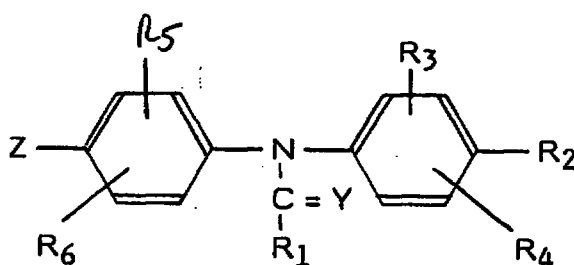


Description

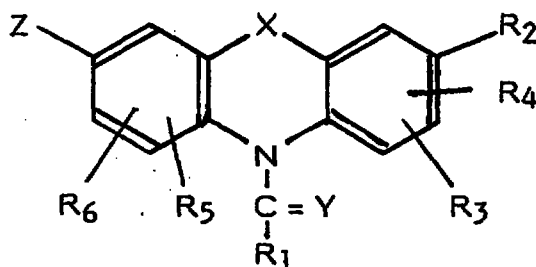
The present invention relates to a method and test composition for the determination of hydrogen peroxide, and more particularly, to a method for the determination of hydrogen peroxide by reacting hydrogen peroxide with a novel chromogen as a hydrogen donor in the presence of peroxidase and determining the amount of pigment formed.

In the medical, diagnostic and biochemical arts, numerous substrates are determined by oxidizing the substrate with an oxidase and determining the amount of hydrogen peroxide produced. For example, cholesterol is determined by reaction with cholesterol oxidase and subsequent determination of the hydrogen peroxide produced, by reaction of the product hydrogen peroxide with a peroxidase in the presence of a chromogen which forms a pigment, and measuring the absorbancy of the reaction solution. In such processes, 4-aminoantipyrine (hereinafter referred to as "4AA") and phenol, 4AA and N,N-dimethylaniline, 4AA and N-ethyl-N(β -hydroxyethyl)-m-toluidine, 3-methylbenzothiazolin hydrazone and N,N-diethylaniline, 4AA and N-ethyl-N-(3-methylphenyl)-N'-acetylenediamine (hereinafter referred to as "EMAE") are commonly used as the chromogen or chromogenic mixture.

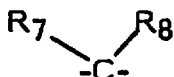
Also known for this purpose are the chromogens disclosed in EP-A-0 045 220, namely compounds of the formulae



and



where Z represents hydroxyl, amino or substituted amino, Y represents an oxygen atom or sulfur atom, R₁ represents hydrogen, alkyl, alkenyl, aryl, amino or mono-substituted amino, R₂ represents hydrogen, hydroxyl, alkyl, alkenyl, aryl, amino, alkylamino, or alkoxy, R₃, R₄, R₅ and R₆ represent hydrogen, alkyl, alkenyl, acyl, aryl, halogen, sulfo, nitro, carboxyl, hydroxyl, hydroxyalkyl or alkoxy, R₃ and R₄ or R₅ and R₆ may form alkenylene, and X represents -S-, -O-,



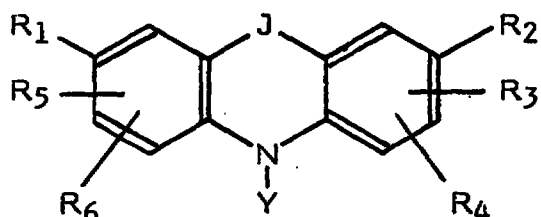
or



R_7 and R_8 represent hydrogen, alkyl, alkenyl or aryl.

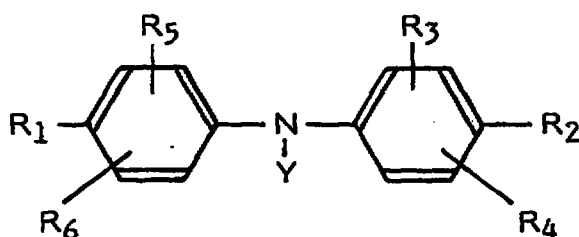
In accordance with the present invention, similar chromogenic derivatives of diphenylamine, phenoxazin and phenothiazin have been found which are superior in sensitivity and which are not affected by components often found *in vivo* such as hemoglobin, bilirubin and uric acid.

These chromogens are compounds of the following formulae (I) and (II):



Formula I

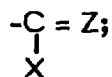
and



Formula II

where:

Y represents hydrogen or



Z represents oxygen or sulfur;

X represents hydrogen, alkyl, alkenyl, aryl, aralkyl, amino or substituted amino;

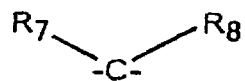
R_1 represents hydroxyl, amino or substituted amino;

R_2 represents hydrogen, hydroxyl, alkyl, alkoxy, aryl, aralkyl, alkenyl, amino or substituted amino;

R_3 represents a group of formula (III), (IV), (V), (VI) or (VII) described hereinafter;

R_4 , R_5 and R_6 are the same or different and represent hydrogen, alkyl, alkenyl, acyl, aryl, aralkyl, halogen atom, nitro, sulfo, carboxyl, hydroxyl, alkoxy or a group of formula (III), (IV), (V), (VI) or (VII) described hereinafter, with the proviso that R_5 and R_6 may also be joined together to form an alkenylene group;

J represents -S-, -O-, or a group of the formula

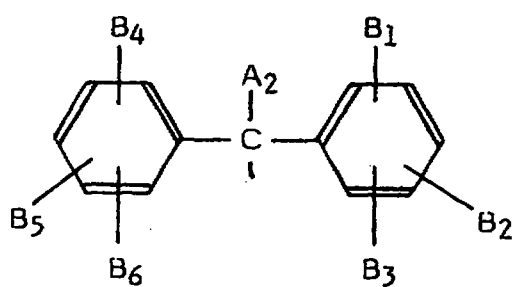


or

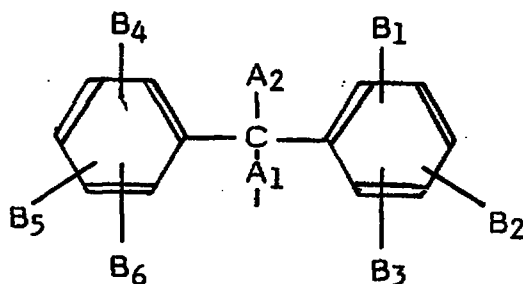


and

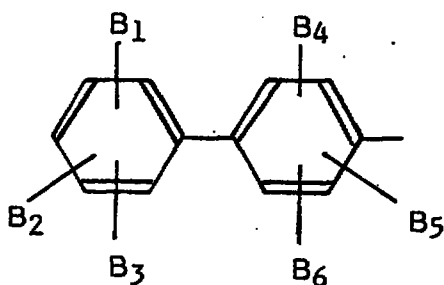
R_7 and R_8 are the same or different and represent hydrogen, alkyl or alkenyl.
Within the above definitions, the groups of formula III to VII are:



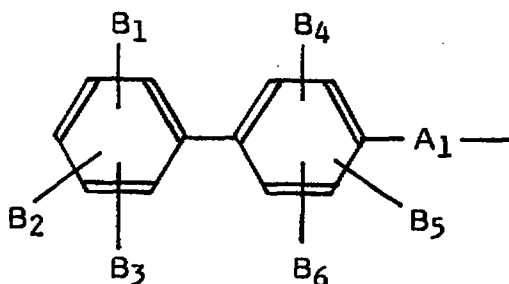
Formula III



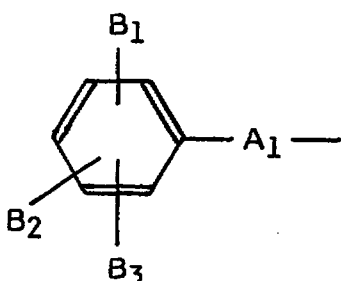
Formula IV



Formula V



Formula VI



Formula VII

wherein:

A₁ represents alkylene;

A₂ has the same meaning as R₂;

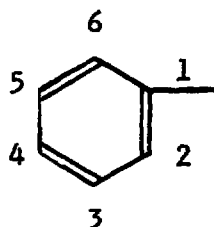
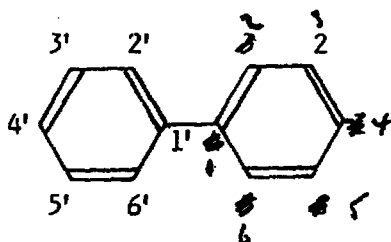
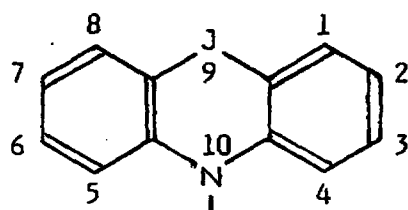
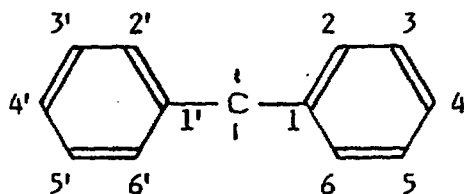
B₁, B₂, B₃, B₄, B₅ and B₆ are the same or different and represent hydrogen, alkyl, alkenyl, acyl, aryl, aralkyl, halogen atom, nitro, sulfo, carboxyl, hydroxyl, alkoxy or hydroxyalkyl.

"Substituted amino" in the above definitions of R₁ and R₂, means amino substituted by alkyl, alkenyl, aryl, hydroxyalkyl, cycloalkyl, acyl, aralkyl, acylalkyl, carboxyl, alkoxy, sulfo or sulfoalkyl.

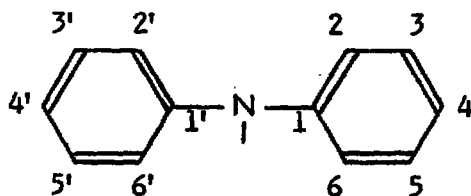
As used herein, alkyl includes alkyl having 1 to 5 carbon atoms, such as methyl, ethyl, propyl and butyl; alkenyl includes alkenyl having 2 to 5 carbon atoms such as vinyl, propylene and butylene; alkoxy includes alkoxy having 1 to 5 carbon atoms such as methoxy, ethoxy, propoxy and butoxy; aryl includes phenyl, naphthyl, and substituted phenyl; substituent of substituted phenyl includes alkyl having 1 to 4 carbon atoms, halogen such as chloro atom and bromo atom, amino, alkoxycarbonylamino, alkoxycarbonylaminoalkyl, alkoxy and acyl; substituted phenyl may have 1 to 5 substituents; aralkyl includes aralkyl having 7 to 10 carbon atoms such as benzyl; acyl includes acyl having 2 to 5 carbon atoms such as acetyl, propionyl and butyryl; halogen includes fluoro atom, chloro atom and bromo atom.

Alkenylene includes alkenylene having 3 to 4 carbon atoms such as $-\text{CH}=\text{CH}-\text{CH}=\text{CH}-$, $-\text{CH}=\text{CH}-\text{CH}_2-$, etc.

Also, where hereinafter reference is made to the numerical position of substituent groups on the aromatic ring structures, the following ring-numbering systems are used, viz:



and



In utilising the chromogens of the present invention in the determination of hydrogen peroxide, the chromogen is added to a sample containing hydrogen peroxide or to the system where hydrogen peroxide is produced (hereinafter referred to as " H_2O_2 -producing system") together with peroxidase. The absorbancy of the resultant coloured solution is measured at a wavelength in the visible or near infra-red ray region, e.g.

in the range 600 to 900 nm. The amount of hydrogen peroxide in the sample is then calculated by comparison with a standard curve.

The reaction is usually carried out at a temperature in the range 5 to 50°C, preferably 25 to 40°C, in a buffer solution having a pH of 2 to 10, and is usually completed within a few minutes.

5 The chromogen is preferably used in an amount of from 10 to 1000 mole equivalents relative to hydrogen peroxide. The peroxidase is used in a concentration of 0.1 to 1000 IU/ml.

As buffers, Good buffer, phosphate buffer, tris-HCl buffer, succinate buffer, citrate buffer, acetate buffer, etc. may be used, at concentrations of from 0.005 to 2 mol/l.

10 A small amount of phenol or EMAE can be added to the reaction system to activate the peroxidase and to promote pigment formation.

Examples of chromogens according to the present invention are shown in Tables 1 and 2, where the following codes apply:

P : Compound of formula I or II

M : -CH₃

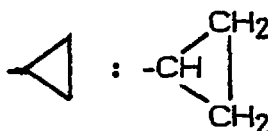
15 E : -C₂H₅

D : -CH₂CH₂CH₂SO₃H

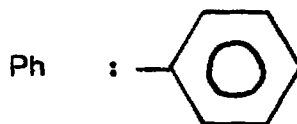
α₁ : -CH₂CH₂CH₃

α₂ : >CHCH₂CH₂CH₃

20



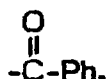
25



30

In each of compounds 1 to 34, R₄, R₅, B₂, B₃, B₅ and B₆ are all hydrogen. In compounds 1 to 27 and 31 to 34 (Table 1), Y is H. In compounds 28, 29 and 30 (Table 2), Y is

35



40

45

50

55

In compounds 18 and 19 (Table 1, compound formula I) J is -S-.

TABLE 1

Compound No.	P	R ₁	R ₂	R ₃	R ₅	A ₁	A ₂	B ₁	B ₄
1	II	NM ₂	NM ₂	III	III	-	H	Cl	H
2	II	NM ₂	NM ₂	III	III	-	H	F	H
3	II	NM ₂	NM ₂	III	III	-	H	H	H
4	II	NM ₂	NM ₂	III	III	-	H	OM	OM
5	II	NM ₂	NM ₂	III	III	-	H	Cl	Cl
6	II	NM ₂	NM ₂	III	III	-	H	F	F
7	II	NM ₂	NM ₂	III	III	-	H	NM ₂	NM ₂
8	II	NM ₂	NM ₂	III	H	-	H	Cl	Cl
9	II	NH ₂	NH ₂	III	III	-	H	H	H
10	II	NM ₂	NM ₂	IV	H	CH ₂	H	Cl	Cl
11	II	NM ₂	NM ₂	IV	H	CH ₂	M	Cl	Cl
12	II	NM ₂	NM ₂	VI	VI	CH ₂	-	H	H
13	II	NM ₂	NM ₂	III	H	-		H	H
14	II	NM ₂	NM ₂	III	H	-	α_1	H	H

TABLE 1 (continued)

Compound No.	P	R ₁	R ₂	R ₃	R ₅	A ₁	A ₂	B ₁	B ₄
5 15	II	NM ₂	NM ₂	VII	H	α_2	-	H	-
16	II	NM ₂	NM ₂	VII	VII	CH ₂	-	Cl	-
17	II	NM ₂	NM ₂	VII	VII	CH ₂	-	Br	-
10 18	I	NM ₂	NM ₂	III	H	-	H	Cl	Cl
19	I	NM ₂	NM ₂	III	NO ₂	-	H	Cl	Cl
20	II	NH ₂	OH	III	H	-	H	Cl	Cl
21	II	NH ₂	OM	III	H	-	H	Cl	Cl
15 22	II	NH ₂	OM	III	H	-	H	F	F
23	II	NH ₂	OH	III	H	-	H	F	F
24	II	NH ₂	NH ₂	III	III	-	H	Cl	Cl
20 25	II	NH ₂	NH ₂	III	III	-	H	F	F
26	II	NH ₂	NH ₂	III	III	-	H	H	Cl
27	II	NH ₂	NH ₂	III	III	-	H	H	F
25 31	II	ND ₂	ND ₂	III	III	-	H	H	H
32	II	ND ₂	ND ₂	III	III	-	H	Cl	Cl
33	II	NHD	NHD	III	III	-	H	H	H
30 34	II	NHD	NHD	III	III	-	H	Cl	Cl

