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 [33] **Germany**
 [31] **P 19 17 997.6**

[56]

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UNITED STATES PATENTS

3,183,173 5/1965 Oakes..... 252/408 X

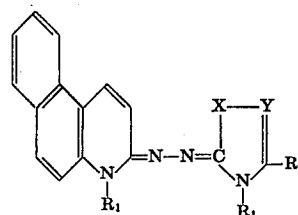
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ABSTRACT:

- [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
 [50] Field of Search..... **252/408;**
23/230 B, 253 TP, 230 R; 195/103.5; 424/7;
260/241



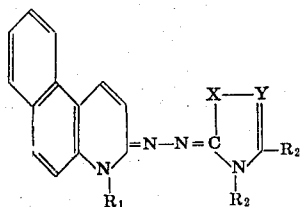
HYDROPEROXIDE DIAGNOSTIC AGENTS CONTAINING A CHROMOGEN INDICATOR

The present invention is concerned with process and diagnostic agent for the determination of hydroperoxides and of substances from which hydrogen peroxide or other hydroperoxides are liberated by a previous reaction, as well as for the determination of peroxidase and of other peroxidatively active substances.

The detection of glucose in urine, blood and serum is, in the case of diabetes, of especial diagnostic interest, as is also the detection of peroxidatively 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 is known which are oxidized to dyestuffs with hydrogen peroxide and peroxidase as catalyst. Preferred compounds of this type include, for example, the Nadi reagents, p-phenylene-diamine derivatives, carboazines, guaiac resins, aldazine and the like. Previously, it has been preferred to use benzidine, o-dianisidine and o-tolidine. However, some of these compounds are not very stable and, according to recent findings, can also be a health hazard so that their use does not appear to be free of danger.

We have now, surprisingly, found that the physiologically harmless compounds of the general formula:



wherein R₁ is lower alkyl; R₂ is hydrogen or alkyl, preferably lower alkyl, or, together with Y, represents a fused benzene or naphthalene nucleus; X is sulfur or oxygen or an alkylated imino group, preferably carrying lower alkyl groups, or a vinylene radical; and Y is a methine radical which, together with R₂, can also form a benzene or naphthalene nucleus, are absolutely stable and are particularly suitable for the determination of hydrogen peroxide and of other hydroperoxides, not only by means of an optical test but also with the help of reagent papers and reagent films.

It is known that some of the compounds of the above-given general formula I can be oxidized by means of comparatively strong oxidizing agents, such as lead tetra-acetate, ceric sulfate and potassium persulfate, and thus give colored oxidation products (cf. S. Hunig et al. Liebig's Ann. Chem., 676, 32-65/1964; H. Lang et al. Z. analyt. Chem., 201, 321/1964). Since, however, no reaction takes place with hydrogen peroxide alone, it could not be foreseen that reproducible and clear colorations can also be obtained in the presence of peroxidase or of peroxidatively active substances.

According to a further feature of the present invention, we have also found, surprisingly, that especially advantageous and clearly graduated color changes are obtained when the compounds of general formula I are used in admixture with reduction agents of the general formula:



wherein Ar is a benzene or naphthalene nucleus; V is amino or hydroxyl; V' is hydrogen or amino or hydroxyl; W is amino or a carboxylic acid or sulfonic acid group; and W' is hydrogen or amino, or a carboxylic acid or sulfonic acid group; as well as with the alkali metal salts thereof.

These compounds of general formula II do not themselves give any coloration or, at most, only give very nonspecific colorations and are, therefore, generally not suitable for use as indicators. However, as already mentioned above, when these

compounds of general formula II are used together with the compounds of general formula I, there are obtained especially 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, with the help of a chromogen, using the reaction of the hydroperoxides with peroxidase or peroxidatively active substances, by evaluation, in known manner, and of the coloration, wherein a compound of general formula I is used as chromogen, preferably in admixture with a compound of general formula II.

The evaluation of the coloration can be carried out, for example, by optical measurement in a spectrophotometer or, in the case of the use of test paper strips and test films, by comparison of the color strength with blank samples of known composition or with color charts.

