autoclaved on the wet cycle for 17 minutes. Saline is replaced with growth media and inoculated with E. coli by injecting a suspension with a sterile needle through the septum. The blood

culture bottle is placed in the instrument and fluorescence emission is measured.

As the concentration of CO_2 increases in the blood culture bottle, the pH sensitive absorbance dye, Xylenol blue, the absorbance of the dye decreases, thus allowing more light to reach the fluorophore, Rhodamine 101, to thus increase the amount of fluorescence emitted at 590nM. This increase in fluorescence intensity v. time is shown in the blood culture growth curve at Fig. 2.

Example 3 Xylenol Blue in Silicone/Rhodamine B in Acrylic

Rhone Poulenc silicone elastomer 141 part A is thoroughly mixed with Rhone Poulenc 141 catalyst part B in a 10:1 ratio, as recommended by the manufacturer. Next 1% w/w of a 100mM xylenol blue solution pH #11, dissolved in 10mM borate buffer containing 1% Tween 80, is added to the silicone and mixed thoroughly with a tongue blade to ensure uniform distribution of the dye. The absorbance layer mixture is then poured into an aluminum square mold to a thickness of 30/1000 of an inch. The mold is allowed to sit out on the countertop at room temperature for about one hour or until the bubbles have disappeared, at which time the mold is placed in the incubator to cure at 55°C for two hours.

Rhone-Poulenc silicone is prepared, as described above. Next, a 40/1,000" thick acrylic disc (Glasflex, Inc.), approximately 1 cm in diameter, containing 0.2 grams/lb of rhodamine B (Sigma) is glued onto the above absorbance layer using the Rhone-Poulenc Silicone at the 10:1 ratio as glue. The double layer sensor is then placed back in the 55°C

incubator for two hours to allow for adherence of the two layers. Following the curing, the double layer sensor is punched out with a cork borer, and glued onto the base of a Wheaton bottle, ensuring that the absorbance layer is face down, using the Rhone Poulenc silicone as mentioned above. The bottle is placed in the 55°C incubator to cure for at least two hours. The bottle is then rehydrated overnite and tested the following day as described in Example 1.

As the concentration of CO_2 increases in the blood culture bottle, the absorbance of the pH sensitive absorbance based dye xylenol blue decreases, thus allowing more light to reach the fluorophore (rhodamine B) doped acrylic, to thus increase the amount of fluorescence emitted at 590nm. This increase in fluorescence intensity v. time is shown in the blood culture growth curve in Fig. 3.

Example 4 Xylenol Blue in Silicone/6213 Red Standard Acrylic

Wacker silicone elastomer 3601 part A is thoroughly mixed with Wacker 3601 catalyst part B in a 9:1 ratio, as recommended with Wacker 3601 catalyst part B in a 9:1 ratio, as recommended by the manufacturer. Next 5% w/w of a 50mM xylenol blue, dissolved in 5mM borate buffer pH 11 containing 1% Tween 80, is added to the silicone and homogenized to ensure a uniform distribution of the dye. The absorbance layer mixture is then poured into an aluminum square mold to a thickness of 30/1000 of an inch and cured at 55°C for two hours.

Next, a 40/1,000" thick acrylic disc (Glasflex, Inc.), approximately 1 cm in diameter, referred to as No. 6213 Red (Glasflex Standard

Product) is glued onto the above absorbance layer using the Wacker silicone at the 9:1 ratio as glue. The double layer sensor is then placed back in the 55°C incubator for two hours to allow for adherence of the two layers. Following the curing, the double layer sensor is punched out with a cork borer, and glued onto the base of a Wheaton bottle, ensuring that the absorbance layer is face down, using the Rhone Poulenc silicone as mentioned above. The bottle is placed in the 55°C incubator to cure for at least two hours. The bottle is then rehydrated overnite and tested the following day as described in Example 1.

As the concentration of CO_2 increases in the blood culture bottle, the absorbance of the pH sensitive absorbance based dye xylenol blue decreases, thus allowing more light to reach the fluorophore (rhodamine B) doped acrylic, to thus increase the amount of fluorescence emitted at 590nm. This increase in fluorescence intensity v. time is shown in the blood culture growth curve in Fig. 4.

Example 5 Bromthymol Blue in Silicone/Rhodamine 101 in Silicone

Wacker silicone elastomer 3601 part A is thoroughly mixed with Wacker 3601 catalyst part B in a 9:1 ratio, as recommended by the manufacturer. Next 5% w/w of 50mM bromthymol blue, dissolved in 5mM tris buffer pH 12 in ethylene glycol, is added to the silicone and homogenized to ensure a uniform distribution of the dye. The absorbance layer mixture is then poured into an aluminum square mold to a thickness of 30/1000 of an inch and cured at 55°C for two hours.