TABLE 2

Compound No.	P	J	R ₁	R ₂	R ₃	R ₅	A ₂	B ₁	B ₄
35 28	II	-	NM ₂	NM ₂	III	III	H	Cl	Cl
29	I	S	NM ₂	NM ₂	III	H	H	Cl	Cl
40 30	I	O	NE ₂	NE ₂	III	H	H	Cl	Cl

45 In the above Tables, in the compounds of Formula II substituent B₁ is at the 4'-position, except in compounds 16 and 17, where it is at the 4-position. Substituent B₄ is at the 4-position in all compounds.

The results of comparative tests between the compounds indicated in Tables 1 and 2 and previously known chromogens are given in Table 3. The test procedure was as follows:

50 A solution containing 0.1 mg/ml of the chromogen in Good buffer solution (pH 6.5) containing 10 U/ml peroxidase, 0.001 mg/ml phenol and 0.1 mg/ml Triton X-100, is prepared. 3 ml of the chromogen solution is then added to 20 μ l of 10.33 mg/dl H₂O₂ solution and the mixture allowed to react.

The OD value of the reaction solution at λ_{max} is then measured and expressed as a relative numerical value taking the OD obtained using 4AA-EMAE as the chromogen as 100.

55 The adverse effect of serum components such as bilirubin, cysteine and uric acid is measured by adding 20 μ g/3 ml bilirubin, 20 μ g/3 ml of cysteine or 10 μ g/3 ml of uric acid to the sample. The symbol " $\pm$ " means that the effect is 3 to 6%, "+" means that the effect is "6 to 20%", "++" means that the effect is more than 20%, and "-" means that the effect is less than 3%.

The degree of the colour in the reagent blank is given in comparison with that of the colour obtained by using Leuco Bindschedler's Green (LBG) as representative prior art chromogen. The following codes are used:

- AA : very little colour;
 A : some colour, but still low;
 B : colouration equal to that of LBG.

Table 3

Compound No.	λ max (nm)	S	F			RB
			B	CY	U	
1	747	390	±	±	±	A
2	740	395	±	±	±	A
3	735	380	-	-	-	AA
4	700	150	+	+	±	AA
5	755	400	-	-	-	AA
6	745	400	-	-	-	AA
7	520	360	±	±	±	AA
8	750	350	±	±	±	A
9	720	200	±	+	+	A
10	728	400	±	+	+	A
11	690	200	±	+	+	A

Table 3 (Continued)

Compound No.	$\lambda_{\max}$ (nm)	S	F			RB
			B	CY	U	
12	733	410	±	+	±	AA
13	728	370	±	+	±	A
14	725	360	±	+	±	A
15	747	390	±	±	±	B
16	737	380	±	+	±	A
17	735	370	±	±	±	A
18	680	700	±	±	±	B
19	670	400	±	±	±	B
20	700	200	±	±	±	A
21	690	160	±	±	±	A
22	723	310	±	±	±	A
23	715	150	±	±	±	A
24	708	270	-	-	-	AA
25	700	270	-	-	-	AA
26	710	250	-	±	-	AA
27	715	260	-	±	-	AA
28	755	395	±	±	±	AA
29	680	700	±	±	±	B
30	685	690	±	±	±	B
31	760	350	-	-	-	AA
32	770	350	-	-	-	AA
33	710	320	-	-	-	A
34	720	320	-	-	-	A
RE ₁	728	410	+	+	+	B
RE ₁	555	100	++	++	++	A

RE₁ : LBGRE₂ : 4AA-EMAE

S : Sensitivity

F : Effect of components in serum

B : Bilirubin

CY : Cysteine

U : Uric acid

RB : Degree of colour development of reagent blank

The results show the better reactivity of the present chromogens as hydrogen donors and their relative insensitivity to contaminants such as bilirubin, cysteine and uric acid.

The chromogens of the present invention are obtained by the condensation reaction of Leuco bases, obtained by the reduction of diphenyl amines, thiazine or oxazine pigments, with a benzhydrol in the presence of sulfuric acid. The condensation reaction is carried out at 30 to 100° C for 1 to 100 hours. The

reaction mixture is then added to an organic solvent such as n-octanol, capable of crystallizing out the desired chromogen. The crude crystals are then re-dissolved in an organic solvent, such as methanol, and the solution subjected to column chromatography through a silica gel column to recover the desired compound.

The preparation of the compounds listed in Tables 1 and 2 is illustrated below.

(1) Preparation of Compound Nos. 1 to 7, 12, 16 and 17

Bindschedler's Green Leuco base [4,4'-bis(dimethylamino)diphenylamine] (hereinafter referred to as "BGLB") was reacted with each of the undermentioned compounds in a molar ratio of 1:2 in the presence of an amount of 60% sulfuric acid equal to 5 times the amount of Leuco base. The reaction mixture was stirred at 80° C for 2 to 3 hours.

At the end of the reaction, n-octanol was added to crystallize the condensation product and the crude crystal product was dissolved in a small amount of methanol. The solution was then subjected to column chromatography in a silica gel column and eluted with a 2:1 mixture of chloroform and n-hexane. The fractions containing the purified product chromogen were collected and evaporated to dryness.

Compound No.	Melting Point (° C)	Reactant
1	102 - 105	4-chlorobenzhydrol
2	98 - 101	4-fluorobenzhydrol
3	120 - 123	benzhydrol
4	152 - 155	4,4'-dimethoxybenzhydrol
5	184 - 187	4,4'-dichlorobenzhydrol
6	111 - 114	4,4'-difluorobenzhydrol
7	89 - 92	4,4'-dimethylaminobenzhydrol
12	108 - 111	4-biphenylmethanol
16	95 - 98	p-chlorobenzylalcohol
17	104 - 107	p-bromobenzylalcohol

(2) Preparation of Compound Nos. 8, 10, 11 and 13 to 15

The same procedure as described in (1) above was carried out except that the BGLB was reacted with the following compounds at a molar ratio of 10:1 at 50° C.

Compound No.	Melting Point (° C)	Reactant
8	78 - 81	4,4'-dichlorobenzhydrol
10	131 - 133	2,2'-bis(p-chlorophenyl)-ethanol
11	128 - 131	2,2'-bis(p-chlorophenyl)-propanol
13	82 - 85	cyclopropyl-benzhydrol
14	84 - 87	1,1'-diphenylbutanol
15	172 - 175	1-phenylbutanol

(3) Preparation of Compound Nos. 9 and 24 to 27

Following the same general procedure as described in (1) above, 4,4'-diamino-diphenyl amine sulfate salt was reacted respectively with diphenylmethanol, 4,4'-dichlorobenzhydrol, 4,4'-difluorobenzhydrol, 4-chlorobenzhydrol and 4-fluorobenzhydrol. The resulting compounds 9 and 24 to 27 have melting points of 158 to 161° C, 173 to 175° C, 89 to 92° C, 155 to 158° C and 104 to 106° C respectively.

