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[54] **HYDROPEROXIDE DIAGNOSTIC AGENTS CONTAINING A CHROMOGEN INDICATOR**
18 Claims, No Drawings

[52] **U.S. Cl.** ..... **252/408,** 23/230 B, 23/253 TP, 195/103.5, 260/241
[51] **Int. Cl.** **G01n 31/14,** G01n 31/22
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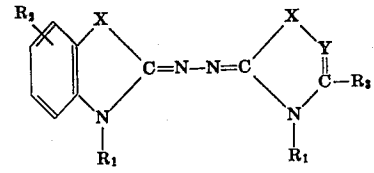
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ABSTRACT:



HYDROPEROXIDE DIAGNOSTIC AGENTS CONTAINING A CHROMOGEN INDICATOR

This application is a continuation-in-part of Ser. No. 762,965 filed by Rey et al. on Sept. 26, 1968.

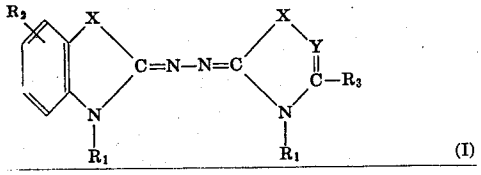
This invention relates to diagnostic agents for use in carrying out rapid analytical determinations and to methods for manufacturing and using such agents.

More particularly this invention relates to diagnostic agents for use in the analytical determination of hydroperoxides and of substances from which hydrogen peroxide and other hydroperoxides can be liberated by a previous reaction, as well as for the determination of peroxidase and other peroxidate-active substances.

The detection of glucose in urine, blood and serum has, in the case of diabetes, acquired considerable clinical importance as has the detection of peroxidate-active substances, such as hemoglobin in urine and blood, and the detection of hydroperoxides in, for example, the milk industry, the cosmetics industry and in polymer chemistry.

A series of compounds are known which are oxidized to dyestuffs by means of hydrogen peroxide and peroxidase as catalyst. Compounds of this type include, for example, the Nadi reagents, *p*-phenylene-diamine derivatives, carboazines, quaiac resin, aldazine and the like. Other compounds previously preferred for this purpose include for example benzidine, *o*-dianisidine, *o*-tolidine and guaiacol. However, it has been established that in practice certain of these compounds are not very stable and further according to recent findings, that they can also be dangerous to the health of the personnel working with them so that their use does not appear to be indicated in view of danger to personnel or reliability of results.

In accordance with the invention it has now surprisingly been found that compounds having the following formula:



(I)

wherein R_1 is lower alkyl; R_2 is hydrogen, a sulfonic acid group or an alkali metal sulfonate group; R_3 is hydrogen, halogen, e.g. chlorine, or lower alkyl; X is sulfur, oxygen, a carbon atom substituted with two lower alkyl groups, substituted or unsubstituted vinylene or imino, wherein said substituent is lower alkyl or aryl; and Y is nitrogen or methine which, when taken together with R_3, can form a benzene ring substituted by R_2; are absolutely stable, physiologically safe and are particularly well suited for use in the determination of hydrogen peroxide, hydroperoxides, peroxidase and peroxidatively active substances carried out by means of colorimetry, optical tests and with test papers and test film.

It is known that compounds having the formula I as set out above can be oxidized by means of comparatively strong oxidation agents, such as lead tetraacetate, ceric sulfate and potassium persulfate, to provide colored oxidation products (*cf.* S. Hunig et al. Liebigs Ann. Chem., 676, 32–65/ 1964; H. Lang et al., Z. Analyt. Chem., 201, 321/1964). However, no reaction takes place in the presence of hydrogen peroxide alone. It was therefore not to have been expected that reproducible and clear colorations could also be obtained for hydrogen peroxide in the presence of peroxidase or of peroxidatively active substances.

According to a further feature of the present invention, it has now been found, that especially advantageous and clearly graduated color changes are realized when the compounds of formula I are used in admixture with reduction agents having the formula:



(II)

wherein *Ar* is benzene or naphthalene, V is hydroxyl or amino, V′ is hydrogen, hydroxyl or amino, which may be alkylated or arylated, W is amino, carboxy, a sulfonic acid group, alkyl, alkoxy or aryl and W′ is hydrogen, amino, carboxy or a sulfonic acid group, an alkali metal carboxylate group or sulfonate group, alkyl, alkoxy or aryl.

These compounds of formula II do not themselves give any coloration or, at most, only give rise to very nonspecific colorations and are, therefore, generally not suitable per se for use as indicators. However, as already mentioned above, when these compounds of formula II are used together with the compounds of formula I, there are realized particularly clear and well-graduated color changes.

