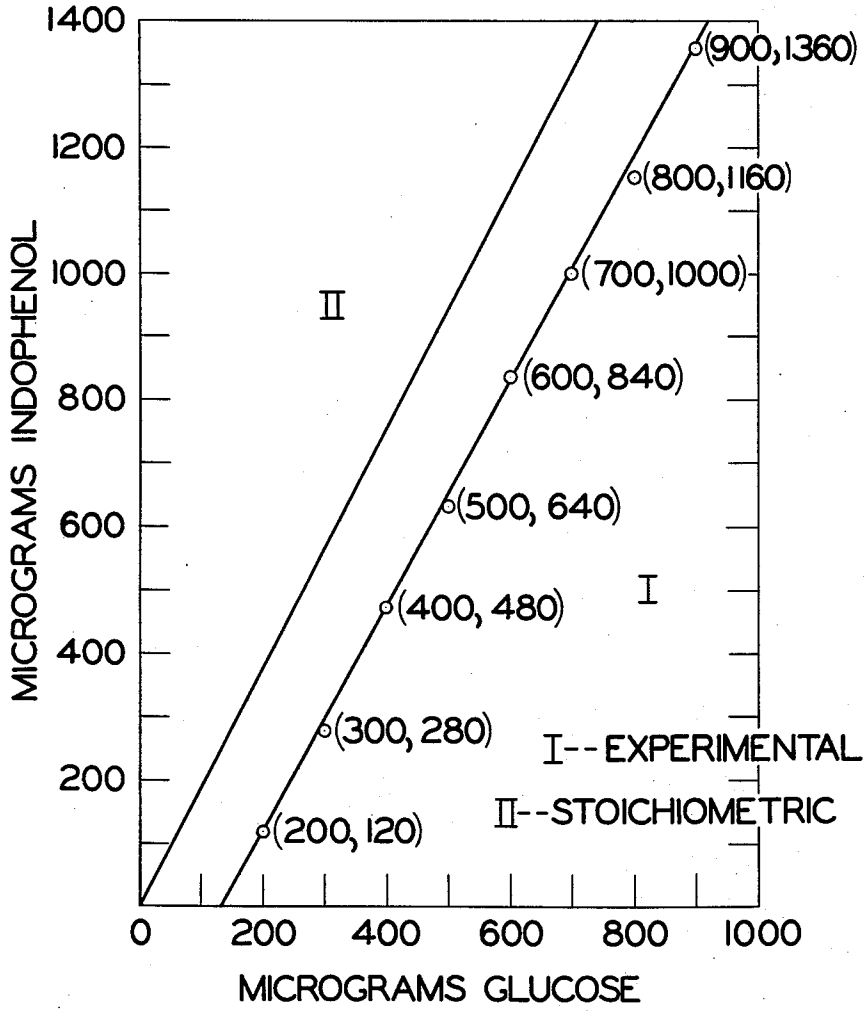
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METHOD FOR DETERMINING GLUCOSE LEVELS IN BLOOD AND OTHER BIOLOGICAL FLUIDS

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METHOD FOR DETERMINING GLUCOSE LEVELS IN BLOOD AND OTHER BIOLOGICAL FLUIDS

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This invention relates generally to a rapid method for determining glucose levels in blood and other biological fluids and more specifically to a rapid method for screening for blood glucose, which method is applied directly to protein-carrying blood serum or plasma.

Heretofore the usual method for determining glucose in biological fluids such as blood has required the preliminary steps (1) obtaining a protein-free filtrate by precipitating the protein with such reagents as sulfuric acid and sodium tungstate, or barium hydroxide and zinc sulfate, and (2) removing the precipitated protein before proceeding to determine the glucose content by the usual method employing copper sulfate or potassium ferricyanide. The requirement for a protein-free filtrate makes such methods time consuming and generally unsuitable for mass screening for glucose.