(4) Preparation of Compound Nos. 18 and 19

Following the same general procedure as described in (1) above, reduction reaction products of Methylene Blue (C.I. 52015) and Methylene Green (C.I. 52020) respectively were reacted with 4,4'-

dichlorobenzhydrol at a molar ratio of 10:1 and at 50 °C. The resulting compounds have melting points of 179 to 180 °C and 148 to 151 °C respectively.

(5) Preparation of Compound No. 29

Following the same general procedure as described in (1) above, benzoyl Leuco Methylene Blue was reacted with 4,4'-dichlorobenzhydrol in a molar ratio of 10:1 at 50 °C to obtain compound No. 29 (m.p. 212 to 215 °C).

(6) Preparation of Compound No. 28

Compound No. 5, prepared as above, was reacted with phenyl isocyanate in the presence of dimethylformamide (DMF) at 50 °C for 3 days to obtain compound No. 28 (m.p. 196 to 200 °C).

(7) Preparation of Compound No. 30

Basic Blue (C.I. 51004) was reduced using sodium borohydride and the product reacted with 4,4'-dichlorobenzhydrol in a molar ratio of 10:1 in 60% sulfuric acid at 50 °C. The condensation product was purified by the same purification procedures as described in (1) above, and the purified product reacted with an equal molar amount of isocyanic acid in the presence of DMF at 50 °C for 3 days. The purification procedures as described in (1) above were repeated to obtain compound No. 30 (m.p. 118 to 120 °C).

(8) Preparation of Compound Nos. 20 and 23

Following the same general procedure as described in (1) above, p-(p-aminoanilino)-phenol was reacted respectively with 4,4'-dichlorobenzhydrol and 4,4'-difluorobenzhydrol at a molar ratio of 10:1 in 60% sulfuric acid at 50 °C. The product compound Nos. 20 and 23 have m.p. 89 to 91 °C and 121 to 123 °C respectively.

(9) Preparation of Compound Nos. 21 and 22

Following the same general procedure as described in (1) above, Variamine Blue hydrochloride was reacted respectively with 4,4'-dichlorobenzhydrol and 4,4'-difluorobenzhydrol at a molar ratio of 10:1 in 60% sulfuric acid at 50 °C. The product compound Nos. 21 and 22 respectively have m.p. 163 to 165 °C and 158 to 160 °C.

(10) Preparation of Compound Nos. 31 to 34

4,4'-diamino-diphenylamine sulfate and propane sultone in a molar ratio of 1:20 were added to chloroform and the mixture stirred at 50 °C for 5 to 7 days. Distilled water was then added to the reaction mixture in equal amount. After vigorous stirring, the water layer was separated and evaporated to dryness. The dried product was then reacted with benzhydrol and 4,4'-dichlorobenzhydrol, respectively, in a weight ratio of 2:1 in 60% sulfuric acid. The reaction mixture was stirred at 80 °C for 2 to 3 hours.

The same purification procedures as described in (1) above were followed except that different polarity solvents were used following initiation of the elution with chloroform. Compound Nos. 33 and 34 are obtained using solvents of low polarity. Compounds Nos. 31 and 32 are subsequently eluted using solvents of higher polarity.

m.p., Compound No. 31 : 93 to 95 °C

m.p., Compound No. 32 : 78 to 82 °C

m.p., Compound No. 33 : 62 to 65 °C

m.p., Compound No. 34 : 45 to 48 °C

The chromogens of the present invention are useful in the determination of substrates or enzymatic activity in the H₂O₂-producing system. In such systems both the H₂O₂-producing reaction and the determination of the product H₂O₂ can proceed at the same time, thus providing a simple and convenient system for the determination of enzyme activity or substrate concentration. Typical examples of such enzyme/substrate systems are the following combinations: uric acid-uricase, cholesterol-cholesterol oxidase, cholesterol ester-cholesterol esterase and cholesterol oxidase, xanthin, hypoxanthin or guanine-xanthin oxidase, phospholipase D-lecithin-choline oxidase, choline-choline oxidase, pyruvic acid-pyruvate oxidase-

phosphoric acid, triglyceride-lipoprotein lipase-ATP-glycerin kinase-glycerin-3-phosphate oxidase, fatty acid-coenzyme A-acyl Co A synthetase-acyl Co A oxidase, triglyceride-lipase-glycerol oxidase, glucose-glucose oxidase and galactose-galactose oxidase.

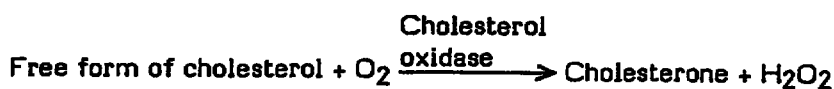
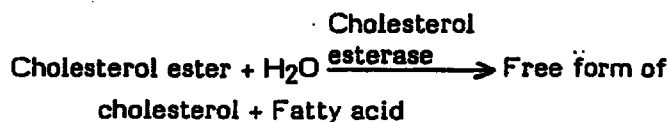
The substrates of these enzymatic reactions are contained in serum, urine, etc., and the determination of the substrates is useful for diagnostic purposes.

Examples of the enzymatic reactions involved are schematically shown as follows.

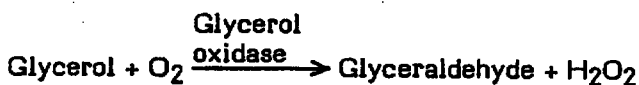
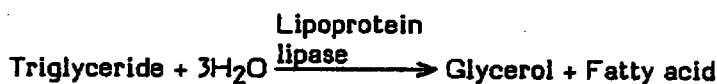
1. Uric acid



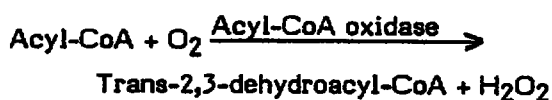
2. Total cholesterol



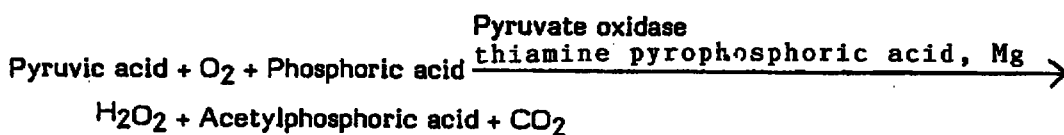
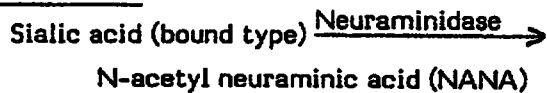
3. Triglyceride

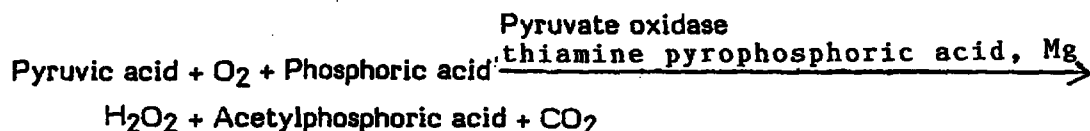
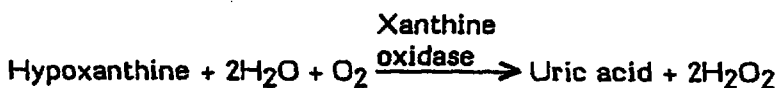
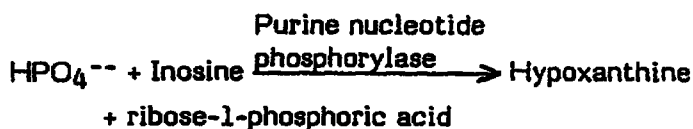
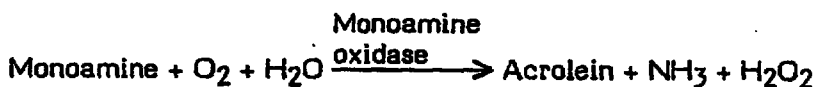


4. Free form of fatty acid



5. Sialic acid



6. Pyruvic acid7. Glucose8. Inorganic phosphorus9. Phospholipid10. Monoamine oxidase11. Choline esterase

The hydrogen peroxide-producing reaction and pigment-producing reaction may be conducted stepwise or more preferably, simultaneously by adding to the sample all the components necessary for the determination of hydrogen peroxide and conducting all the reactions in one step, and measuring the absorbancy of the reaction solution. For this purpose, the various components include the oxidase for the substrate to be determined, a peroxidase, and the chromogen (I) or (II). A buffer solution and surfactant, etc., may be added, if necessary. Of course, if other components are necessary in addition to the oxidase for oxidising the substrate, then these must be added to the H_2O_2 -producing system.