Wacker silicone is prepared, as described above. Next 2% w/w of 7.5mM Rhodamine 101, in 50mM Tris-HCl buffer pH 8.5 in 95% ethylene glycol, is added to the silicone. The mixture is poured over the previously cured xylenol blue layer in the mold, described above to isolate the absorbance layer. This sensor is then cured at 55°C overnight. This cured, dehydrated, double layer sensor consists of two distinct layers, each 30/1000 of an inch thick. Disks may now be punched out of the mold and adhered onto the base of bottles using more silicone, ensuring that the absorbance layer is face down. Finally, the bottles are cured at 55°C for 15 minutes, rehydrated with normal saline and autoclaved on the wet cycle for 17 minutes. Saline is replaced with growth media and inoculated with E. coli by injecting a suspension with a sterile needle through the septum. The blood culture bottle is placed in the instrument and fluorescence emission is measured. The increase in fluorescence intensity v. time is shown in blood culture growth curve in Fig. 5.

Although this invention has been described with respect to specific embodiments, the details thereof are not to be construed as limitations, for it will be apparent that various equivalents, changes and modifications may be resorted to without departing from the spirit and scope thereof and it is understood that such equivalent embodiments are intended to be included herein.

WHAT IS CLAIMED IS:

1. A microorganism growth monitoring apparatus for collected bodily fluid to be transfused into a patient comprising:

a. a flexible bag;

b. means to add said bodily fluid to said bag;

c. a sensor for detecting microbial growth in said bag, said sensor comprising a pH sensitive absorbance based dye encapsulated in a light transmissive, gas permeable, proton impermeable matrix, and a pH insensitive fluorescent dye in an inert light transparent matrix, said first and second matrices being spectrally coupled, said sensor being attached to the inside wall of said bag.

2. The apparatus of claim 1 wherein said bag is a blood collection bag.

3. The apparatus of claim 2 wherein said bag is a sample test bag.

4. A method to detect microbial growth, in collected bodily fluid to be transfused into a patient, in a flexible bag containing a microbial growth sensor attached to the inside of said bag comprising:

a. adding said bodily fluid to said bag;

b. storing said bag at about 4°C for a sufficient period of time to allow for microbial growth;

c. detecting microbial growth to obtain a baseline reading of growth using said sensor;

d. detecting microbial growth in said bag prior to transfusion to obtain reading prior to transfusion;

e. comparing said baseline reading to said reading prior to transfusion to determine if microbial growth has occurred in said bodily fluid.

5. The method of claim 4 wherein said microbial growth sensor is comprised of a pH sensitive absorbance based dye encapsulated in a light transmissive, gas permeable, proton impermeable matrix, and a pH insensitive fluorescent dye in an inert light transparent matrix, said first and second matrices being spectrally coupled.

6. The method of claim 4 wherein said microbial growth is detected by measuring an increase in CO_2 production through a change in fluorescence emitted from said sensor.

7. The method of claim 6 wherein a handheld fluorometer is used to detect changes in emitted fluorescence.

8. The method of claim 4 wherein said bag is a blood collection bag.

9. The method of claim 4 wherein said bag is a sample test bag.

10. The method of claim 9 wherein said bag contains bacterial growth media.

11. The method of claim 9 wherein said bag contains an inert substance.

12. The method of claim 4 wherein said fluid is blood.

13. A noninvasive method to detect microbial growth in a flexible bag comprising:

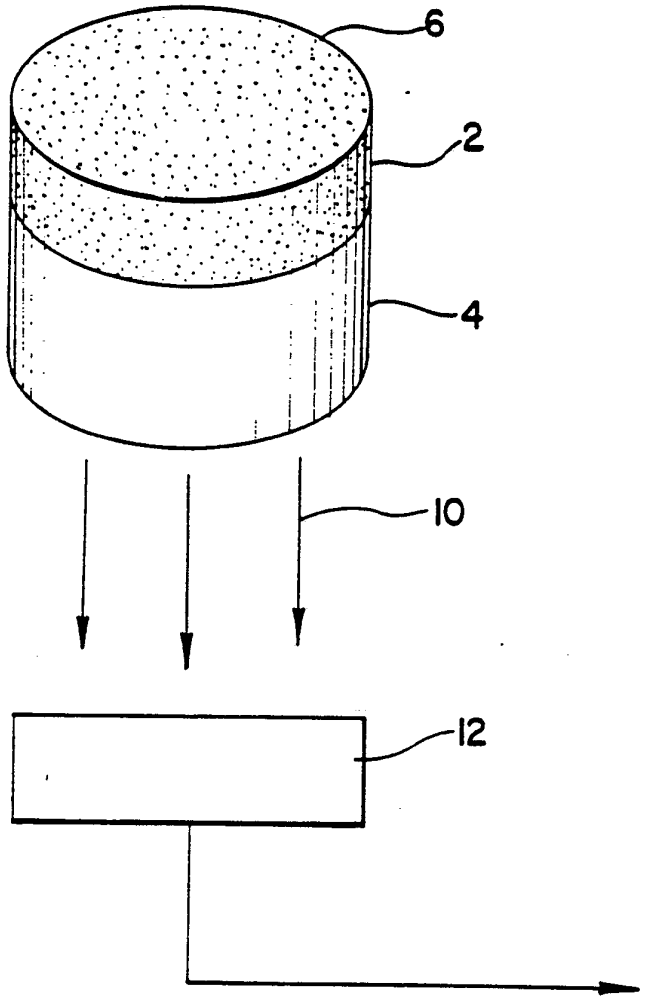
a. adding blood to a said bag containing a sensor for CO_2 production;

b. means to noninvasively monitor said sensor to determine microbial growth.