Another method heretofore used to determine glucose in biological fluids such as blood serum employs the property of the enzyme, glucose oxidase, to catalyze the oxidation of glucose to gluconic acid. The amount of glucose present in the blood serum is determined by manometrically measuring the oxygen uptake during the reaction. However, this technique is too complex for general use and therefore is totally unsuitable for mass screening of large numbers of blood samples.

Another method heretofore used employs a paper tape which because of the hydrogen peroxide liberated by the glucose-glucose oxidase reaction yields a chromogenic oxidation product, the color intensity of which is semi-quantitatively proportional to glucose concentration in urine and blood plasma. However, this method is not sufficiently quantitatively accurate, particularly when applied to blood.

It is therefore an object of this invention to provide an improved method for determining glucose levels directly in biological fluids such as blood serum or plasma which method does not require removal of protein from the serum or plasma for determination of glucose.

Another object of this invention is to provide an improved method for rapidly screening for glucose in biological fluids such as blood serum or plasma which method does not require comparison of the reactant with a standard, or comparison of the color of the reactant with a color chart.

Another object of this invention is to provide an improved method for mass screening for glucose in biological fluids such as blood serum or plasma which is simple to perform, does not require special laboratory skills or techniques, has a high degree of accuracy, and which overcomes the disadvantages of the prior art methods.

Another object of the invention is to provide in a glucose-glucose oxidase reaction a hydrogen acceptor which will itself indicate the course of the reaction.

Still another object of this invention is to provide an improved method for mass screening for glucose in biological fluids such as blood serum which method may be performed under either anaerobic or aerobic conditions.

Other objects and advantages will become readily apparent from the following description of the invention taken together with the accompanying drawing.

The inventor hereof found upon bringing together a

biological fluid such as blood serum or plasma, an oxidation-reduction indicator, such as sodium 2,6-dichlorobenzenoneindophenol, and a catalytic enzyme, such as glucose oxidase, in the foregoing order, that he obtained a reaction which if buffered at a pH of from about 4.0 to 5.6, with a preferred pH of 4.6, gave a solution varying from colorless to a deep blue. The presence or absence of color was found to depend on the quantities of glucose, indicator, and enzyme present, and the pH of the solution. If the quantities of indicator and enzyme, the value of pH, and the reaction time are fixed within predetermined limits so as to establish a screening level for glucose of a predetermined amount, then a colorless solution indicates glucose above this amount and a colored solution glucose below this amount.

If the foregoing method is performed under anaerobic conditions, it is possible to determine glucose concentrations varying as little as 10 milligrams per 100 milliliters above or below a set screening level. Thus glucose in a biological fluid such as blood serum or plasma can be classified as being greater or less than any set screening level with an accuracy 10 milligrams per 100 milliliters.

Anaerobic conditions may be obtained without the use of special apparatus by the addition of a light oil, such as a light petroleum oil, to the blood serum or plasma before adding the indicator and enzyme. Under aerobic conditions the foregoing method also gives consistent results with a probable error of about plus or minus 20 milligrams per 100 milliliters.

The method of this invention owes its efficacy to the fact that the oxidation-reduction indicator, sodium 2,6-dichlorobenzenone-indophenol

$$(NaOC_6H_4N{:}C_6H_2(Cl_2){:}O)$$

replaces molecular oxygen as the hydrogen acceptor in the glucose-glucose oxidase reaction. Under anaerobic conditions, it has been established experimentally that a sharply defined quantitative relationship exists between the amount of glucose oxidized and the amount of sodium 2,6-dichlorobenzenone-indophenol reduced. It has also been confirmed experimentally that for every increment of 100 parts of glucose oxidized, there is a corresponding increment of 180 parts of sodium 2,6-dichlorobenzenone-indophenol (hereinafter referred to as indophenol) reduced. Whereas 1 mole of glucose will theoretically reduce 1 mole of indophenol, the incremental ratio between glucose oxidized and indophenol reduced approaches stoichiometric proportions.