Thus, according to the present invention, there is provided a process for the determination of hydroperoxides and of substances which react with the liberation of hydrogen peroxide, as well as of peroxidase and of peroxidatively active substances, comprising a chromogen which is oxidized by hydrogen peroxide or hydroperoxide in the presence of peroxidase or peroxidatively active substance to form a colored dyestuff, the color intensity of which can be evaluated in the conventional manner to indicate the quantity of hydrogen peroxide, hydroperoxide, peroxidase or peroxidatively active substance present in the specimen being analyzed wherein a compound of formula I is used as chromogen, preferably in admixture with a compound of formula II.

The evaluation of the coloration can be carried out, for example, by optical measurement using therefor a spectrophotometer or, when test paper strips and test films are involved, by comparison of the color intensity with standard color scales or standard comparison solutions or with color charts.

The determination of the presence of hydroperoxides by the process according to the present invention is particularly useful for coupled and uncoupled enzyme reactions, as for example, for the determination of glucose, galactose, amino acids, uric acid, peroxides, hemoglobin, peroxidase or other peroxidatively active substances, as well as of enzyme activities. Because of the increasing importance of such determinations, the routine determination of substances of this type is now an essential feature of clinical chemistry and of foodstuff chemistry.

In the case of the determination of glucose, the latter is, for example, oxidized by glucose-oxidase to gluconic acid, atmospheric oxygen thereby being reduced to hydrogen peroxide. In the presence of peroxidase or of a peroxidatively active substance, the hydrogen peroxide then oxidizes the indicator or chromogen (formula I) used according to the present invention to provide the corresponding color dyestuff.

Further examples of analytically useful enzyme systems of this type, which react with the liberation of hydrogen peroxide, include L-amino acid oxidase +L-amino acids, uricase +uric acid, xanthine oxidase +hypoxanthine or xanthine, glycine oxidase +glycine, monoamine oxidase +monoamine (such as adrenaline, mescaline and the like), diamine oxidase +diamine (such as histamine) luciferase +luciferin, D-aspartic acid oxidase +D-aspartic acid, liver aldehyde oxidase +aldehyde, galactose oxidase +galactose, Edson's flavine enzyme +lactic acid.

According to a further feature of the present invention, there is provided a diagnostic agent for the determination of hydroperoxides and of substances which react with the liberation of hydrogen peroxide, which comprises peroxidase or a peroxidatively active substance and a chromogen having the formula I alone or in admixture with a compound of formula II.

According to yet another feature of the present invention, there is provided a diagnostic agent for the determination of hemoglobin and of other peroxidatively active substances, which comprises hydrogen peroxide or a substance forming hydrogen peroxide and a chromogen of formula I alone or in admixture with a compound corresponding to formula II.

It is to be understood that, within the context of the present invention, the expression "a substance forming hydrogen peroxide" is intended to mean not only a single compound, such as an organic peroxide, but also a mixture, such as glucose and glucose-oxidase.

It is further to be understood that the new diagnostic agents according to the present invention can be prepared in the form of solutions in appropriate solvents, if necessary with the addition of conventional adjuvants, such as buffers. Alternatively, the new diagnostic agents can be prepared in the form of test papers by the impregnation of suitable adsorbent materials, such as filter papers, with solutions of the components of the diagnostic reagents or such solutions can be used for making reagent films. In this connection, as in the case of the diagnostic solutions, it is frequently advantageous for the test papers and films to be prepared from solutions which contain conventional adjuvants, such as buffers.

Finally, mention should be made of the fact that the process according to the present invention can, in addition, be used for the determination of the chromogens of formula I as well as of the compounds of formula II, utilizing therefor a hydroperoxide and a peroxidate-active substance. This has proved to be very useful for control purposes in the preparation of these diagnostic materials.

The preparation of the compounds I can be carried out in known manner by the reaction of 2-halo- or 2-mercapto-quaternary salts with an appropriate N-alkylated hydrazone salt, with the addition of an amine, preferably of triethylamine, in a polar solvent, such as methanol, at a temperature of 15°–100° C. Depending upon the reactivity of the individual reaction components, the reaction is finished with a period of 10 minutes to 2 hours (S. Hünig et al., Liebig's Ann. Chem., 676, 32–65/1964). The preparation of the hydrazones used as starting materials, as well as of the N-quaternized salts, takes place in the manner described by S. Hünig Ann. Chem., 609, 160–180/1957) and by H. Balli (Liebig's Ann. Chem., 647, 1–8/1961).

A more comprehensive understanding of the invention may be obtained by referring to the following illustrative examples which are not intended, however, to be unduly limitative of the invention.

Example 1

Reagent Film for the Detection of Glucose

45 g. Propiofan (BASF), 35 g. of a solution of 37 g. Algipon in 2 liters of a 0.5M phosphate or citrate buffer having a pH of 5.7, 1 g. Texapon P (Henkel, Düsseldorf), 10 ml. water, 0.075 g. peroxidase and 0.15 g. glucose oxidase were stirred together to give a homogeneous slurry. To this mixture, there were added 6 ml. of a 5 percent aqueous solution of 2,2'-azino-di-(3-ethylbenzthiazolone-6-sulfonic acid). Mixtures prepared in this manner, contain 0.2–0.3 percent of indicator. Foils were then coated with the mixture with a coating thickness of 300$\mu$and thereafter dried.