The present invention also provides a test composition for the determination of hydrogen peroxide comprising a peroxidase and a chromogen represented by the formula (I) or (II). The composition may also contain a suitable buffer as well as surfactants such as polyoxyethylenealkylether, antiseptics such as sodium azide, and ascorbate oxidase for decomposing ascorbic acid. Also included are such test compositions which additionally contain a second enzyme, e.g. an oxidase which is capable of producing hydrogen peroxide in situ in the sample from, and proportional to, the concentration of a substrate for said second

enzyme in the said sample, thus enabling the quantitative determination of said substrate in said sample via the determination of the H_2O_2 produced in the sample by the second enzyme.

Example 1

In this Example 18 U of uricase, 100 U of peroxidase, 100 mg of Triton X-100, 0.1 mg of phenol and (A) 100 mg of 4AA and 100 mg of EMAE, (B) 50 mg of LBG, (C) 22.5 mg of Compound No.3, (D) 22.5 mg of Compound No. 5, (E) 22.5 mg of Compound No. 6, (F) 22.5 mg of Compound No. 32 or (G) 22.5 mg of Compound No. 33, are dissolved in 100 ml of 50 mM Good buffer solution (pH 6.5) to prepare a reagent solution. 20 μl of serum and 3 ml of the reagent solution are mixed in a test tube and the mixture incubated at 37°C for 10 minutes.

The absorbancy of each reaction solution at λ_{max} is measured using blank reagent as a control. The concentration of uric acid in the serum is calculated by using calibration curve prepared in advance.

For comparison, the same test sample is analysed by ultra violet spectrophotometry. The results are shown in Table 4.

TABLE 4

Serum No.	Control	Uric acid content (mg/dl)						
		A	B	C	D	E	F	G
1	5.7	5.9	5.0	5.7	5.6	5.7	5.7	5.6
2	2.8	3.8	4.0	2.9	2.7	2.7	2.7	2.8
3	11.0	9.2	12.8	11.0	11.0	11.0	11.1	11.1
4	4.0	5.8	4.9	4.1	4.1	3.9	4.0	3.9
5	6.2	6.5	6.5	6.1	6.1	6.3	6.1	6.0

Example 2

In this Example, 15 U of cholesterol oxidase, 20 U of cholesterol esterase, 150 U of peroxidase, 100 mg of Triton X-100, 0.1 mg of phenol and the chromogens (A to G) in Example 1, are dissolved in 100 ml of 50 mM Good buffer solution (pH 5.8) to prepare a reagent solution. 10 μl of serum and 3 ml of the reagent solution are poured into a test tube and the mixture incubated at 37°C for 10 minutes. The absorbancy of the reaction solution at λ_{max} is measured using blank reagent as a control. The concentration of cholesterol in serum is calculated by using a calibration curve prepared in advance.

For comparison, the same sample is analysed by gas chromatography (GC). The results are shown in Table 5.

TABLE 5

Serum No.	GC	Cholesterol content (mg/dl)						
		A	B	C	D	E	F	G
1	92	82	102	90	91	89	89	93
2	243	248	280	240	242	242	241	242
3	100	90	115	99	100	98	100	100
4	178	169	193	178	176	179	177	178
5	112	120	140	111	111	112	111	112

Example 3

In this Example 9 U of acyl CoA synthetase, 57 U of acyl-CoA oxidase, 833 U of peroxidase, 200 mg of ATP, 27 mg of CoA, 70 mg of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 100 mg of Triton X-100, 0.1 mg of phenol and the chromogens (A to G) used in Example 1, are dissolved in 100 ml of 0.1 M Good buffer solution (pH 6.75) to prepare a

reagent solution. To a 20 μ l serum sample is added 3 ml of the reagent solution and the mixture heated to 37°C for 10 minutes. The absorbancy of the reaction solution at λ_{max} is measured using blank reagent as a control and the concentration of free fatty acid in serum is calculated from a calibration curve. For comparison, the same samples are analysed by gas chromatography (GC). The results are shown in Table 6.

TABLE 6

Serum No.	GC	Free fatty acid content (mg/dl)						
		A	B	C	D	E	F	G
1	80	53	102	82	80	79	79	81
2	115	180	160	115	118	116	114	115
3	102	42	280	101	100	101	102	101
4	258	290	295	260	261	259	259	257
5	180	300	220	180	179	179	181	179

Example 4

In this Example, 100 ml of Good buffer solution (pH 6.0) containing 140 mg of potassium dihydrogen phosphate, 23 mg of thiamine pyrophosphoric acid, 27 mg of $MgCl \cdot 6H_2O$, 300 U of pyruvate oxidase, 100 U of peroxidase, 10 mg of Triton X-100, 0.1 mg of phenol and (A) 100 mg of 4AA and 100 mg of EMAE, (B) 50 mg of LBG, (C) 22.5 mg of Compound No. 5, (D) 22.5 mg of Compound No. 24, (E) 22.5 mg of Compound No. 25, (F) 22.5 mg of Compound No. 26 or (G) 22.5 mg of Compound No. 27, is prepared as a reagent solution. To a 50 μ l serum sample is added 3 ml of the reagent solution and the mixture reacted at 37°C for 10 minutes. The absorbancy of the reaction solution at λ_{max} is measured using blank reagent as a control.

The concentration of pyruvate in the sample is calculated from a calibration curve and the results are shown in Table 7. For comparison, the same sample is analysed by a UV method using lactate dehydrogenase.

TABLE 7

Serum No.	UV	Pyruvic acid content (mg/dl)						
		A	B	C	D	E	F	G
1	0.38	0.53	0.69	0.38	0.38	0.36	0.37	0.38
2	0.91	1.81	0.76	0.93	0.90	0.91	0.91	0.89
3	0.55	0.22	1.10	0.57	0.53	0.57	0.55	0.53
4	0.72	1.01	1.31	0.70	0.71	0.70	0.74	0.70
5	0.50	0.63	0.33	0.49	0.49	0.50	0.48	0.52

Claims

1. A method for the determination of hydrogen peroxide in a sample which comprises reacting the hydrogen peroxide with a peroxidase in the presence of a chromogen and measuring the absorbancy of the reaction solution in the visible or near infra-red region of the spectrum, characterised in that the chromogen is a compound of the formula



reagent solution. To a 20 μ l serum sample is added 3 ml of the reagent solution and the mixture heated to 37°C for 10 minutes. The absorbancy of the reaction solution at λ_{max} is measured using blank reagent as a control and the concentration of free fatty acid in serum is calculated from a calibration curve. For comparison, the same samples are analysed by gas chromatography (GC). The results are shown in Table 6.

