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COMPOSITION FOR DETERMINATION OF GLUCOSE

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This invention relates to a composition providing means for testing biological and chemical fluids for the presence of glucose. More particularly, it relates to a method of high sensitivity for testing body fluids for small quantities of glucose. Still more particularly, it relates to an enzyme system useful in the testing for glucose characterized by the absence of catalase activity.

A simple, rapid and reliable test for detecting the presence of glucose has been sought as an aid for rapid screening of samples as well as for assaying glucose concentration.

The most widely known and conventional method for determination of glucose is based upon the reduction of a metal ion by the glucose when the solution is rendered alkaline and subjected to heat. The Somogyi and Benedict tests are based upon the use of alkaline copper solutions.

Recently a composition has been used in the testing for glucose consisting of two enzymes, an indicator, a buffer and a stabilizer such as gelatin. The enzymes used are known as glucose oxidase and peroxidase. The glucose oxidase enzyme in the presence of moisture and oxygen converts glucose to gluconic acid and at the same time produces hydrogen peroxide. The peroxidase enzyme acts to catalyze the oxidation of certain indicators such as oxidizable dyes when present together with such dye and hydrogen peroxide. Such a system is limited to the use of indicators which are oxygen acceptors.

Peroxidase is a relatively difficult and expensive enzyme to prepare. Elimination of peroxidase enzyme from test compositions so as to have a single enzyme system, offers a chance for a less expensive composition operating in a more reliable manner.

It has now been discovered that an effective means for detecting glucose in various materials including body fluids such as urine, blood, cervical mucus, as well as in starch solutions and glucose in splittable complexes, etc., involving the reaction of only one enzyme, i.e., glucose oxidase, exists which is free of many of the disadvantages characterizing prior compositions, testing means and procedures.

In practicing the invention, a composition is prepared comprising glucose oxidase enzyme substantially free of proteins functioning to produce peroxidase activity, an iodide salt and compound which undergoes a color reaction with iodine liberated by oxidation of iodide present in salts such as sodium iodide and potassium iodide or mixtures thereof.

Such a composition may be made into a suspension or solution and used to impregnate bibulous material such as paper, wood, fiber or the like having any desired size or shape. Compositions of this invention may be utilized by segregating a test quantity of aqueous solution of test material and adding a drop of the suspect glucose containing material. If the test solution develops color in accordance with the system used, glucose is present. A

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distinct color change will also be developed if glucose containing material is brought in contact with wet bibulous material impregnated with composition, or with dry impregnated bibulous material in the presence of an activating amount of moisture, as well as when a portion of composition is brought in contact with bibulous paper impregnated with test sample. In this situation color develops over the wetted area on the paper.

In general, the compositions may comprise a system containing glucose oxidase, iodide salt and an indicator system, and optional constituents such as buffers and contrasting background colors. Buffers serve a useful purpose through maintenance of an optimum pH for reaction, color change, and the like. Background colors make changes more apparent and thereby add to the sensitivity of the test.

Iodide salts useful in the test indicator compositions for detecting glucose are water soluble salts such as ammonium iodide, alkali metal iodides such as sodium iodide, potassium iodide, multivalent-metal iodides such as copper iodide, magnesium iodide, calcium iodide, ferrous iodide, cobaltous iodide, and nickel iodide.

The purpose of buffers in the compositions is to maintain reaction conditions generally in a specific range. When using an iodide salt such as sodium iodide, potassium iodide, etc., the oxidation of iodide ion to iodine takes place at any pH. However, a solution containing iodide salt is generally buffered to maintain a pH between about 4 and about 7.5, which range is optimum for glucose oxidase activity. Suitable buffers for use in these compositions are sodium phosphates, a mixture of citric acid and sodium citrate dihydrate, etc.

When contrast dyes are used in the compositions, the amount should not be such as to mask color changes. About 2-5 parts by weight of dye such as erythrosine, tartrazine, etc., usually produce sufficient contrast. Almost any contrasting color can be used which is different from the color exhibited by the indicator before and after the color change.

The iodide component of an iodide salt, for example, potassium iodide, is converted to the free iodine state by hydrogen peroxide formed during the reaction of glucose and glucose oxidase. In the presence of starch, free iodine reacts to produce a characteristic color varying from black to blue to red depending upon the type of starch, starch derivative such as cationic starch or starch degradation products such as erythrodextrine. Other compounds which undergo a color reaction when contacted with iodine are orthotolidine dihydrochloride, ortho dianisidine, meta-toluidine, guaiacol, 2,7 diamino fluorene, benzidine and analogues thereof, and the like.

In preparing the test compositions, a sequestering agent may be used which ties up heavy metal ions having a tendency to promote non-specific oxidations or to minimize side reactions. For this purpose, compounds such as salts of ethylene diamine tetraacetic acid, and the like may be used.