When glucose solutions of varying concentrations were evaluated with the above-described foils poorly differentiated green colorations were observed.

On the other hand, if the above-described mixture contained, in place of the aqueous solution of 2,2'-azino-di-(3-ethyl-benzthiazolone-6-sulfonic acid), 6 ml. of a 5 percent solution of *p*-amino-salicylic acid, then the test strips, upon being moistened with aqueous solutions of glucose, only became pale yellow in color and further showed no graduation in coloration.

However, if there was prepared a mixture analogous to the one described above but so it contained 6 ml. of an aqueous methanolic solution of a mixture of 5 percent 2,2 '-azino-di-(3-ethyl-benzthiazolone-6-sulfonic acid) and 5 percent *p*-amino-salicylic acid, then using this mixture there could be prepared test strips which, depending upon the glucose concentration in the solutions tested produced colors in graduations from green to violet.

Example 2

Detection of Glucose in Blood, Urine or Serum

Using a procedure analogous to that described in example 1, reagent films were produced with the following compounds (I):

A. 2,2'-azino-di-(3-ethyl-benzthiazolone-6-sulfonic acid)
B. 2,2'-azino-di-(1-ethyl-quinoline-6-sulfonic acid)
C. 2,2'-azino-di-(1,3-diethyl-benzimidazolone-6-sulfonic acid)
D. 1-[3-methyl-benzthiazolone-(2)]-2-[1-ethyl-pyridone-(2)]-azine
E. 1-[3-ethyl-benzthiazolone-(2)]-2-[1-phenyl-3-methyl-4-ethyl-1,2,4-triazolone-(5)]-azine
F. 1-[3-ethyl-benzthiazolone-(2)]-2-[1-ethyl-quinolinone-(2)]-azine
G. 1-[3-methyl-benzthiazolone-(2)]-2-[1-phenyl-3-methyl-4-ethyl-1,2,4-triazolone-(5)]-azine
H. 1-[1-ethyl-quinolinone-(2)]-2-[1ethyl-pyridone-(2)]-azine
I. 1-[3-ethyl-benzthiazolone-(2)]-2-[1-ethyl-pyridone-(2)]-azine
J. 1-[3-methyl-benzthiazolone-(2)]-2-[1-ethyl-quinolinone-2)]-azine
K. 1-[3-methyl-benzthiazolone-(2)]-2-[1-ethyl-6-chloropyridone-(2)]-azine
L. 1-[1-ethyl-quinolinone-(2)]-2-[1-ethyl-6-chloropyridone-2)]-azine
M. 2,2'-azino-di-(3-ethyl-benzthiazolone)
N. 2,2'-azino-di-(3-ethyl-benzoxazol-2-one)
O. 1-(3-ethyl-benzoxazol-2-one)-2-(1-ethyl-pyrid-2-one)-azine
P. 1-(3-ethyl-benzoxazol-2-one)-2-(3-ethyl-benzthiazol-2-one)-azine
Q. 1-(3-ethyl-benzoxazol-2-one)-2-(1-ethyl-quinoline-2-one)-azine
R. 1-(1-ethyl-3,3-dimethyl-indoline-2-one)-2-(3-ethyl-benzthiazol-2-one)-azine

The above-mentioned compounds coming within the scope of formula I were used in combination with a number of different compounds within the scope of formula II.

The resulting color changes produced when the corresponding reagent films were dipped into or moistened with various glucose-containing solutions, such as blood, urine, or serum, were evaluated after a short period of time. The specific details of the color reagents used and the color changes obtained therewith are summarized in the following table.

TABLE I

Indicator (I)	Indicator conc. percent	Modifier (II)	Modifier conc. percent	Dissolved in—	Color reaction depending on glucose conc.
A	0.2				Green (graduated).
A	0.15	o-Aminophenol-p-sulfonic acid	0.2	Water	Yellow-green.
A	0.2	(1-amino naphthol-8)-2,4-disulfon8c acid	0.2	do	Yellow-green to grey-pink.
A	0.3	(7-aminonaphthol-1)-3,6-disodium sulfonate	0.05	do	Pink-green.
A	0.3	Naphthyl-1-amine-6-sulfonic acid	0.05	N/50 NaOH soln	Do.
A	0.3	Naphthyl-1-amine-8-sulfonic acid	0.1	Dimethyl formamide	Do.
A	0.3	p-Aminosalicylic acid	0.1	Methanol	Green-violet.
A	0.3	o-Amino-phenol	0.01	do	Yellow-green.
A	0.3	m-Amino-phenol	0.007	Water	Red-brown red.
A	0.3	p-Phenetidine	0.05	Methanol	Violet-green.
A	0.15	2-hydroxy-4-amino-4'-methoxy-diphenyl-amine	0.15	N/50 NaOH Soln	Green-gray.
A	0.3	α-Naphthol-2-sulfonic acid	0.1	Water	Red-violet to dark red-violet.
B	0.01				Violet (graduated).