TABLE 6

Serum No.	GC	Free fatty acid content (mg/dl)						
		A	B	C	D	E	F	G
1	80	53	102	82	80	79	79	81
2	115	180	160	115	118	116	114	115
3	102	42	280	101	100	101	102	101
4	258	290	295	260	261	259	259	257
5	180	300	220	180	179	179	181	179

Example 4

In this Example, 100 ml of Good buffer solution (pH 6.0) containing 140 mg of potassium dihydrogen phosphate, 23 mg of thiamine pyrophosphoric acid, 27 mg of $MgCl \cdot 6H_2O$, 300 U of pyruvate oxidase, 100 U of peroxidase, 10 mg of Triton X-100, 0.1 mg of phenol and (A) 100 mg of 4AA and 100 mg of EMAE, (B) 50 mg of LBG, (C) 22.5 mg of Compound No. 5, (D) 22.5 mg of Compound No. 24, (E) 22.5 mg of Compound No. 25, (F) 22.5 mg of Compound No. 26 or (G) 22.5 mg of Compound No. 27, is prepared as a reagent solution. To a 50 μ l serum sample is added 3 ml of the reagent solution and the mixture reacted at 37°C for 10 minutes. The absorbancy of the reaction solution at λ_{max} is measured using blank reagent as a control.

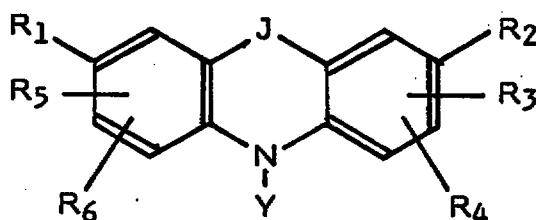
The concentration of pyruvate in the sample is calculated from a calibration curve and the results are shown in Table 7. For comparison, the same sample is analysed by a UV method using lactate dehydrogenase.

TABLE 7

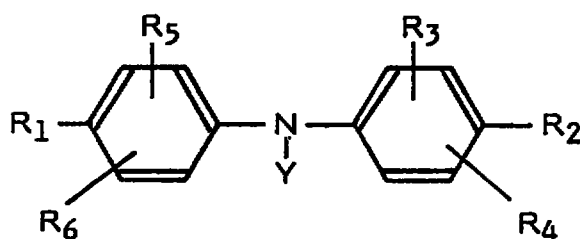
Serum No.	UV	Pyruvic acid content (mg/dl)						
		A	B	C	D	E	F	G
1	0.38	0.53	0.69	0.38	0.38	0.36	0.37	0.38
2	0.91	1.81	0.76	0.93	0.90	0.91	0.91	0.89
3	0.55	0.22	1.10	0.57	0.53	0.57	0.55	0.53
4	0.72	1.01	1.31	0.70	0.71	0.70	0.74	0.70
5	0.50	0.63	0.33	0.49	0.49	0.50	0.48	0.52

Claims

1. A method for the determination of hydrogen peroxide in a sample which comprises reacting the hydrogen peroxide with a peroxidase in the presence of a chromogen and measuring the absorbancy of the reaction solution in the visible or near infra-red region of the spectrum, characterised in that the chromogen is a compound of the formula

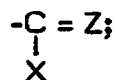


or



where:

Y represents hydrogen or



Z represents oxygen or sulfur;

X represents hydrogen, alkyl, alkenyl, aryl, aralkyl, amino or substituted amino;

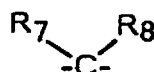
R₁ represents hydroxyl, amino or substituted amino;

R₂ represents hydrogen, hydroxyl, alkyl, alkoxy, aryl, aralkyl, alkenyl, amino or substituted amino;

R₃ represents a group of formula (III), (IV), (V), (VI) or (VII) described hereinafter;

R₄, R₅ and R₆ are the same or different and represent hydrogen, alkyl, alkenyl, acyl, aryl, aralkyl, halogen atom, nitro, sulfo, carboxyl, hydroxyl, alkoxy or a group of formula (III), (IV), (V), (VI) or (VII) described hereinafter, with the proviso that R₅ and R₆ may also be joined together to form an alkenylene group;

J represents -S-, -O-, or a group of the formula



or



and

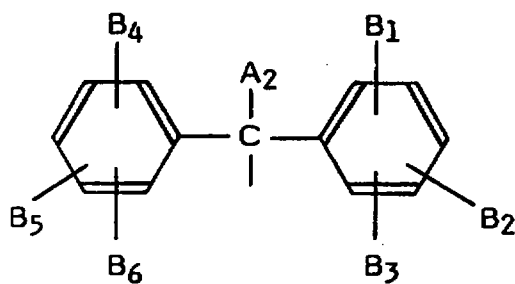
R₇ and R₈ are the same or different and represent hydrogen, alkyl or alkenyl;



the groups of formula III to VII being:

5

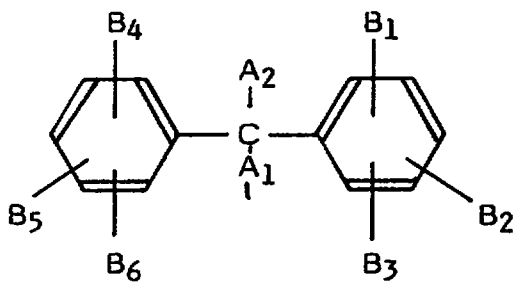
10



Formula III

15

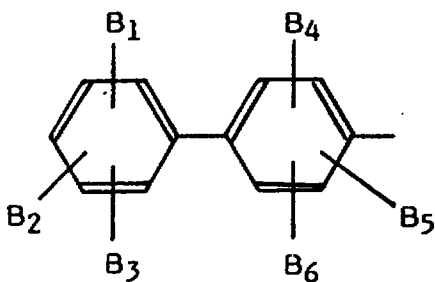
20



Formula IV

25

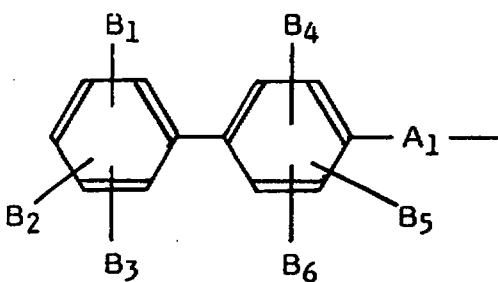
30



Formula V

35

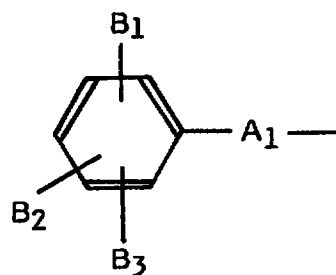
40



Formula VI

45

50



Formula VII

55

wherein:



A₁ represents alkylene;

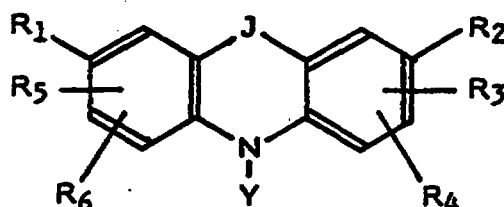
A₂ has the same meaning as R₂;

B₁, B₂, B₃, B₄, B₅ and B₆ are the same or different and represent hydrogen, alkyl, alkenyl, acyl, aryl, aralkyl, halogen atom, nitro, sulfo, carboxyl, hydroxyl, alkoxy or hydroxyalkyl.