In the drawing curve I shows maximal amount of indophenol having a formula weight of 340, as determined iodometrically, reduced to colorless state with increasing amounts of glucose, as determined experimentally. Curve II shows the stoichiometric relationship between indophenol having a formula weight of 340, as determined iodometrically, reduced to colorless state with increasing amounts of glucose.

The use of indophenol as an indicator gives a convenient means of following the glucose-glucose oxidase reaction since the dye is intensely colored in its oxidized state and totally colorless in its reduced state.

The indicator indophenol may be prepared as a stock solution or it may be prepared in powdered form by mixing with a filler such as sodium chloride. Such powdered mixture may be formed into a stable standardized tablet, vial or capsule. In any of the foregoing forms the indicator is diluted with a suitable dilution reagent comprising an aqueous solution containing 40 percent by volume of propylene glycol, 1 percent by volume of ethyl alcohol and 0.2 percent by weight of sodium bicarbonate before use in the method of the invention. When diluted to a predetermined amount as described

hereinafter, the indicator is ready for use as a screening indicator.

The quantity of indophenol which the screening indicator solution is caused to contain will depend upon the glucose screening level chosen. If the glucose screening level selected is 100 milligrams per 100 milliliters, the indicator is diluted with the dilution reagent until the screening indicator solution contains an indophenol concentration (formula weight 340 as determined iodometrically) of 700 micrograms per 1.5 milliliters, which is 60 micrograms of indophenol in excess of the maximal amount of indophenol that is visibly reduced to a colorless state by 0.5 milliliter of a standard 100 milligrams per 100 milliliters aqueous glucose solution. When a screening solution with this concentration of indophenol is added to a biological fluid such as blood serum or plasma (in the presence of an appropriate catalytic enzyme), only serum or plasma containing glucose in excess of the screening level will exhibit colorless reaction products.

For the stock indicator solution 100 milligrams of indophenol is diluted to 100 milliliters with the dilution reagent. By further diluting appropriate aliquots of this stock indicator solution with the dilution reagent an indicator screening solution is prepared. Each milliliter of indicator screening solution so prepared will contain a predetermined number of micrograms of indophenol which has a formula weight, 340 (sodium, 2,6-dichlorobenzenone-indophenel·$7.78H_2O$ as determined iodometrically).

An alternate method of preparing the indicator screening solution directly by weight is to utilize the powdered mixture of indophenol and sodium chloride. For example, for the preparation of a screening indicator solution at the 100 milligram per 100 milliliters glucose level, 3.7900 grams of sodium chloride are intimately mixed with 0.2100 gram of indophenol to give a total weight of 4.0000 grams. Of the foregoing mixture, 0.4000 gram is diluted to 45 milliliters with the dilution reagent to directly give the screening indicator solution.

If screening levels other than 100 milligrams per 100 milliliters are desired, the indicator is diluted with the dilution reagent until the solution contains 60 micrograms of indophenol in excess of that which is visibly reduced to the colorless state by 0.5 milliliter of glucose of the desired standard. For example, in a screening indicator solution prepared for a 120 milligrams per 100 milliliters glucose level, the amount of indophenol which is visibly reduced to colorless state is 840 micrograms. The total amount of indophenol, therefore, in such a screening solution is 900 micrograms.

The dilution reagent used to dilute the indicator contains proplylene glycol to insure that the indophenol dye in the presence of the enzyme and the glucose remains soluble at the pH of from about 4.0 to 5.6, and particularly at the preferred pH of about 4.6. Ethylene glycol may be substituted for the propylene glycol to perform the same function.

Ethyl alchol is included in the dilution reagent to retard in the screening reaction reoxidation of the reduced indophenol by any hydrogen peroxide which may be present.