14. The method of claim 13 wherein said sensor emits fluorescence light upon interrogation with a fluorometer as a function of microbial growth.

15. The method of claim 13 wherein said sensor changes color in response to microbial growth.

Fig. 1



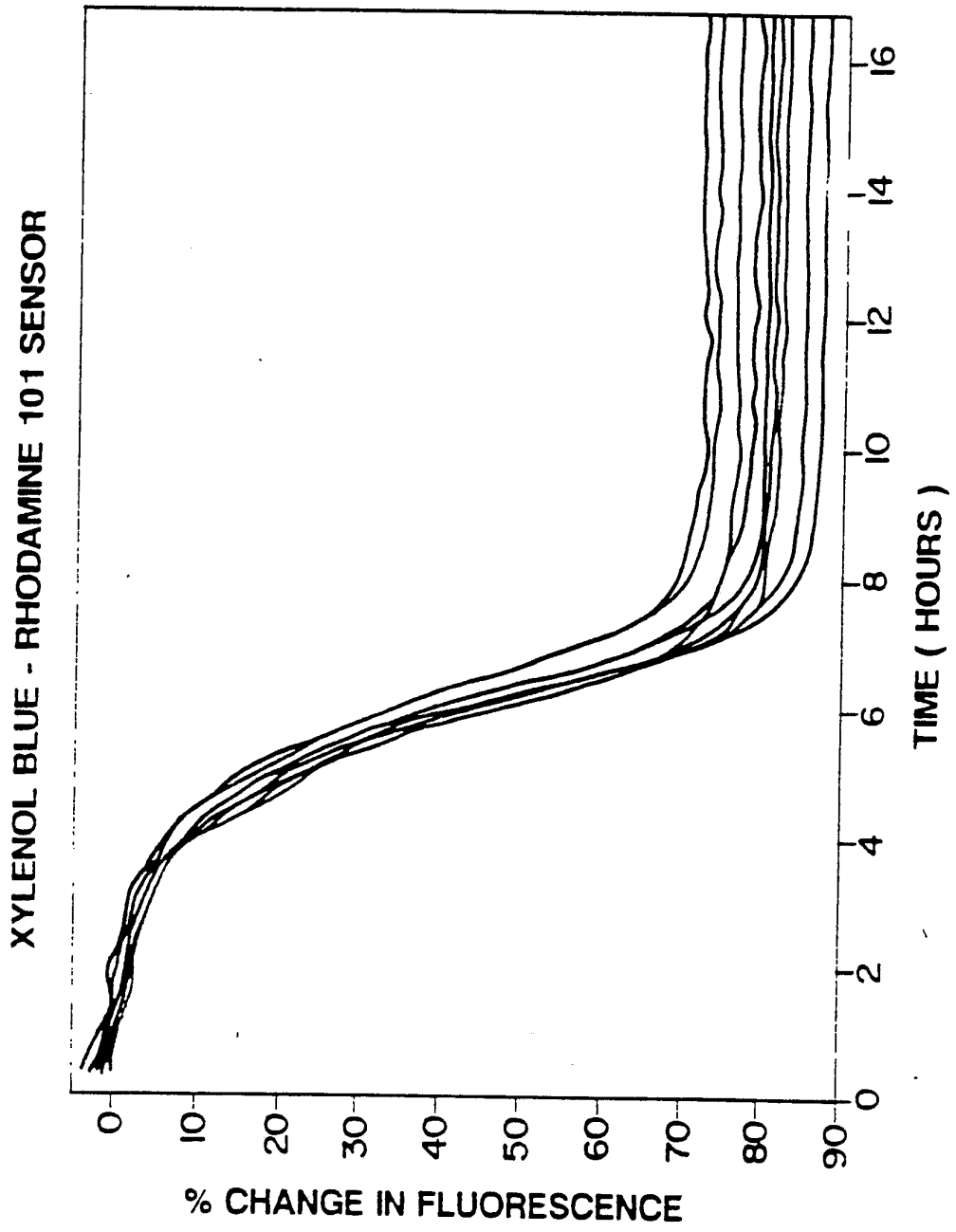

XYLENOL BLUE - RHODAMINE 101 SENSOR
% CHANGE IN FLUORESCENCE
0
10
20
30
40
50
60
70
80
90
TIME (HOURS)
0
2
4
6
8
10
12
14
16