TABLE I

Indicator (I)	Indicator conc. percent	Modifier (II)	Modifier conc. percent	Dissolved in—	Color reaction depending on glucose conc.
B	0.01	o-Amino-phenol-p-sulfonic acid	0.1	Water	Yellow brown to violet.
B	0.01	(1-amino-naphthol-8)-2,4-disulfonic acid	0.1	do	Grey-violet to blue.
B	0.01	Naphthyl-1-amine-6-sulfonic acid	0.1	N/50 NaOH soln	Grey-violet.
C	0.1	p-Amino-salicylic acid	0.1	Methanol	Blue-red.
D	0.3				Green to dark green.
D	0.3	p-Amino-salicylic acid	0.3	Methanol	Grey-violet to violet.
E	0.3				Pink.
E	0.3	p-Amino-salicylic acid	0.25	Methanol	Rose to red-violet.
F	0.3				Lilac.
F	0.3	p-Amino-salicylic acid	0.3	Methanol	Rose to red-violet.
G	0.2				Blue.
G	0.2	p-Amino-salicylic acid	0.2	Methanol	Blue-violet.
H	0.3				Lilac.
H	0.3	p-Amino-salicylic acid	0.2	Methanol	Pale to dark violet.
I	0.2				Pale pink.
I	0.2	p-Amino-salicylic acid	0.3	Methanol	Pink to dark pink.
J	0.3				Grey-green.
J	0.3	p-Amino-salicylic acid	0.2	Methanol	Red-brown.
K	0.1				Yellow-green to green.
K	0.1	p-Amino-salicylic acid	0.1	Methanol	Yellow-green.
L	0.1				Brown.
L	0.1	p-Amino-salicylic acid	0.1	Methanol	Do.
M	0.2				Green.
M	0.2	p-Amino-salicylic acid	0.2	Methanol	Grey-green to violet.
N	0.3				Lilac.
N	0.3	(1-amino-naphthol-8)-2,4-disulphonic acid	0.2	Water	Green.
N	0.3	Naphthyl-1-amine-6-sulphonic acid	0.05	N/50 NaOH	Pink.
N	0.3	p-Aminosalicylic acid	0.1	Methanol	Orange.
N	0.3	m-Aminophenol	0.007	Water	Do.
O	0.3	0-Aminophenol-p-sulphonic acid	0.2	do	Blue.
O	0.3	(1-aminonaphthol-8)-2,4-disulphonic acid	0.2	do	Green.
O	0.3	p-Aminosalicylic acid	0.1	Methanol	Pink.
O	0.3	m-Aminophenol	0.007	Water	Do.
O	0.3	p-Phenetidine	0.05	Methanol	Red-brown.
P	0.3	Naphthyl-1-amine-6-sulphonic acid	0.05	N/50 NaOH	Flesh colored.
P	0.3	Naphthyl-1-amine-8-sulphonic acid	0.1	Dimethyl formamide	Pink violet.
P	0.3	p-Aminosalicylic acid	0.1	Methanol	Orange.
Q	0.3	o-Aminophenol-p-sulphonic acid	0.2	Water	Violet.
Q	0.3	(1-aminonaphthol-8)-2,4-disulphonic acid	0.2	do	Green.
Q	0.3	p-Aminosalicylic acid	0.1	Methanol	Pink.
R	0.3				Red.
R	0.3	p-Aminosalicylic acid	0.1	Methanol	Lilac.
R	0.3	m-Aminophenol	0.007	Water	Violet.
R	0.3	(1-aminonaphthol-8)-2,4-disulphonic acid	0.2	do	Bright green-grey green.

The percentages given in the above table refer to the whole mixture; the compounds (I) A–C were dissolved in water and D–R were dissolved in methanol.

Example 3

Detection of Blood in Urine

Filter paper (Schleicher & Schüll No. 2316) was impregnated with a 0.1M phosphate buffer solution having a pH of 7.0 in which 1 percent Texapon P (Henkel, Düsseldorf) had been dissolved and then the impregnated paper was dried. The dried paper was thereafter impregnated a second time with an aqueous solution of an indicator mixture (0.03 percent, 2,2'-azino- di-[1-ethyl-quinoline-6-sulfonic acid] and 0.1 percent *o*-amino-phenol- *p*-sulfonic acid) and dried.

When, to a paper thus prepared, there was applied a drop of a blood-containing urine and a drop of 3 percent hydrogen peroxide solution then, even at a dilution of 1:50,000, the paper became red-violet in color.

If, instead of *o*-amino-phenol-*p*-sulfonic acid there was used 1-amino-8-naphthol -2,4-disulfonic acid, then, at a dilution of 1:50,000, the paper became blue-violet in color.