The enzyme used in the method of the invention may be prepared from a stable stock solution or it may be prepared directly from the anhydrous form. The stock solution used contained substantially 750 units per milliliter of glucose oxidase. The glucose oxidase is buffered preferably to the pH of about 4.6, by the addition of any suitable buffer such as citric acid and anhydrous disodium hydrogen phosphate. For example, 14.0 grams of citric acid·$1H_2O$ and 16.6 grams of anhydrous disodium phosphate are diluted to 100 milliliters with water and mixed with slight warming until dissolved to give a stock buffer of a pH of about 4.6. To obtain a buffer-enzyme mixture of 75 units per milliliter, for example, 6 parts by volume of the stock buffer are mixed with 3 parts of water and one part of glucose oxidase. Other proportions of glucose oxidase may be used to give buffer-enzyme mixtures of higher enzyme concentrations.

With the foregoing materials on hand, the method of this invention may be performed for the mass screening of biological fluids such as blood serum or plasma. The term biological fluid as used herein means any fluid containing living or dead plant or animal matter, and is not limited to fluid derived from man or other animals.

If mass screening for glucose at a level of 100 milligrams per 100 milliliters is desired, the method of the invention will be performed as follows:

A plurality of biological fluid samples such as blood serum or plasma specimens are placed in respective test tubes so that each tube contains 0.5 milliliter of a particular specimen. Prior to placing the specimen in the tube, each tube is equipped with a short length of glass rod. To each tube is further added in the following order, 1 milliliter of light peroleum oil (if anaerobic conditions are desired), 1.5 milliliters of screening indicator solution, and 0.5 milliliter of a 75-unit buffer-enzyme mixture, all previously prepared as described.

The test tubes are agitated gently, as by placing them in a rack and rotating the rack so as to insure mixing of the reagents. As the rack is rotated the glass rods rotate in the tubes and assist in the mixing. The tubes are then placed in a water bath at preferably 37 to 40 degrees centigrade, although the reaction will proceed, though more slowly, at room temperature, and are again gently agitated at fifteen minute intervals. The temperature 37 to 40 degrees centrigrade is the preferred temperature because at this temperature the results attain the greatest degree of accuracy.

After a reaction time, preferably of thirty minutes, but which may be as little as five minutes if a buffer-enzyme mixture having a concentration greater than 75 units per milliliter of glucose oxidase is used and if the glucose concentration in the specimen being tested is above the predetermined limit, a point of delineation between colored and colorless solutions is observable. The tubes are then scanned, as by visual observation or by means of an electro-mechanical color scanning device of conventional type, for changes in color. A substantially unchanged deep blue indicates glucose levels below 100 milligrams per 100 milliliters. A light blue color indicates glucose levels approaching 100 milligrams per 100 milliliters, whereas a colorless solution indicates glucose levels above 100 milligrams per 100 milliliters.

Using the method of this invention, a single individual is at least able to visually screen approximately 100 blood specimens in one hour. With the aid of automatic equipment to deliver the reagents and to scan the serum specimens, the number of specimens screened may be greatly increased.

Experimentally it has been shown that protein does not affect the over-all reaction between indophenol and glucose. For that reason, no protein correction is needed in the final results.

While indophenol of formula weight 340 as determined iodometrically has been used in the examples described herein, indophenol of other purity may be used for the indicator in the method of this invention with similar results.

Although in the examples described herein sodium 2,6-dichlorobenzenone-indophenol was the indicator used, sodium 2,6-dibromobenzenone-indophenol may be substituted for sodium 2,6-dichlorobezenone-indophenol in the method of this invention with equivalent results, provided proper allowance is made for the difference in formula weight and purity of the two indophenols.

It will be apparent to one skilled in the art that various changes and modifications, singly or collectively, including the use of equivalent reagents and materials, may be made in the method of the invention without

departing from the scope thereof as defined in the appended claims and without departing from the essence of the invention.