Fig. 2

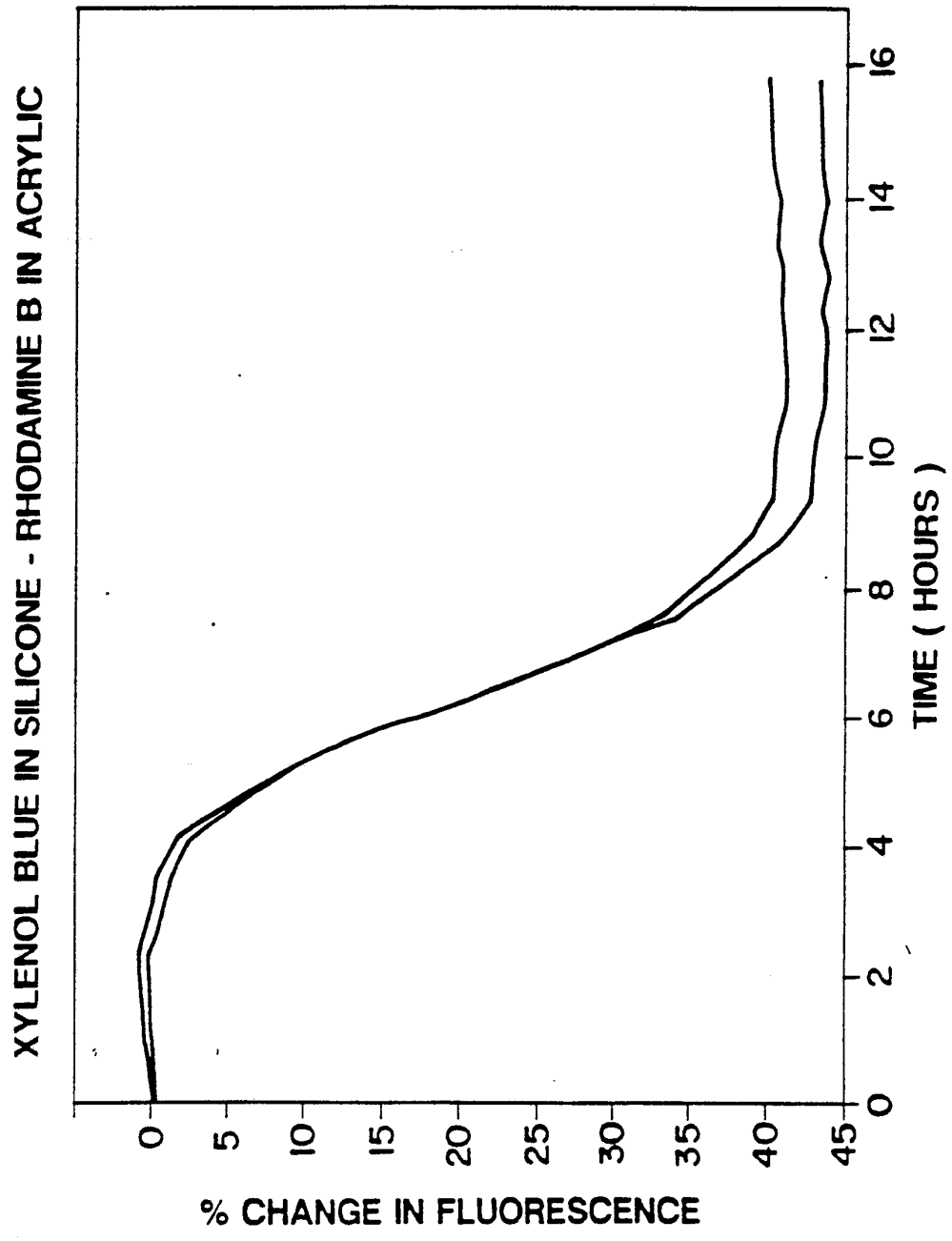

XYLENOL BLUE IN SILICONE - RHODAMINE B IN ACRYLIC
% CHANGE IN FLUORESCENCE
0
5
10
15
20
25
30
35
40
45
TIME (HOURS)
0
2
4
6
8
10
12
14
16