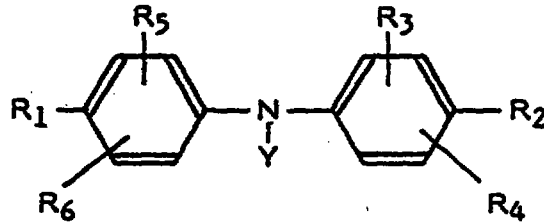
2. A method according to claim 1, wherein the hydrogen peroxide is produced in said sample as the result of an enzymatic reaction.
3. A method according to claim 2, wherein the hydrogen peroxide is produced in said sample as the result of an enzymatic reaction involving the reaction of an oxidase.
4. A method according to claim 3, wherein said oxidase is uricase, cholesterol oxidase, xanthine oxidase, choline oxidase, pyruvate oxidase, glycerin-3-phosphate oxidase, acyl CoA oxidase, glycerol oxidase, glucose oxidase or galactose oxidase.
5. A method according to claim 2, 3 or 4, wherein the hydrogen peroxide-producing enzymatic reaction and the reaction of hydrogen peroxide with said chromogen and the peroxidase are conducted simultaneously.
6. A method according to any one of claims 1 to 5, wherein the reaction(s) is or are carried out in a buffer solution.
7. A test composition for the determination of hydrogen peroxide, comprising a chromogen of formula I or II as defined in claim 1, and a peroxidase.
8. A test composition according to claim 7, which additionally contains a buffer.
9. A test composition according to claim 7 or 8, additionally containing a surfactant, antiseptic or ascorbate oxidase.
10. A test composition according to claim 7, 8 or 9, additionally containing a second enzyme capable of producing hydrogen peroxide *in situ* in the sample from, and proportional to the concentration of a substrate for said second enzyme in the said sample.
11. A test composition according to claim 10, wherein said second enzyme is an oxidase.
12. A test composition according to claim 11, wherein said oxidase is an oxidase as listed in claim 4.

Patentansprüche

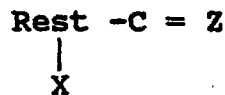
1. Verfahren zur Bestimmung von Wasserstoffperoxid in einer Probe, das das Umsetzen des Wasserstoffperoxids mit einer Peroxidase in Gegenwart eines Chromogens und die Messung der Absorption der Reaktionslösung im sichtbaren oder nahen infraroten Spektralbereich umfaßt, dadurch gekennzeichnet, daß das Chromogen eine Verbindung der Formel



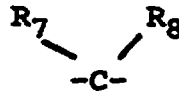
oder



ist, wobei
Y ein Wasserstoffatom oder der



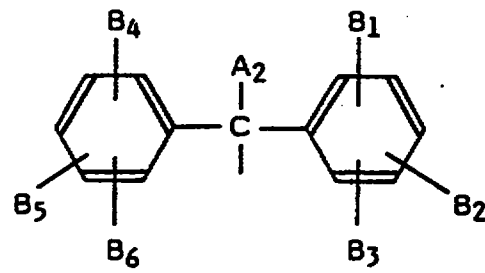
ist;
Z ein Sauerstoff- oder Schwefelatom ist;
X ein Wasserstoffatom, ein Alkyl-, Alkenyl-, Aryl-, Aralkyl-, Amino- oder substituierter Aminorest ist;
R₁ ein Hydroxyl-, Amino- oder substituierter Aminorest ist;
R₂ ein Wasserstoffatom, ein Hydroxyl-, ein Alkyl-, Alkoxy-, Aryl-, Aralkyl-, Alkenyl-, Amino-, oder substituierter Aminorest ist;
R₃ eine Gruppe der Formel (III), (IV), (V), (VI) oder (VII) die nachstehend beschrieben sind, ist;
R₄, R₅ und R₆ gleich oder verschieden sind und ein Wasserstoffatom, einen Alkyl-, Alkenyl-, Acyl-, Aryl-, Aralkylrest, ein Halogenatom, eine Nitro-, Sulfo-, Carboxyl-, Hydroxylgruppe, einen Alkoxyrest oder eine Gruppe der Formel (III), (IV), (V), (VI) oder (VII), die nachstehend beschrieben sind, darstellen, mit der Maßgabe, daß R₅ und R₆ auch miteinander zu einem Alkenylenrest verbunden sein können;
J ein Schwefel- oder Sauerstoffatom oder eine Gruppe der Formel



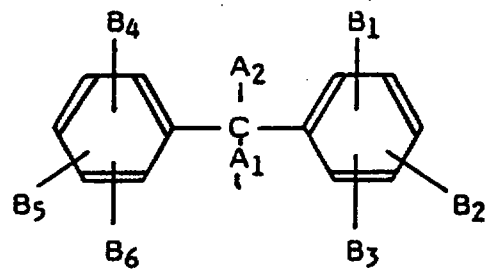
oder



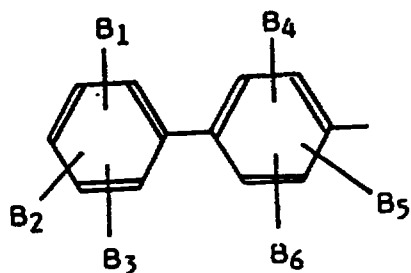
ist und
R₇ und R₈ gleich oder verschieden sind und ein Wasserstoffatom, einen Alkyl- oder Alkenylrest darstellen;
die Gruppen der Formel III bis VII



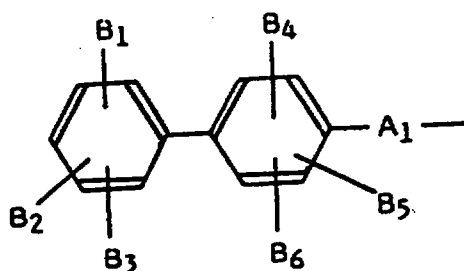
Formel III



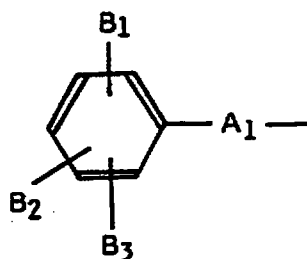
Formel IV



Formel V



Formel VI



Formel VII

sind, wobei

A₁ ein Alkylrest ist;

A₂ die gleiche Bedeutung wie R₂ hat;

B₁, B₂, B₃, B₄, B₅ und B₆ gleich oder verschieden sind und ein Wasserstoffatom, einen Alkyl-, Alkenyl-, Acyl-, Aryl-, Aralkylrest, ein Halogenatom, Nitro-, Sulfo-, Carboxyl-, Hydroxylgruppen, Alkoxy- oder Hydroyalkylreste sind.

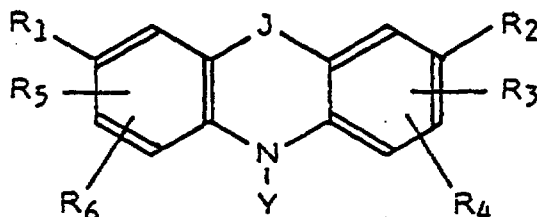
2. Verfahren nach Anspruch 1, wobei das Wasserstoffperoxid in der Probe als Ergebnis einer enzymatischen Reaktion hergestellt wird.
3. Verfahren nach Anspruch 2, wobei das Wasserstoffperoxid in der Probe als Ergebnis einer enzymatischen Reaktion unter Beteiligung der Reaktion einer Oxidase hergestellt wird.
4. Verfahren nach Anspruch 3, wobei die Oxidase Uricase, Cholesterin-Oxidase, Xanthin-Oxidase, Cholin-Oxidase, Pyruvat-Oxidase, Glycerin-3-phosphat-Oxidase, Acyl-CoA-Oxidase, Glycerin-Oxidase, Glucose-Oxidase oder Galactose-Oxidase ist.
5. Verfahren nach Anspruch 2, 3 oder 4, wobei die Wasserstoffperoxid erzeugende enzymatische Reaktion und die Reaktion des Wasserstoffperoxids mit dem Chromogen und der Peroxidase gleichzeitig durchgeführt werden.