I claim:

1. A self-indicating method for directly screening for glucose at a predetermined level in a biological fluid comprising the steps of bringing together in solution a predetermined amount of glucose-containing biological fluid, an amount of an oxidation-reduction indicator selected from the class consisting of sodium 2,6-dichlorobezenone-indophenol and sodium 2,6-dibromobezenone-indophenol, said amount of said indicator being sufficient to change color as it is reduced in response to levels of glucose in said biological fluid equal to and above said predetermined level, and an amount of a catalytic enzyme consisting of glucose oxidase buffered to a pH of from about 4.0 to about 5.6 to prevent said indicator from being reoxidized sufficiently to interfere with said color change of said indicator at said predetermined glucose level, said amount of said endyme being sufficient to act as a catalyst in the reduction of said indicator by said glucose, and warming said solution to a predetermined temperature for a period sufficient to reduce said indicator.

2. A self-indicating method for directly screening for glucose at a predetermined level in a biological fluid comprising the steps of bringing together in solution in the following order a predetermined amount of glucose-containing biological fluid, an amount of a light petroleum oil sufficient to establish substantially anaerobic conditions for said solution an amount of an oxidation-reduction indicator selected from the class consisting of sodium 2,6-dichlorobenzenone-indophenol and sodium 2,6-dibromobenzenone-indophenol, said amount of said indicator being sufficient to change color as it is reduced in response to levels of glucose in said biological fluid equal to and above said predetermined level, and an amount of catalytic enzyme consisting of glucose oxidase buffered to a pH in the range of from about 4.0 to 5.6, said pH range cooperating with said oil to prevent said indicator from being reoxidized sufficiently to interfere with said color change at said predetermined level, said amount of said enzyme being sufficient to act as a catalyst in the reduction of said indicator by said glucose, and warming said solution to a predetermined temperature for a period sufficient to reduce said indicator.

3. A self-indicating method for directly screening for glucose at a predetermined level in a biological fluid selected from the class consisting of blood serum and plasma comprising the steps of bringing together in solution a predetermined amount of glucose-containing biological fluid selected from the class consisting of blood serum and plasma, an amount of an indicator consisting of sodium 2,6-dichlorobenzenone-indophenol sufficient as it acts to indicate while acting as a hydrogen acceptor to indicate directly by color change levels of glucose in said biological fluid equal to and above said predetermined level, and an amount of a catalytic enzyme consisting of glucose oxidase buffered to a pH of 4.6 to prevent said indicator from being reoxidized sufficiently to interfere with said color change of said indicator at said predetermined glucose level, said amount of said enzyme being sufficient to act as a catalyst in the reduction of said indophenol by said glucose, and warming said solution at a temperature of from about 37 to about 40 degrees centigrade for a period sufficient to complete said reduction of said indicator.

4. A self-indicating method for directly screening for glucose at a predetermined level in a biological fluid selected from the class consisting of blood serum and plasma comprising the steps of bringing together in solution in the following order a predetermined amount of glucose-containing biological fluid selected from the class consisting of blood serum and plasma, an amount of a light petroleum oil sufficient to establish substantially anaerobic conditions for said solution, an amount of sodium 2,6-dichlorobenzenone-indophenol sufficient while acting as a hydrogen acceptor to indicate by changing color in response to the glucose in said biological fluid levels of glucose equal to and above said predetermined level, and an amount of a catalytic enzyme consisting of glucose oxidase buffered to a pH of 4.6 to prevent said indicator from being reoxidized sufficiently to interfere with said color change of said indicator at said predetermined glucose level, said amount of said enzyme being sufficient to act as a catalyst in the reduction of said sodium 2,6-dichlorobenzenone-indophenol by said glucose, and warming said solution at a temperature of from about 37 to about 40 degrees centigrade for a period sufficient to reduce said indicator.