Fig. 3

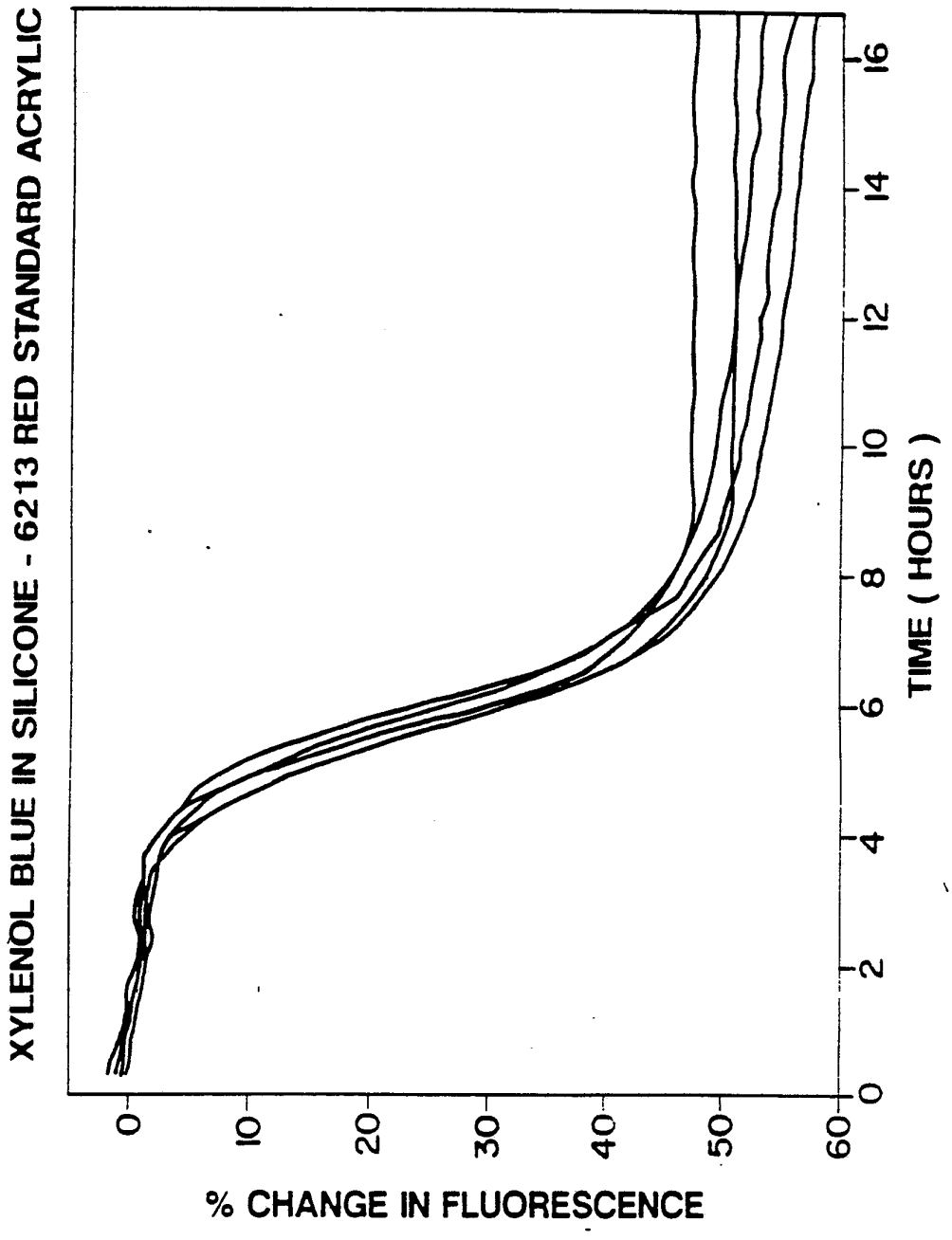

XYLENOL BLUE IN SILICONE - 6213 RED STANDARD ACRYLIC
% CHANGE IN FLUORESCENCE
0
10
20
30
40
50
60
TIME (HOURS)
0
2
4
6
8
10
12
14
16