Example 4

Detection of Hydroperoxide in Liquids

a. Filter paper (Schleicher & Schüll No. 2316) was impregnated with a 0.1M phosphate buffer solution having a pH of 6.0 and in which there had been dissolved, per 50 ml. of buffer solution, 25 mg. peroxidase and 0.25 ml. Texapon P (Henkel, Düsseldorf). The impregnated paper was then dried. Thereafter, the paper was impregnated a further time using a solution of 0.01 g. 2,2'-azino-di-(1-ethyl-quinoline-6-sulfonic acid) and 0.1 g. *o*-amino-phenol-*p*-sulfonic acid in 100 ml. water and the paper dried.

When there was applied to a paper thus prepared a drop of an aqueous hydroperoxide solution, then, even at a concentration of 0.2 γ/ml. the paper assumed a red-violet coloration.

b. A filter paper (2316 of Schleicher & Schüll) was impregnated with a solution of 40 mg. peroxidase and 0.25 ml. Texapon P in 100 ml. of a 0.1M phosphate buffer of pH 5.6 and dried. The paper prepared in this manner was subsequently impregnated with a cold, saturated solution in methanol of 1-(3-ethyl-benzoxazol-2-one)-2-(3-ethyl-benzthiazol- 2-one)-azine and dried. When solutions of hydrogen peroxide of various concentrations were applied to a reagent paper prepared in this manner, then the paper became green-blue, the strength of the color depending upon the hydrogen peroxide concentration. The lower-most detection limit was 0.01 γhydrogen peroxide/ml.

c. One ml. of a 0.2 percent methanolic solution of 1-(1-ethyl-3,3-dimethyl-indolin-2-one)-2-(3-ethyl-benzthiazol-2-one)-azine, 1 ml. of a 0.1 percent methanolic solution of *p*-amino-salicylic acid, 0.05 ml. of a 1 percent solution of peroxidase in a 0.1M phosphate buffer of pH 5.7 and 0.05 ml. of a solution of hydrogen peroxide were pipetted together, stirred up and measured at a wavelength of 530 mm. The hydrogen peroxide concentration was determined with the help of a previously prepared calibrated curve.

Example 5

Detection of Glucose in Liquids

0.01 ml. of glucose solutions having concentrations of 0–300 mg.-percent were each pipetted into 2 ml. of an aqueous-methanolic 0.00125M indicator solution containing equimolar amounts of 2,2'-azino-di-(3-ethyl-benzthiazolone-6-sulfonic acid) and *p*-amino-salicylic acid. There were then added to the resulting mixture 2 ml. of a solution of 10 mg. peroxidase and 20 mg. glucose oxidase in 100 ml. of a 0.5M phosphate buffer, the pH of which was 5.7. Measurements were then carried out at a wavelength of 490 nm. and the corresponding glucose values calculated using therefor a calibration curve.

When, as the indicator mixture, there was used 2,2'-azino-di-(1-ethyl-quinoline-6-sulfonic acid) and *m*-amino-phenol, the absorption maximum was established at 510 nm.

Example 6

A reagent film was prepared using the procedures described in example 1, using an indicator mixture which contained 0.02 percent 2,2'-azino-di-(1-ethyl-quinoline-6-sulfonic acid) and 0.3 percent 2,2'-azino-di-(3-ethyl-benzthiazolone-6-sulfonic acid). When these strips were employed in tests, as the glucose concentration increased, the color changed from violet to green. This color change could be very readily observed because the human eye is extremely sensitive to changes in this color range.

Example 7

Detection of Glucose in Blood

0.1 ml. blood was deproteinized with 1 ml. of 0.3M perchloric acid. 0.2 ml. of the supernatant liquid were pipetted into 5 ml. of a solution having the following composition: 0.1M phosphate buffer having a pH of 7.0 which contained per ml. 40γg. peroxidase, 250γg. glucose oxidase and 500γg. of the diammonium salt of 2,2'-azino-di-(3-ethyl-benzthiazolone-6-sulfonic acid). The extinction was measured at a wavelength of 420 nm. and the corresponding glucose value read off from a calibration curve.

Example 8

Blood Glucose Test Strips

Forty-five g. of an aqueous dispersion of polyvinyl propionate (Propiofan, BASF), 35 g. of a solution of 37 g. sodium alginate (Algipon) in 2 liters of a 0.5M phosphate or citrate buffer having a pH of 5.7, 1 g. Texapon P (Henkel, Düsseldorf), 5 ml. water, 0.07 g. peroxidase and 0.16 g. glucose oxidase were stirred together to form a homogeneous slurry. There were then added to this slurry 0.3 g. of the ammonium salt of 2,2'-azino- di-(3-ethyl-benzthiazolone-6-sulfonic acid) and 0.04 g. of the ammonium salt of *o*-aminophenol-*p*-sulfonic acid dissolved in 7 ml. water, as well as 0.3 g. N,N,N', N'-tetramethyl-4,4'di-amino-diphenyl-methane dissolved in 4 ml. acetone. The mixture prepared in this manner was applied, at a coating thickness of 300γ, to a foil and thereafter dried.