When conducting a test with treated paper, for example, it is desirable that there be a well defined area to provide contrast with unreacting portions of the paper. Such a well defined area can be produced by incorporating an agent such as higher molecular weight polyethylene glycols, methyl cellulose, hydroxy propyl methyl cellulose, sodium polypectate, pectin, peptone, gelatin, gelatinized

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starch, or mixture thereof in the impregnant for the paper or in solution applied to the paper in conducting the test which increases solution viscosity and/or acts as humectant. Upon wetting papers treated to have these agents present, a well defined wetted area is obtained which promotes uniformity and intensity of color development over a desired test period of time. Of the agents disclosed, derivatives such as methyl cellulose, hydroxypropylmethylcellulose and the like are preferred because they produce a more uniform colored area than the peptones or gelatin.

Compositions are standardized so as to provide qualitative and/or quantitative tests. Enzyme preparations containing glucose oxidase substantially free of catalase and amylase may be prepared in accordance with known procedures.

Components of the composition may be varied within wide limits. In general, impregnating solution should contain not less than 10 units/ml. of glucose oxidase activity unless extended reactions may be employed. Lesser concentrations are usually avoided because of a desire to maintain an appreciable color reaction within a 1 to 10 minute period. Glucose oxidase will generally have an activity between 200 and 1000 units per ml., a unit being determined by standard test as a quantity of enzyme giving predetermined rate of oxygen uptake at 30° C.

When desirable, the sample to be tested may be pretreated with an absorbent such as charcoal and the like to remove interfering substances prior to bringing it into contact with the compositions herein disclosed.

The invention will be more fully understood from the following examples which are given by way of illustration and without any intention of the invention being limited thereto.

EXAMPLE I

A solution was prepared as follows:

- 5 ml. of solution containing 400 glucose oxidase units/ml. essentially devoid of both catalase and amylase
 - 5 ml. 10% potassium iodide
 - 10 ml. soluble starch solution (1.2% Lintner in pH 4.6 0.02 M acetate buffer)
- Paper was impregnated therewith

A second solution, prepared with substantially catalase free glucose oxidase enzyme 750 units/ml. containing amylase, turned blue virtually instantly and was discarded.

On testing solution A paper with varying glucose concentrations, the following results were obtained:

Table I

Time in Minutes	Glucose Concentration					
	0	0.125%	0.25%	0.5%	1.0%	2.0%
0.-----	-	-	-	-	-	-
0.5.-----	-	-	-	-	-	-
1.0.-----	-	+	+	+	+	+
1.5.-----	-	+	+	+	+	+
5.-----	-	+	+	+	+	+
10.-----	-	+	+	+	+	+

+ signs indicate extent of color change.

EXAMPLE II

Two solutions were prepared as follows:

- A. 10 ml. distilled water
- 5 ml. 10% potassium iodide
- 2 ml. o-tolidine solution (4% in 3A ethanol)
- 3 ml. glucose oxidase 750 units/ml. substantially catalase-free
- 0.1 ml. of 0.5% amaranth red added to give pink background color
- B. As above but using an enzyme containing 750 units glucose oxidase and 330 units catalase per ml.

Each solution was used to impregnate Whatman #1

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paper and the paper air-dried. Six strips measuring about 1 x 2 cm. were then cut from each paper and these were arranged on a white porcelain surface. Glucose solutions of Merck's anhydrous dextrose were prepared ranging in concentration from 0 to 2%. One drop of each concentration was then placed on separate pieces of each type of paper almost simultaneously. In the following table the degree of color development is given by the number of plus signs: a negative sign shows no color change.

Table II

PAPER A

Time in Minutes	Glucose Concentration					
	0	0.125%	0.25%	0.5%	1.0%	2.0%
0.-----	-	-	-	-	-	-
0.5.-----	-	+	+	+	+	+
1.0.-----	-	+	+	+	+	+
1.5.-----	-	+	+	+	+	+
5.-----	-	+	+	+	+	+
10.-----	-	+	+	+	+	+

PAPER B

Time in Minutes	Glucose Concentration					
	0	0.125%	0.25%	0.5%	1.0%	2.0%
0.-----	-	-	-	-	-	-
0.5.-----	-	+	+	+	+	+
1.0.-----	-	+	+	+	+	+
1.5.-----	-	+	+	+	+	+
5.-----	-	+	+	+	+	+
10.-----	-	+	+	+	+	+

At the end of 10 minutes, Paper B with 2% glucose had developed only as much color as the paper, impregnated with solution A (the low catalase paper) containing 0.25% glucose developed in 1½ minutes.