Fig. 4

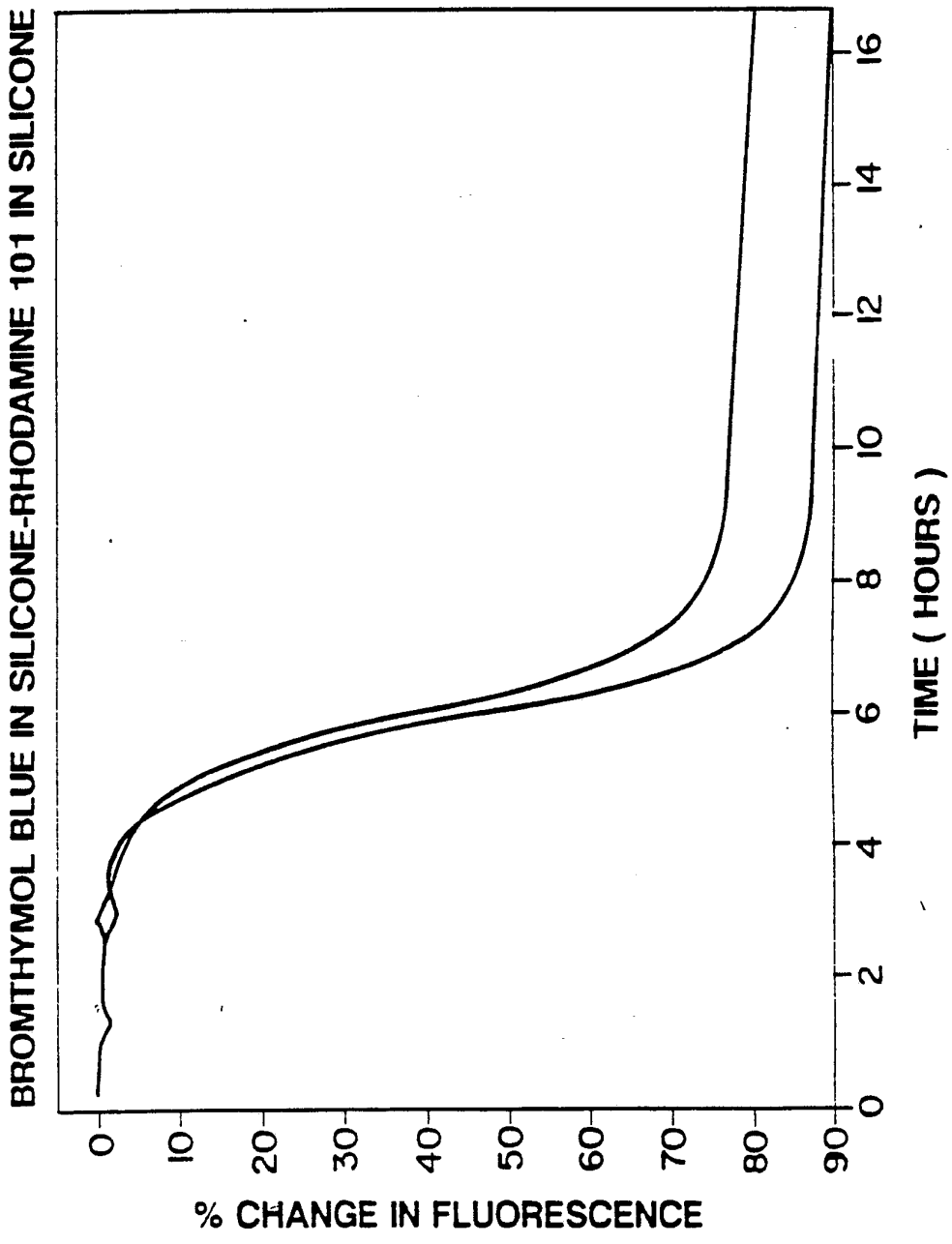

BROMTHYMOL BLUE IN SILICONE-RHODAMINE 101 IN SILICONE
0
10
20
30
40
50
60
70
80
90
% CHANGE IN FLUORESCENCE
0
2
4
6
8
10
12
14
16
TIME (HOURS)