5. A self-indicating method for directly screening for glucose at a predetermined level in a biological fluid comprising the steps of bringing together in solution in the following order a predetermined amount of glucose-containing biological fluid, an amount of light petroleum oil sufficient to establish substantially anaerobic conditions for said solution, a quantity of an aqueous screening indicator reagent containing a predetermined quantity of sodium 2,6-dichlorobenzenone-indophenol capable of changing color when it is reduced in response to levels of glucose in said biological fluid equal to and above said predetermined level, said predetermined quantity exceeding by about 60 micrograms the amount of said sodium 2,6-dichlorobenzenone-indophenol required to oxidize an amount of glucose corresponding to said predetermined level, and an amount of glucose oxidase buffered to a pH of 4.6, said pH cooperating with said oil to prevent said indicator from being reoxidized sufficiently to interfere with said color change of said indicator at said predetermined level, said amount of said glucose being sufficient to act as a catalyst in the reduction of said indophenol, and warming said solution at a temperature of from about 37 to about 40 degrees centigrade for a period sufficient to reduce said indicator.

6. A self-indicating method for directly screening for glucose at a predetermined level in a biological fluid comprising the steps of bringing together in solution a predetermined amount of glucose-containing biological fluid, a quantity of an aqueous screening indicator reagent containing a predetermined quantity of sodium 2,6-dichlorobenzenone-indophenol capable of changing color after it is reduced in response to levels of glucose in said biological fluid equal to and above said predetermined level, said predetermined quantity exceeding by about 60 micrograms the amount of said sodium 2,6-dichlorobenzenone-indophenol required to theoretically oxidize an amount of glucose corresponding to said predetermined level, and an amount of glucose oxidase buffered to a pH of 4.6 to prevent said indicator from being reoxidized sufficiently to interfere with said color change at said predetermined level, said amount of said glucose oxidase being sufficient to act as a catalyst in the reduction of said indophenol, and warming said solution at a temperature of from about 37 to about 40 degrees centigrade for a period sufficient to reduce said indicator.

7. A self-indicating method for directly screening for glucose at a predetermined level in a biological fluid selected from the class consisting of blod serum and plasma comprising the steps of bringing together in solution in the following order one part by volume of glucose-containing biological fluid selected from the class consisting of blood serum and plasma, two parts by volume of light petroleum oil to provide substantially anaerobic conditions for said solution, three parts by volume of an aqueous screening indicator reagent containing a predetermined quantity of sodium 2,6-dichlorobenzenone-indophenol capable of changing color when it is reduced in response to levels of glucose in said biological fluid equal to and above said predetermined level, said predetermined

quantity exceeding by about 60 micrograms the amount of said indophenol required to oxidize an amount of glucose corresponding to said predetermined level, and one part glucose oxidase enzyme buffered to a pH of 4.6, said pH cooperating with said oil to prevent said indicator from being reoxidized sufficiently to interfere with said color change of said indicator at said predetermined level and warming said solution at a temperature of from about 37 to about 40 degrees centigrade for a period sufficient to reduce said indicator.

8. A self-indicating method for directly screening for glucose at a predetermined level in a biological fluid selected from the class consisting of blood serum and plasma comprising the steps of bringing together in solution one part by volume of glucose-containing biological fluid selected from the class consisting of blood serum and plasma, three parts by volume of an aqueous screening indicator reagent containing a predetermined quantity of sodium 2,6-dichlorobenzenone-indophenol capable of changing color when it is reduced in response to levels of glucose in said biological fluid equal to and above said predetermined level, said predetermined quantity exceeding by about 60 micrograms the amount of said indophenol required to oxidize an amount of glucose corresponding to said predetermined level, and one part of glucose oxidase enzyme buffered to a pH of 4.6 to substantially prevent reoxidation of said indophenol, and warming said solution at a temperature of from about 37 to 40 degrees centigrade for a period sufficient to reduce said indicator.