When blood samples of varying glucose concentrations were applied to the thus prepared reagent films and the blood left thereon for about 1 minute and thereafter wiped off, then, in the physiological range, there were observed color graduations from yellow to green to blue. (Graduations: at 60 milligram-percent yellow; at 120 milligram-percent green; at 180 milligram-percent blue-green; at 240 milligram-percent blue; at 300 milligram-percent blue-violet).

The Texapon P used in the above examples is a detergent-active product. In place of Texapon P, there can be used, for example, sodium lauryl sulfate with equally good results.

Example 9

Detection of Glucose in Urine

A filter paper (2316 of Schleicher & Schuil) was impregnated with a solution of 75 mg. peroxidase, 143 mg. glucose oxidase and 0.25 ml. Texapon P (Henkel, Düsseldorf) in 100 ml. of a 0.1M phosphate buffer of pH 5.6 and then dried. The paper impregnated in this manner was dipped into a solution of 0.2 g. 1-(3-ethyl-benzoxazol-2-one)-2-(1-ethyl-quinolin-2-one)-azine and 0.1 g. *p*-aminosalicylic acid 100 ml. methanol and dried.

Upon moistening a strip of a reagent paper produced in this manner with urine which contains a pathological amount of glucose, there was obtained a red-violet color, the strength of which depends upon the glucose concentration.

Example 10

Detection of Blood in Body Fluids

A filter paper (23 S of Schleicher & Schüll) was impregnated with a solution of 0.25 ml. Texapon P and 100 mg. sodium alginate in 100 ml. of a 0.1M phosphate buffer of pH 5.6 and dried. The paper thus prepared was then impregnated with a solution of 0.2 g. 1-(3-ethyl-benzoxazol-2-one)-2-(1-ethyl-quinolin-2-one)-azine and 0.1 g. *p*-aminosalicylic acid in 100 ml. methanol and dried.

When a drop of a body fluid which contains blood was applied to a reagent paper prepared in this manner and a drop of 3 percent hydrogen peroxide solution subsequently applied, then the paper became red-violet even at a blood concentration of 1:50,000.

Example 11

Detection of Glucose in Solutions

One ml. of a methanolic solution which contained one of the indicators (I) set out in the following table II in an amount of up to 1 percent, depending upon the solubility, was mixed with 1 ml. of a solution of 150 mg. glucose oxidase and 75 mg. peroxidase in 100 ml. of a 0.1M phosphate buffer of pH 5.6 and 1 ml. of a glucose solution. There were obtained the color reactions indicated in table II. As modifier, there was, in each case, used 0.1 ml. of a 1 percent methanolic solution of *p*-aminosalicylic acid.

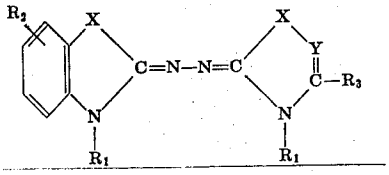
TABLE II

Indicator compound designation	Indicator compound name	M.P., °C.	Color reaction	
			Without modifier	With modifier
N	2,2'-azino-di-(3-ethyl-benzoxazol-2-one)	216–217	Blue	Red-brown.
O	1-(3-ethyl-benzoxazol-2-one)-2-(1-ethyl-pyrid-2-one)-azine	145–146	Violet	Red.
Q	1-(3-ethyl-benzoxazol-2-one)-2-(1-ethyl-quinolin-2-one)-azine	199	do	Red.
P	1-(3-ethyl-benzoxazol-2-one)-2-(3-ethyl-benzthiazol-2-one)-azine	193–194	Blue	Red.
S	1-(3-ethyl-benzoxazol-2-one)-2-(1,3-diethyl-benzimidazol-2-one)-azine	215–216	Violet	Brown-orange.
T	1-(3-ethyl-benzoxazol-2-one)-2-(1-ethyl-6-chloropyrid-2-one)-azine	145	do	Red.
R	1-(1-ethyl-3,3-dimethyl-indolin-2-one)-2-(3-ethylbenzthiazol-2-one)-azine	208–209	Red-brown	Red-violet.
U	1-(1-ethyl-3,3-dimethyl-indolin-2-one)-2-(3-methylbenzthiazol-2-one)-azine		do	Do.
V	1-(1-ethyl-3,3-dimethyl-indolin-2-one)-2-(1-ethyl-quinolin-2-one)-azine	199	Red-orange	Brown-orange.
W	2,2'-azino-di-(3-ethyl-benzoxazol-2-one-6-sulphonic acid)	[1] 258–260	Blue	Red-brown.
X	2,2'-azino-di-(3-ethyl-benzoxazol-2-one-6-sulphonic acid) ammonium salt	[1] 300	do	Do
Y	1-(1-ethyl-3,3-dimethyl-indolin-2-one)-2-(1-ethylpyrid-2-one)-azine	165	Red-violet	Pale brown.
Z	1-(1-ethyl-3,3-dimethyl-indolin-2-one)-2-(1-ethyl-6-chloro-pyrid-2-one)-azine	146	Blue-violet	Do.
AA	1-(1-ethyl-3,3-dimethyl-indolin-2-one)-2-(1,3-diethylbenzimidazol-2-one)-azine	177	Brown-orange	Brown-orange.
BB	1-(1,3,3-trimethylindolin-2-one)-2-(1-phenyl-3-methyl-4-ethyl-1,2,4-triazol-5-one)-azine	244	Green	Brown.
CC	1-(1,3,3-trimethylindolin-2-one)-2-(3-ethyl-benzoxazol-2-one)-azine	225	do	Blue-grey.
DD	1-(3-ethyl-benzoxazol-2-one)-2-(1-phenyl-3-methyl-4-ethyl-1,2,4-triazol-5-one)-azine	227	Blue	Red.