Fig. 5

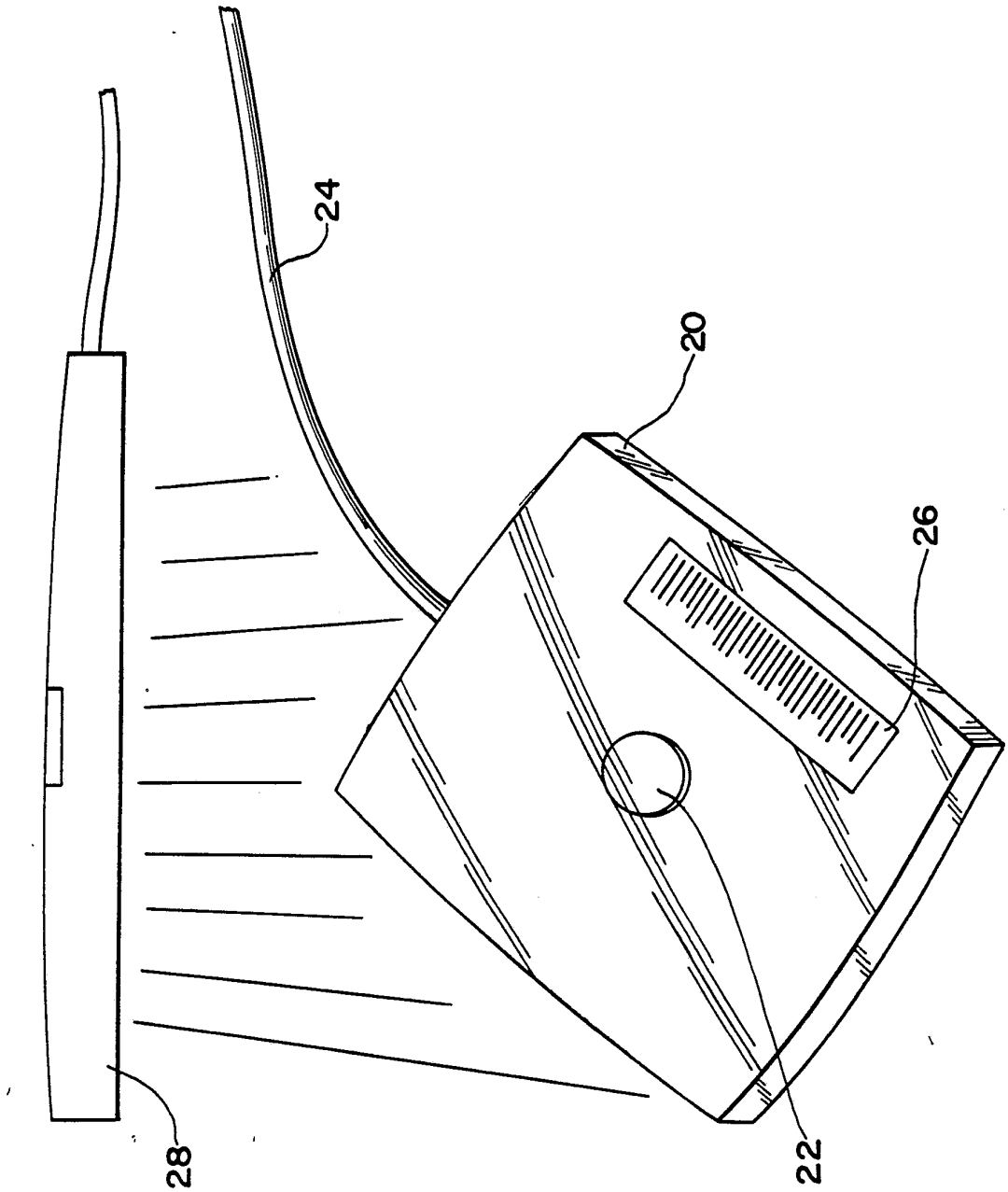


Fig. 6

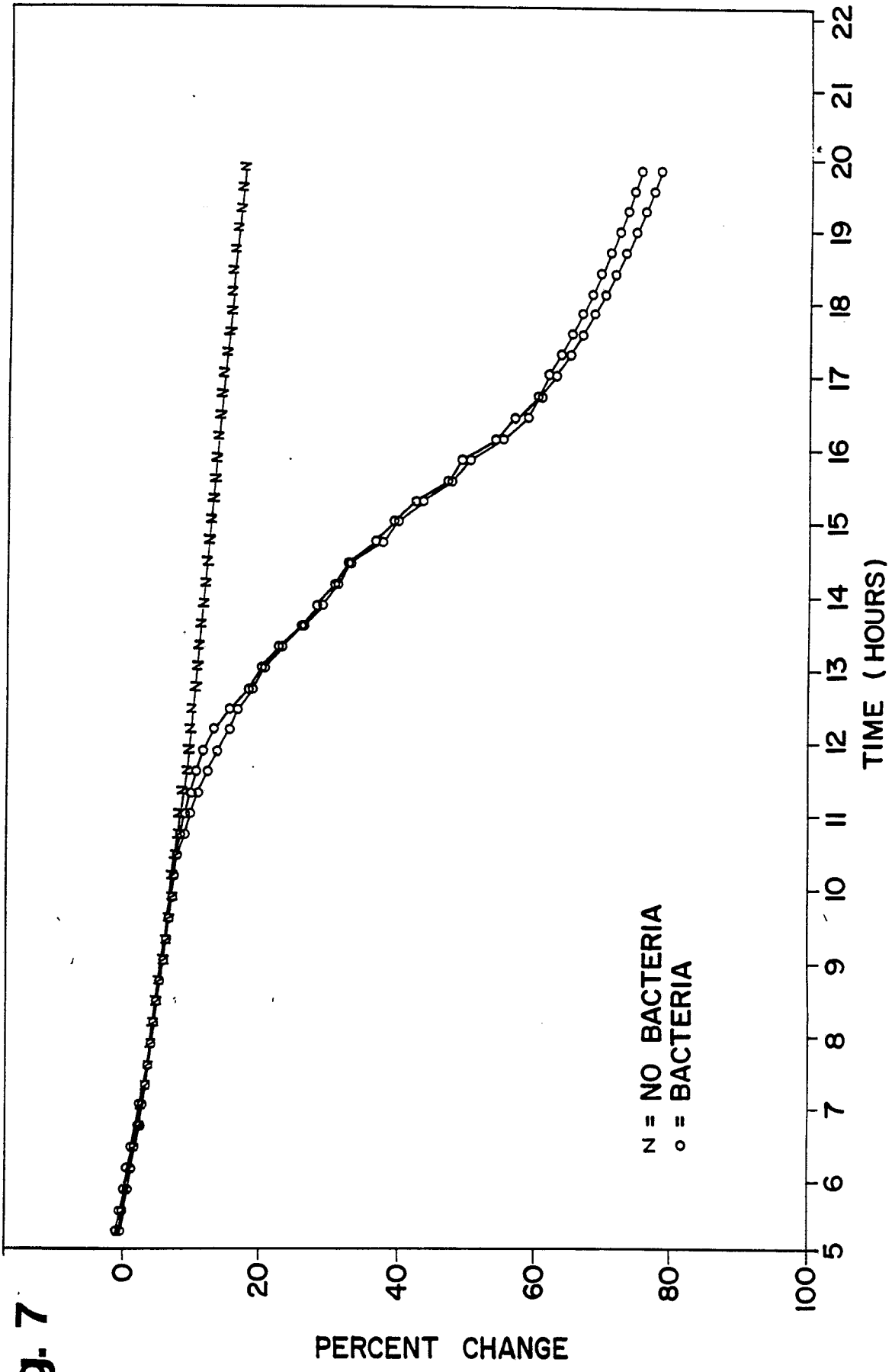

PERCENT CHANGE
0
20
40
60
80
100
TIME (HOURS)
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
N = NO BACTERIA
o = BACTERIA

Fig. 7

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/03637

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

IPC (5): C12Q 1/04; G01N 21/75, 21/76, 21/77
US CL : 435/34, 291, 807; 436/167, 170, 172; 604/404

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/34, 287, 291, 807; 436/167, 169, 170, 172; 422/56, 60, 68.1; 604/403, 404