The determination of hydroperoxides by the process according to the present invention is particularly useful for coupled and uncoupled enzyme reactions, 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 their outstanding importance, the routine determination of substrates 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. By means of peroxidase or of a peroxidatively active substance, the hydrogen peroxide then oxidizes the indicator used according to the present invention to give 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 of general formula I alone or in admixture with a compound of general 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 general formula I alone or in admixture with a compound of general 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 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 absorbent materials, such as filter paper, with solutions of the components of the diagnostic reagents or can be used for making reagent films. Here again, in the case of such test papers and test films, it is frequently advantageous for them to contain conventional adjuvants, such as buffers.

Finally, mention should also 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 general formula I, as well as of the compounds of general formula II, with the help of hydroperoxides and of peroxidatively active substances, which is very useful for control purposes in the preparation of these diagnostic materials.

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

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

EXAMPLE 1

Reagent Film for the Detection of Glucose in Blood

Forty-Five g. Propiofan (BASF), 35 g. of a solution of 37 g. algipon in 2 liters of a 0.5 M phosphate or citrate buffer of pH 5.7, 1 g. Texapon P (Henkel, Dusseldorf), 10 ml. water, 75 mg. peroxidase and 150 mg. glucose oxidase were stirred up to give a homogeneous slurry. To this solution there was added 8 ml. of a concentrated methanolic solution of 1-[1-methyl-benzo(f)quinoline-2-one]-2-[3-ethyl-benzthiazol-2-one]-azine, as well as 4 ml. of a 2 percent solution of p-aminosalicylic acid. A foil was coated with the mixture prepared in this manner with a layer thickness of 300 μ and then dried. When glucose-containing blood is dropped onto the reagent strip thus produced, left to react therewith and the blood then again removed, there was obtained a red-brown color reaction.

When, to the indicator mixture, instead of a 2 percent solution of p-aminosalicylic acid, there was added a solution of (1-amino-naphthol-8)-2,4-disulfonic acid, then a green color reaction was obtained. When naphthyl-1-amine-6-sulfonic acid or m-aminophenol was added, then the reagent film, by the action of glucose-containing blood, produced color graduations from pink to red-brown, depending upon the glucose concentration.

EXAMPLE 2

To a mixture of Propiofan, algipon, buffer, Texapon P, water, peroxidase and glucose oxidase prepared in the manner described in example 1, there were added 8 ml. of a concentrated methanolic solution of 1-[1-methyl-benzo(f)quinoline-2-one]-2-[1-ethyl-quinoline-2-one]-azine, as well as 4 ml. of a 2 percent solution of p-aminosalicylic acid. A foil was coated with the mixture prepared in this manner with a layer thickness of 300 μ and then of When glucose-containing blood was dropped onto the reagent film thus produced, left to react therewith and the blood then again removed, there was obtained a pink color reaction.

EXAMPLE 3

Detection of Hydrogen Peroxide in Liquids

Three ml. of a 0.005 percent methanolic solution of 1-[1-methyl-benzo(f)quinoline-2-one]-2-[3-ethyl-benzoxazol-2-one]-azine, 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, any precipitate

formed then centrifuged off and the supernatant measured at a wavelength of 443 nm. The hydrogen peroxide concentration was subsequently determined with the help of a previously prepared calibrated curve.

EXAMPLE 4

A filter paper (Schleicher & Schüll 23 S) was impregnated with a solution of 0.25 ml. Texapon P (Henkel, Dusseldorf) and 100 mg. sodium alginate in 100 ml. of a 0.1M phosphate buffer of pH 5.7 and then dried. The buffered paper thus prepared was further impregnated with a 0.01 percent methanolic solution of 1-[1-methyl-benzo(f)quinoline-2-one]-2-[1-ethyl-quinoline-2-one]-azine and a 0.01 percent methanolic solution of p-aminosalicylic acid and again dried. When a drop of a body fluid which contained blood was applied to a test paper strip prepared in this manner, together with a drop of a 3 percent hydrogen peroxide solution, then the paper became blue-violet colored.