[1] Decomposition.

What is claimed is:

1. Diagnostic agent for use in the analytical determination of hydroperoxide, substances which react with the liberation of hydroperoxide, peroxidase and peroxidatively active substances comprising a first member selected from the group consisting of hydroperoxide, substances which react with the liberation of hydrogen peroxide, peroxidase and peroxidate-active substances and a second member comprising a chromogen of the formula:



wherein R_1 is lower alkyl, R_2 is a member selected from the group consisting of hydrogen, alkali metal sulfonate groups and sulfonic acid groups, R_3 is a member selected from the group consisting of hydrogen, halogen and lower alkyl, X is a member selected from the group consisting of sulfur, oxygen, a carbon atom substituted with two lower alkyl groups, substituted and unsubstituted vinylene and imino, wherein said substituents are a member selected from the group of lower alkyl and aryl and Y is a member selected from the group consisting of nitrogen and methine, wherein said methine group member can be joined together with R_3 to form a benzene ring substituted by R_2.

2. Diagnostic agent according to claim 1 for use in the determination of hydroperoxide or a substance which reacts with the liberation of hydrogen peroxide comprising a member selected from the group consisting of peroxidase and a peroxidatively active substance and a chromogen as defined in claim 1.

3. Diagnostic agent according to claim 1 for use in the determination of peroxidatively active substances comprising a member selected from the group consisting of hydrogen peroxide and substances forming hydrogen peroxide and a chromogen as defined in claim 1.

4. Diagnostic agent as claimed in claim 1 wherein one, and only one, of the X groups in the formula is oxygen or a carbon atom substituted with two lower alkyl groups.

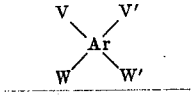
5. Diagnostic agent as claimed in claim 1 wherein neither of the X groups in the formula is oxygen or a carbon atom substituted with two lower alkyl groups.

6. Diagnostic agent according to claim 1 in the form of a test paper impregnated with said diagnostic agent.

7. Diagnostic agent according to claim 1 in the form of a test film impregnated with said diagnostic agent.

8. Diagnostic agent according to claim 1 in the form of a solution thereof.

9. Diagnostic agent according to claim 1 additionally containing a compound having the formula:



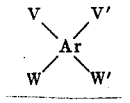
wherein Ar is a member selected from the group consisting of benzene and naphthalene nuclei, V is a member selected from the group consisting of hydroxyl and amino, v' is a member selected from the group consisting of hydrogen, hydroxy, amino, alkylated amino and arylated amino, W is a member selected from the group consisting of amino, carboxy, alkyl, alkoxy, aryl and sulfonic acid groups and W' is a member selected from the group consisting of hydrogen, amino, carboxy, alkyl, alkoxy, aryl, sulfonic acid groups and alkali metal carboxylate or sulfonate groups, as modifier.

10. Diagnostic agent according to claim 2 additionally containing a compound having the formula:



wherein Ar is a member selected from the group consisting of benzene and naphthalene nuclei, V is a member selected from the group consisting of hydroxyl and amino, V' is a member selected from the group consisting of hydrogen, hydroxy, amino, alkylated amino and arylated amino, W is a member selected from the group consisting of amino, carboxy, alkyl, aryl, alkoxy and sulfonic acid groups and W' is a member selected from the group consisting of hydrogen, amino, carboxy, alkyl alkoxy, aryl, sulfonic acid groups and alkali metal carboxylate or sulfonate groups, as modifier.