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

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]
Y	US, A, 4,945,060 (Turner et al.) 31 July 1990, see column 2, lines 25-34, column 3, lines 48-52 and 65-68, and column 4, lines 1-3, 30, 39-41, and 50-53.	1-15
Y	US, A, 4,182,656 (Ahnell et al.) 08 January 1980, see column 2, lines 24-26 and 30-35.	4-12
A	US, A, 4,798,788 (Sands) 17 January 1989, see entire document.	1-15
A	US, A, 4,784,947 (Noeller) 15 November 1988, see entire document.	4-15
A	US, A, 4,780,191 (Romette et al.) 25 October 1988, see entire document.	1-15
A	US, A, 4,772,558 (Hammann) 20 September 1988, see entire document.	1-15
A	US, A, 4,698,308 (Ikeda) 06 October 1987, see entire document.	1-3
A	US, A, 4,672,039 (Lundblom) 09 June 1987, see entire document.	1-3

* 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 understand 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]
22 June 1992	17 JUL 1992
International Searching Authority[1]	Signature of Authorized Officer[20]
ISA/US	THERESA A. TREMBLEY

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET		
A	US, A, 4,653,907 (Freundlich) 31 March 1987, see entire document.	1-3
A	US, A, 4,495,293 (Shaffar) 22 January 1985, see entire document.	4-15
A	US, A, 4,152,213 (Ahnell) 01 May 1979, see entire document.	4-15
A	US, A, 4,073,691 (Ahnell et al.) 14 February 1978, see entire document.	4-15
A	US, A, 3,676,679 (Waters) 11 July 1972, see entire document.	1-3

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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	US, A, RE 31879 (Lubbers et al.) 07 May 1985, see entire document.	1-15
A	US, A, 4,867,919 (Yafuso et al.) 19 January 1989, see entire document.	1,5
A	US, A, 4,824,789 (Yafuso et al.) 25 April 1989, see entire document.	1,5
A	US, A, 4,657,736 (Marsoner et al.) 14 April 1987, see entire document.	1,5
A	US, A, 4,587,101 (Marsoner et al.) 06 May 1986, see entire document.	1,5
A	US, A, 4,557,900 (Heitzmann) 10 December 1985, see entire document.	1,5
A	US, A, 4,568,518 (Wolfbeis et al.) 04 February 1986, see entire document.	1,5
A	GB, A, 1,601,689 (Mariel) 04 November 1981, see entire document.	1-15
A	EP, A, 0,124,193 (Swaine et al.) 07 November 1984, see entire document.	1-15
A	EP, A, 0,171,158 (Boggs et al.) 12 February 1986, see entire document.	1-15
A	EP, A, 0,104,463 (Sussman et al.) 04 April 1984, see entire document.	1-15
A	EP, A, 0,091,837 (Carr et al.) 19 October 1983, see entire document.	1-15
A	GB, A, 2,132,348 (Bacon et al.) 28 May 1987, see entire document.	1-15
A	Analytical Biology, Volume 129, issued 1983, Ando et al., "Pyruvate as a Fluorescence Quencher: A New Spectroscopic Assay for Pyruvate Reactions", pages 170-175, see entire document.	1-15
A	Methods in Microbiology, Volume 19, issued 1987, Bascomb, "Enzyme Tests in Bacterial Identification", pages 104-160, see entire document.	1-15
A	Clinical Chemistry, Volume 26, No. 3,issued 1980, Blumberg et al., "Hemoglobin Determined in 15 μL of Whole Blood by "Front-Face" Fluorometry", pages 409-413, see entire document.	1-15
A	European Journal of Biochemistry, Volume 73, issued 1978, Carmel et al., "Intramolecularly-Quenched Fluorescent Peptides as Fluorogenic Substrates of Leucine Aminopeptidase and Inhibitors of Clostridial Aminopeptidase", pages 617-625, see entire document.	1-15
A	European Journel of Biochemistry, Volume 125, issued 1982, Fleminger et al., "Fluoregenic Substrates for Bacterial Aminopeptidase P and Its Analogs Detected in Human Serum and Calf Lung", pages 609-615, see entire document.	1,5

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	Analytical Biochemistry, Volume 141, issued 1984, Florentin et al., "A Highly Sensitive Fluorometric Assay for "Enkephalinase," a Neutral Metalloendopeptidase that Releases Tyrosine-Glycine-Glycine from Enkephalins", pages 62-69, see entire document.	4-15
A	Analytical Chemistry, Volume 60, issued 1988, Rhines et al., "Simplex Optimization of a Fiber-Optic Ammonia Sensor Based on Multiple Indicators", pages 76-81, see entire document.	1-15
A	Analytical Chimica Acta, Volume 185, issued 1986, Wolfbeis et al., "Fibre-Optic Fluorescing Sensor for Ammonia", pages 321-327, see entire document.	1-15
A	Analytical Biochemistry, Volume 95, issued 1979, Yaron et al., "Intramolecularly Quenched Fluorogenic Substrates for Hydrolytic Enzymes", pages 228-235, see entire document.	1-15
Y,E	US, A, 5,094,955 (Calandra et al.) 10 March 1992, see entire document.	1-15