9. A self-indicating method for directly screening for glucose at a predetermined level thereof in a biological fluid comprising the steps of bringing together in solution a predetermined amount of glucose-containing biological fluid, an amount of an oxidation-reduction indicator selected from the class consisting of sodium 2,6-dichlorobezenone-indophenol and sodium 2,6-dibromobenzenone-indophenol, said amount of said indicator being sufficient to change color when it is reduced in response to levels of glucose in said biological fluid equal to and above said predetermined level, an amount of a solvent for said indicator selected from the class consisting of propylene glycol and ethylene glycol, said amount of said solvent being sufficient to maintain the solubility of said indicator in said solution at a pH range of from about 4.0 to about 5.6, and an amount of a catalytic enzyme consisting of glucose oxidase buffered to a pH in the range of from about 4.0 to about 5.6 to prevent said indicator from being reoxidized sufficiently to interfere with said color change of said indicator at said predetermined glucose level, said amount of said enzyme being sufficient to act as a catalyst in the reduction of said indicator by said glucose, and warming said solution to a predetermined temperature for a period sufficient to reduce said indicator.

10. A self-indicating method for directly screening for glucose at a predetermined level thereof in a biological fluid comprising the steps of bringing together in solution a predetermined amount of a glucose-containing biological fluid, an amount of a light petroleum oil sufficient to establish substantially anaerobic conditions for said solution, an amount of an oxidation-reduction indicator selected from the class consisting of sodium 2,6-dichlorobenzenone-indophenol and sodium 2,6-dibromobenzenone-indophenol, said amount of said indicator being sufficient to change color when it is reduced in response to levels of glucose in said biological fluid equal to and above said predetermined level, an amount of a solvent for said indicator selected from the class consisting of propylene glycol and ethylene glycol, said amount of said solvent being sufficient to maintain the solubility of said indicator in said solution at a pH range of from about 4.0 to about 5.6, and an amount of catalytic enzyme consisting of glucose oxidase buffered to a pH in the range of from about 4.0 to about 5.6, said pH range cooperating with said oil to prevent said indicator from being reoxidized sufficiently to interfere with said color change at said predetermined glucose level, said amount of said enzyme being sufficient to act as a catalyst in the reduction of said indicator by said glucose, and warming said solution to a predetermined temperature for a period sufficient to reduce said indicator.

11. A self-indicating method for directly screening for glucose at a predetermined level thereof in a biological fluid comprising the steps of bringing together in solution under substantially anaerobic conditions a predetermined amount of glucose-containing biological fluid, an amount of an oxidation-reduction indicator selected from the class consisting of sodium 2,6-dichlorobenzenone-indophenol and sodium 2,6-dibromobenzenone-indophenol, said amount of said indicator being sufficient to change color when it is reduced in response to levels of glucose in said biological fluid equal to and above said predetermined level, an amount of a solvent for said indicator selected from the class consisting of propylene glycol and ethylene glycol, said amount of said solvent being sufficient to maintain the solubility of said indicator in said solution at a pH range of from about 4.0 to about 5.6, and an amount of a catalytic enzyme consisting of glucose oxidase buffered to a pH in the range of from about 4.0 to about 5.6 to prevent said indicator from being reoxidized sufficiently to interfere with said color change of said indicator at said predetermined glucose level, said amount of said enzyme being sufficient to act as a catalyst in the reduction of said indicator by said glucose, and warming said solution to a predetermined temperature for a period sufficient to reduce said indicator.

12. A self-indicating method for directly screening for glucose at a predetermined level in a biological fluid comprising the steps of bringing together in solution under substantially anaerobic conditions a predetermined amount of glucose-containing biological fluid, an amount of an oxidation-reduction indicator selected from the class consisting of sodium 2,6-dichlorobenzenone-indophenol and sodium 2,6-dibromobenzenone-indophenol, said amount of said indicator being sufficient to change color as it is reduced in response to levels of glucose in said biological fluid equal to and above said predetermined level, and an amount of a catalytic enzyme consisting of glucose oxidase buffered to a pH of from about 4.0 to about 5.6 to prevent said indicator from being reoxidized sufficiently to interfere with said color change of said indicator at said predetermined glucose level, said amount of said enzyme being sufficient to act as a catalyst in the reduction of said indicator by said glucose, and warming said solution to a predetermined temperature for a period sufficient to reduce said indicator.

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