EXAMPLE 5

Detection of Glucose in Urine

Filter paper (Schleicher & Schüll 2316) was impregnated with a solution of 75 mg. peroxidase, 143 mg. glucose oxidase and 0.25 ml. Texapon P in 100 ml. of a 0.1M phosphate buffer of pH 5.6 and dried. The enzyme-buffer paper thus prepared was subsequently impregnated with a 0.01 percent solution of 1-[1-methyl-benzo(f)quinoline-2-one]-2-[3-ethyl-benzoxazol-2-one]-azine in chloroform and with a 0.01 percent methanolic solution of p-aminosalicylic acid and again dried. When a reagent paper prepared in this manner was dipped into a urine with a pathological glucose concentration, then the reagent paper assumed a red-brown coloration.

EXAMPLE 6

Detection of Glucose with and without a Modifier Substance of General Formula II

One hundred mg. amounts of the indicators set out in the following table were slurried in 10 ml. methanol, which, if desired, contained 0.1 percent p-aminosalicylic acid as modifier, 2 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. glucose solution are mixed with 1 ml. of the indicator slurry and stirred up. There were obtained the color reactions set out in the following table.

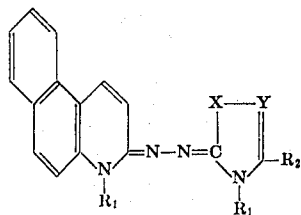
TABLE I

Indicator (I)	M.P. (° C.)	Color reaction	
		Without modifier	With modifier
1-[1-methyl-benzo(f)-quinoline-2-one]-2-[3-ethyl-benzthiazol-2-one]-azine.	230	Green.....	Red.
1-[1-methyl-benzo(f)-quinoline-2-one]-2-[1-ethyl-quinoline-2-one]-azine.	250–251	Blue-gray....	Red.
1-[1-methyl-benzo(f)-quinoline-2-one]-2-[3-ethyl-benzoxazol-2-one]-azine.	296	Green.....	Red.
1-[1-methyl-benzo(f)-quinolin-2-one]-2-[1,3-diethyl-benzimidazol-2-one]-azine.	354–355	Blue-green....	Violet.
2,2'-azino-di-[1-methyl-benzo(f)-quinoline].	339–340	Green.....	Red-violet.

What is claimed is:

1. Diagnostic agent for use in the analytical determination of (a) hydroperoxide and substances which react with the liberation of hydroperoxide or (b) peroxidase and peroxidatively active substances which agent comprises (a) a member selected from the group consisting of peroxidase and peroxidatively active substances; or (b) a member selected from the group consisting of hydroperoxide and substances which react with the liberation of hydrogen peroxide; respectively, and a chromogen of the formula

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wherein R_1 is lower alkyl; R_2 is hydrogen or alkyl and, together with Y, can represent a fused benzene or naphthalene nucleus; X is sulfur, oxygen, an alkylated imino group, or a vinylene radical; and Y is a methine radical which, together with R_2 , can also form a benzene or naphthalene nucleus.

2. Diagnostic agent as claimed in claim 1 for use in the determination of hydroperoxide or a substance which reacts with the liberation of hydrogen peroxide, which agent comprises (1) a member selected from the group consisting of peroxidase and a peroxidatively active substance, and (b) a chromogen as defined in claim 1.

3. Diagnostic agent as claimed in claim 2 additionally containing a modifier compound having the formula:



wherein Ar is a benzene or naphthalene nucleus; V is amino or hydroxyl; V' is hydrogen or amino or hydroxyl; W is amino or a carboxylic acid or sulfonic acid group; and W' is hydrogen or amino, or a carboxylic acid or sulfonic acid group; as well as with the alkali metal salts thereof.

4. Diagnostic agent as claimed in claim 1 for use in the determination of peroxidase or peroxidatively active substances which agent comprises (1) a member selected from the group consisting of hydrogen peroxide and substances forming hydrogen peroxide and (2) a chromogen as defined in claim 1.

5. Diagnostic agent as claimed in claim 4 additionally containing a modifier compound having the formula:



wherein Ar is a benzene or naphthalene nucleus; V is amino or hydroxyl; V' is hydrogen or amino or hydroxyl; W is amino or a carboxylic acid or sulfonic acid group; and W' is hydrogen or amino, or a carboxylic acid or sulfonic acid group; as well as with the alkali metal salts thereof.