11. Diagnostic agent according to claim 3 additionally containing a compound having the formula:



wherein Ar is a member selected from the group consisting of benzene and naphthalene nuclei, V is a member selected from the group consisting of hydroxyl and amino, V' is a member selected from the group consisting of hydrogen, hydroxy, amino, alkylated amino and arylated amino, W is a member selected from the group consisting of amino, carboxy, alkyl, alkoxy, aryl and sulfonic acid groups and W' is a member selected from the group consisting of hydrogen, amino, carboxy, alkyl, alkoxy, aryl, sulfonic acid groups and alkali metal carboxylate or sulfonate groups, as modifier.

12. Diagnostic agent according to claim 1 wherein said chromogen (I) is 2,2'-azino-di-(3-ethyl-benzthiazolove-6-sulfonic acid).

13. Diagnostic agent according to claim 1 wherein said chromogen (I) is 2,2'-azino-di-[1-ethyl-quinoline-6-sulfonic-acid].

14. Diagnostic agent according to claim 9 wherein said chromogen (I) is 2,2'-azino-di-[1-ethyl-quinoline-6-sulfonic acid] and said modifier is *o*-amino-phenol-*p*-sulfonic acid.

15. Diagnostic agent according to claim 9 wherein said chromogen (I) is 2,2'-azino-di-(3-ethyl-benzthiazolone-6-sulfonic acid) and said modifier is *p*-amino-salicylic acid.

16. Diagnostic agent according to claim 9 wherein said modifier is a member selected from the group consisting of *o*-amino-phenol-*p*-sulfonic acid, (1-amino-naphthol-8)-2, 4-disulfonic acid, (7-amino-naphthol-1)-3,6-disodium sulfonate, naphthyl-1-amino-6-sulfonic acid, naphthyl-1-amino-8-sulfonic acid, *p*-aminosalicylic acid, *o*-amino-phenol, *m*-amino-phenol, *p*-phenetidene, 2-hydroxy-4-amino-4-methoxy-diphenylamine, and α-naphthol-2-sulfonic acid.

17. Process for the analytical determination of hydroperoxide, a substance which reacts with the liberation of hydroperoxide, peroxidase and a peroxidate-active substance which comprises contacting a liquid containing the substance of interest with a reagent comprising a diagnostic agent according to claim 1, producing a color with respect to said liquid as visual evidence of the presence of said substance of interest in said liquid.

18. Process for the analytical determination of hydroperoxide, a substance which reacts with the liberation of hydroperoxide, peroxidase and a peroxidate-active substance which comprises contacting a liquid containing the substance of interest with a reagent comprising a diagnostic agent according to claim 9, producing a color with respect to said liquid as visual evidence of the presence of said substance of interest in said liquid.

* * * * *

343.1 CIP PO-1050 (5/69)

UNITED STATES PATENT OFFICE
CERTIFICATE OF CORRECTION

Patent No. 3,627,698 Dated December 14, 1971

Inventor(s) Hans-Georg Rey, Hans Wielinger, Peter Rieckmann

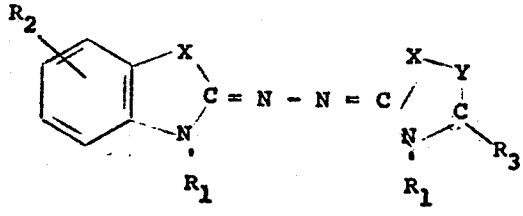
It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Abstract - to read as follows:

DIAGNOSTIC AGENTS

ABSTRACT OF THE DISCLOSURE

Diagnostic agents suitable for use in carrying out rapid analytical determinations of the presence and/or concentration of hydroperoxides, substances which react with the liberation of hydrogen peroxide or hydroperoxide, peroxidase or peroxidatively active substances, comprising an indicator, i.e., chromogen, which is oxidized by hydrogen peroxide or hydroperoxide in the presence of peroxidase or peroxidate active substance to form a dyestuff, the color intensity of which is dependent on the quantity of peroxide, peroxidase or peroxidate active substance present in the test sample, wherein the chromogen is a compound having the formula:



wherein R_1 is lower alkyl; R_2 is hydrogen, a sulfonic acid group or an alkali metal sulfonate group; R_3 is hydrogen, halogen, e.g. chlorine, or lower alkyl; X is sulfur, oxygen, a carbon atom substituted with two lower alkyl groups, substituted or unsubstituted vinylene or imino, wherein said substituent is lower alkyl or aryl; and Y is nitrogen or methine which, when taken together with R_3, can form a benzene ring substituted by R_2.

343.1 CIP

PO-1050
(5/69)

UNITED STATES PATENT OFFICE
CERTIFICATE OF CORRECTION

Patent No. 3,627,698 Dated December 14, 1971

Inventor(s) Hans-Georg Rey, Hans Wielinger, Peter Riechmann

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Col. 6, Table 1, last column, 3rd item from bottom

"L lac" should be --Lilac--

Signed and sealed this 11th day of July 1972.

(SEAL)
Attest:

EDWARD M.FLETCHER,JR.
Attesting Officer

ROBERT GOTTSCHALK
Commissioner of Patents