EXAMPLE III

A mixture was made up as follows:

- 3 ml. a solution contg. 750 units glucose oxidase and 70 units catalase/ml.
- 10 ml. de-ionized water
- 5 ml. 10% KI solution
- 2 ml. o-tolidine solution (4% in 3A ethanol)

Eaton-Dikeman papers #609 and 613 were impregnated with this solution and allowed to dry. Both developed dark blue coloring on addition of 3% glucose solution.

EXAMPLE IV

A mixture was prepared as follows:

- 7.5 ml. 2% soluble starch solution (Merck #74881 iodometry starch)
- 0.25 gm. citrate mixture (35 g. citric acid monohydrate 66 g. trisodium citrate duohydrate)
- 0.25 gm. disodium salt of ethylene diamine tetraacetic acid (Na₂ EDTA)
- 0.75 ml. 40% potassium iodide solution
- 375 units glucose oxidase essentially free of catalase and amylase adjusted to pH 6.0 with NaOH
- Water to 15 ml.

This was then split into 5 portions of 3 ml. each; gelatin was added as follows:

Table III

	Ml. above mix	Ml. 5% gelatin	Ml. H ₂ O	Final percent gelatin
A.-----	3.0	2.0	0.0	2.0
B.-----	3.0	1.0	1.0	1.0
C.-----	3.0	0.4	1.6	0.4
D.-----	3.0	0.2	1.8	0.2
E.-----	3.0	0	2.0	0

E and D paper #613 was impregnated by dipping and air-dried.

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A 0.25% glucose solution was dropped on each. This drop remained intact on A and B, spread on C, D, E.

Color development in test with 0.25% glucose solution is as follows:

Table IV

Time in Minutes	Paper				
	A	B	C	D	E
1.....	-	-	[+]	[+]	[+]
2.....	-	-	[++]	[++]	[+]
3.....	-	[+]	[+++]	[+++]	[+]
4.....	[+]	[+]	[++++]	[++++]	[+]
5.....	[+]	[++]	[++++]	[++++]	[+]
6.....	All gave dark borders and dark centers				

Bracketed values represent border color. Unbracketed values represent center color. Minus signs indicate no visible color change.

From this data it is apparent that there is a minimum concentration of material for iodide-containing compositions required to impart the limited hydrophobic properties to the test paper to maintain a well defined test area and to avoid diffusion of composition into migratory fringe areas of inconclusive color quality, shade and depth. For gelatin, concentrations between about 0.2 and about 0.7% are preferred for buffered compositions.

EXAMPLE V

A mixture was prepared as follows:

7.5 ml. 2% soluble starch (Merck #74881)
0.25 gm. sodium salt of ethylene diamine tetraacetic acid
0.75 ml. 40% KI
2.0 ml. 5% gelatin
375 units-glucose oxidase essentially free of catalase and amylase
Adjusted pH to 6.1

Citrate buffer was added to portions of the above composition as follows:

Table V

	Ml. above mixture	Ml. buffer solution ¹	Ml. H ₂ O	Approx. final molarity citrate
A.....	3.0	2.0	0	0.4
B.....	3.0	0.2	1.8	0.04
C.....	3.0	0.02	1.98	0.004
D.....	3.0	0	2.0	0

¹ Buffer solution consisted of 25% solution of citrate mixture of 33 grams citric acid monohydrate with 66 grams trisodium citrate dihydrate.

Eaton-Dikeman #613 paper impregnated as before and air dried. Pieces of each were spotted with 0.25% glucose solution.

Color development in tests with 0.25% glucose solution was as follows:

Table VI

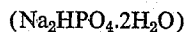
Time in Minutes	Paper			
	A	B	C	D
1.....	-	[+]	[+]	[+]
2.....	-	[++]	[++]	[+]
3.....	[+]	[+++]	[+++]	[+++]
5.....	[+]	[++++]	[++++]	[++++]

Consistent test results require the presence of some buffer to overcome the effect of acid formed in the reac-

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tion. Such buffering action, however, should not be such as to slow down the reaction. Buffer concentration in impregnating solution and for impregnated paper of between about 0.04 molar and about 0.004 molar is preferred.

Solutions were prepared as above except for the substitution of phosphate buffer for citrate buffer in equimolar amounts. A typical phosphate buffer consists of 13.4 grams of disodium hydrogen phosphate



mixed with 58.5 grams of sodium dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$).

This inorganic buffer shows substantially the same concentration effect as the buffer containing salts of organic acids.

EXAMPLE VI

A mixture was prepared as follows:

7.5 ml. 2% soluble starch (Merck #74881)
2.0 ml. 5% gelatin
0.36 gm. phosphate buffer prepared as in Example IV
1.25 ml. 40% KI
375 units glucose oxidase essentially free of catalase and amylase
Water to 15 ml.