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

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

8. Diagnostic agent as claimed in claim 1 in the form of a solution thereof.

9. Diagnostic agent as claimed in claim 1 additionally containing a modifier compound having the formula:



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15 wherein Ar is a benzene or naphthalene nucleus; V is amino or hydroxyl; V' is hydrogen or amino or hydroxyl; W is amino or a carboxylic acid or sulfonic acid group; and W' is hydrogen or amino, or a carboxylic acid or sulfonic acid group; as well as with the alkali metal salts thereof.

20 10. Diagnostic agent as claimed in claim 9 wherein said modifier compound is o-amino-phenol-p-sulfonic acid.

11. Diagnostic agent as claimed in claim 9 wherein said modifier compound is p-amino-salicylic acid.

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

13. Diagnostic agent as claimed in claim 1 wherein said chromogen (I) is 1-methyl-benzo(f)quinoline-2-one]-2-[3-ethyl-benzthiazol-2-one]-azine.

14. Diagnostic agent as claimed in claim 1 wherein said chromogen (I) is 1-[1-methyl-benzo(f)quinoline-2-one]-2-[1-ethyl-quinoline-2-one]-azine.

15. Diagnostic agent as claimed in claim 1 wherein said chromogen (I) is 1-[1-methyl-benzo(f)quinoline-2-one]-2-[3-ethyl-benzoxazol-2-one]-azine.

16. Diagnostic agent as claimed in claim 1 wherein said chromogen (I) is 1-[1-methyl-benzo(f)quinoline-2-one]-2-[1,3-diethyl-benzimidazol-2-one]-azine.

17. Diagnostic agent as claimed in claim 1 wherein said chromogen (I) is 2,2'-azino-di-[1-methyl-benzo(f)quinoline].

18. Process for the analytical determination of (a) hydroperoxide and a substance which reacts with the liberation of hydroperoxide or (b) peroxidase and a peroxidatively active substance which comprises contacting a liquid containing the substance of interest with a reagent comprising a diagnostic agent according to claim 1 and producing a color with respect to said liquid as visual evidence of the presence of said substance of interest in said liquid.

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UNITED STATES PATENT OFFICE
CERTIFICATE OF CORRECTIONPatent No. 3,627,697 Dated December 14, 1971Inventor(s) Hans-Georg Rey, Hans Wielinger and Peter Rieckmann

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

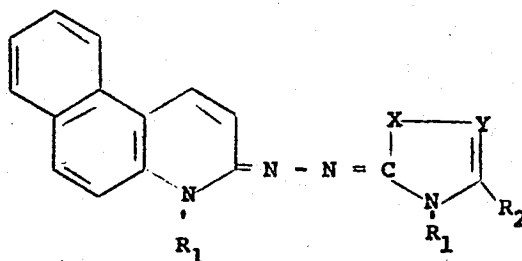
Title Page

Abstract, consists only of a formula. - should read:

HYDROPEROXIDE DIAGNOSTIC AGENTS
CONTAINING A CHROMOGEN INDICATOR

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 peroxidatively-active substance to form a dyestuff, the color intensity of which is dependent on the quantity of peroxide, peroxidase or peroxidatively-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 or alkyl, preferably lower alkyl, or, together with Y, represents a fused benzene or naphthalene nucleus; X is sulfur or oxygen or an alkylated imino group, preferably carrying lower alkyl groups, or a vinylene radical; and Y is a methino radical which, together with R_2 , can also form a benzene or naphthalene nucleus.

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PAGE - 2

Col. 3, line 10

"merapto" should be --mercapto--

Col. 3, line 63

"of" (second occurrence) should read --dried.--

Claim 13, line 2

"1-methyl" should read --1-[1-methyl--

Claim 18, line 1

Delete close parenthesis at end of line

Signed and sealed this 11th day of July 1972.

(SEAL)
Attest:

EDWARD M. FLETCHER, JR.
Attesting Officer

ROBERT GOTTSCHALK
Commissioner of Patents