The mixture was split into four portions of 3 ml. each. Concentration of the sodium salt of ethylenediaminetetraacetic acid (indicated below by Na_2EDTA) was varied as follows:

Table VII

	Ml. above mixture	Ml. 2.5% Na_2EDTA	Ml. H ₂ O	Final percent Na_2EDTA in impregnating solution
A.....	3.0	2.0	0	1.0
B.....	3.0	1.0	1.0	0.5
C.....	3.0	0.5	1.5	0.25
D.....	3.0	0	2.0	0

Color development in tests with 0.25% glucose solution was as follows:

Table VIII

Time in Minutes	Paper			
	A	B	C	D
1.....	[+]	[+]	[+]	[+]
2.....	[+]	[+]	[+]	[+]
3.....	[+++]	[+++]	[+++]	[+++]
5.....	[++++]	[++++]	[+++]	[+++]

The sodium salt of ethylenediaminetetraacetic acid acts as a sequestering agent. The presence of sequestering agents tend to stabilize impregnated paper. Paper impregnating with solutions containing in excess of about 0.5% of sodium salt of ethylenediaminetetraacetic acid are preferred.

It is apparent that the glucose oxidase concentration as well as other components of the composition can be varied to give a positive test at any predetermined glucose level within a specific test interval. In general, an impregnating solution should contain not less than about 10 units per ml. although greater amounts as evidenced by the examples can be used to produce a faster reaction. In the above examples, starch was used in amounts which would develop a strong depth of color in the period of 3 to 10 minutes. In general, soluble starch may be incorporated in the impregnating solutions in amounts of between 0.2 and 2% concentration.

EXAMPLE VII

A stock solution was prepared as follows:

16.5 soluble starch solution (2% Lintner)
720 mg. phosphate buffer (solid mixture of disodium
and mono sodium phosphate)
2.5 ml. 40% potassium iodide
250 mg. sodium salt of ethylenediaminetetraacetic acid
Adjust pH to 6.0 and then add
750 units glucose oxidase essentially free of catalase and
amylase
11 ml. water

Paper impregnating solution was prepared as follows:

3 ml. stock solution
1.2 ml. of water
0.8 ml. of 2.5% solution of each of the materials listed
below

Four types of papers indicated E, F, G and H were
prepared by impregnating Eaton-Dikeman #613 paper
with solution containing hydroxypropylmethylcellulose
(representative of methyl cellulose materials), sodium
polypectate, peptone and gelatin, respectively.

Color development in test with 0.25% glucose solution
is as follows:

Table IX

Time in Minutes	E	F	G	H
1.....	[+]	[+]	[+]	[+]
2.....	[++]	[+]	[++]	[++]
3.....	[+++]	[++]	[+++]	[+++]
5.....	[++++]	[+++]	[++++]	[++++]

From the above data, it is apparent that compositions
containing hydroxypropylmethylcellulose develop color
on a more uniform basis, i.e., less difference at the end
of 5 minutes between peripheral area (color depth in-
dicated within brackets) of the test spot and the center
of the test spot.

Although the present invention has been described in
connection with various examples of its use, it is not in-
tended that the details of the examples shall be regarded
as limitations upon the scope of the invention, except
insofar as they are included in the accompanying claims.

I claim:

1. A composition for detecting the presence of glucose
which comprises glucose oxidase enzyme substantially
free of proteinaceous material functioning to produce
peroxidase and catalase activity, iodide salt, a compound
which undergoes a color reaction when contacted with
iodine produced from the iodide of the salt, and a
sequestering agent.
2. A composition for detecting the presence of glucose
which comprises glucose oxidase substantially free of
proteinaceous material functioning to produce peroxi-
dase and catalase activity, iodide salt, a compound which
undergoes a color reaction when contacted with iodine
produced by oxidation of the iodide and the sodium salt
of ethylenediaminetetraacetic acid.
3. A composition for detecting the presence of glucose
which comprises glucose oxidase enzyme substantially
free of proteinaceous material functioning to produce
peroxidase and catalase activity, iodide salt, a compound
which undergoes a color reaction when contacted with
iodine produced from the iodide of the salt, a compound
selected from the group consisting of higher molecular
weight polyethylene glycols, methyl cellulose, hydroxy
propyl methyl cellulose, sodium polypectate, pectin, pep-
tone, gelatin, gelatinized starch, and mixtures thereof in
quantities adapted to impart limited hydrophobic prop-
erties to bibulous material, and a sequestering agent.
4. A composition for detecting the presence of glucose
which comprises glucose oxidase substantially free of
proteinaceous material functioning to produce peroxidase
and catalase activity, alkali metal iodide, a buffer for
maintaining a pH in the range between about 4 and about
7.5, ortho toluidine dihydrochloride and a sequestering
agent.

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