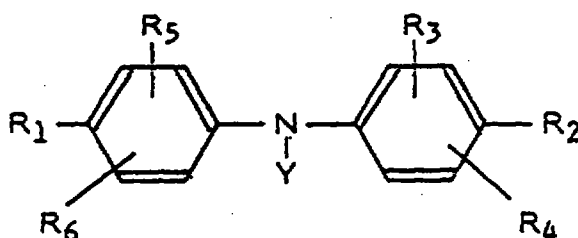
6. Verfahren nach einem der Ansprüche 1 bis 5, wobei die Reaktion(en) in einer Pufferlösung ausgeführt wird (werden).
7. Prüfungszusammensetzung zur Bestimmung von Wasserstoffperoxid, umfassend ein Chromogen der Formel I oder II nach Anspruch 1 und eine Peroxidase.
8. Prüfungszusammensetzung nach Anspruch 7, die zusätzlich einen Puffer enthält.
9. Prüfungszusammensetzung nach Anspruch 7 oder 8, die zusätzlich ein Tensid, ein Antiseptikum oder Ascorbat-oxidase enthält.
10. Prüfungszusammensetzung nach Anspruch 7, 8 oder 9, die zusätzlich ein zweites Enzym enthält, das dazu fähig ist, in der Probe in situ Wasserstoffperoxid aus einem Substrat für dieses zweite Enzym und proportional zur Konzentration dieses Substrats herzustellen.
11. Prüfungszusammensetzung nach Anspruch 10, wobei das zweite Enzym eine Oxidase ist.
12. Prüfungszusammensetzung nach Anspruch 11, wobei die Oxidase eine der in Anspruch 4 aufgeführten Oxidasen ist.

Revendications

1. Procédé de dosage du peroxyde d'hydrogène dans un échantillon, qui comporte la réaction du peroxyde d'hydrogène avec une peroxydase en présence d'un chromogène, et la mesure de l'absorbance de la solution réactionnelle dans le domaine visible ou proche infrarouge du spectre, caractérisé en ce que le chromogène est un composé de formule :



ou



dans lesquelles :

Y représente un atome d'hydrogène ou un groupe $-C(=Z)X$, où Z représente un atome d'oxygène ou de soufre et X représente un atome d'hydrogène ou un groupe alkyle, alcényle, aryle, aralkyle, amino ou amino substitué ;

R₁ représente un groupe hydroxyle, amino ou amino substitué ;

R₂ représente un atome d'hydrogène ou un groupe hydroxyle, alkyle, alcoxy, aryle, aralkyle, alcényle, amino ou amino substitué ;

R₃ représente un groupe de formule (III), (IV), (V), (VI) ou (VII), formules qui sont décrites plus loin ;

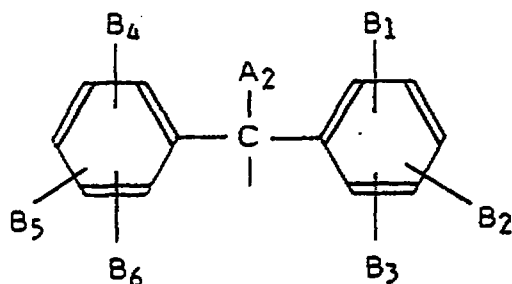
R₄, R₅ et R₆ sont identiques ou différents et représentent des atomes d'hydrogène ou d'halogène ou



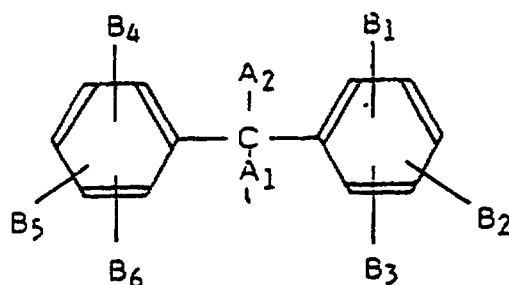
des groupes alkyle, alcényle, acyle, aryle, aralkyle, nitro, sulfo, carboxyle, hydroxyle ou alcoxy, ou encore des groupes de formule (III), (IV), (V), (VI) ou (VII), formules qui seront décrites plus loin, R_5 et R_6 pouvant également être réunis l'un à l'autre pour former un groupe alcénylène ;

J représente -S-, -O- ou un groupe de formule $-CR_7R_8-$ ou $-NR_7-$, R_7 et R_8 étant identiques ou différents et représentant des atomes d'hydrogène ou des groupes alkyle ou alcényle ;

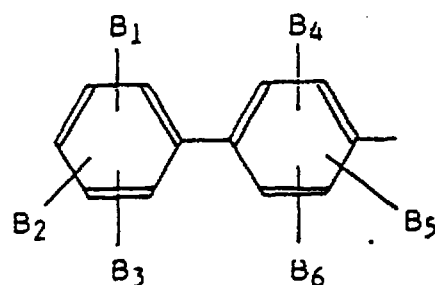
les groupes de formules (III) à (VII) étant les suivants :



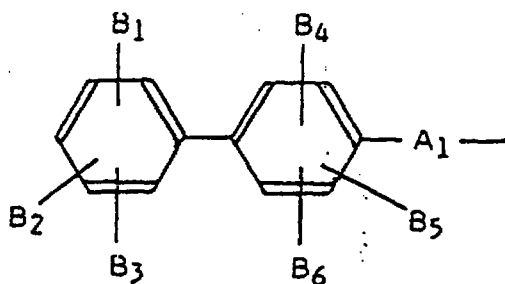
Formule III



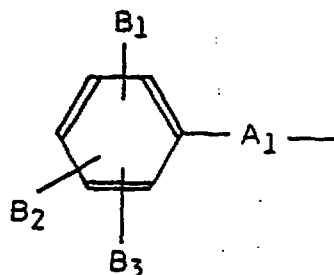
Formule IV



Formule V



Formule VI



Formule VII

dans lesquelles :

A₁ représente un groupe alkylène ;

A₂ a la même signification que R₂ ;

B₁, B₂, B₃, B₄, B₅ et B₆ sont identiques ou différents et représentent des atomes d'hydrogène ou d'halogène ou des groupes alkyle, alcényle, acyle, aryle, aralkyle, nitro, sulfo, carboxyle, hydroxyle, alcoxy ou hydroxyalkyle.

2. Procédé conforme à la revendication 1, dans lequel le peroxyde d'hydrogène est produit dans ledit échantillon en résultat d'une réaction enzymatique.
3. Procédé conforme à la revendication 2, dans lequel le peroxyde d'hydrogène est produit dans ledit échantillon en résultat d'une réaction enzymatique impliquant la réaction d'une oxydase.
4. Procédé conforme à la revendication 3, dans lequel ladite oxydase est une uricase, une cholestérol oxydase, une xanthine oxydase, une choline oxydase, une pyruvate oxydase, une glycérine-3-phosphate oxydase, une acyl-CoA oxydase, une glycérol oxydase, une glucose oxydase ou une galactose oxydase.
5. Procédé conforme à la revendication 2, 3 ou 4, dans lequel la réaction enzymatique produisant le peroxyde d'hydrogène et la réaction du peroxyde d'hydrogène avec ledit chromogène et la peroxydase sont effectuées simultanément.
6. Procédé conforme à l'une quelconque des revendications 1 à 5, dans lequel la ou les réaction(s) est ou sont effectuée(s) dans une solution tampon.
7. Composition de test pour le dosage du peroxyde d'hydrogène, comprenant un chromogène de formule I ou II définie dans la revendication 1, et une peroxydase.
8. Composition de test conforme à la revendication 7, qui contient en outre un tampon.
9. Composition de test conforme à la revendication 7 ou 8, qui contient en outre en tensioactif, un antiseptique ou une ascorbate oxydase.
10. Composition de test conforme à la revendication 7, 8 ou 9, qui contient en outre une seconde enzyme capable de produire du peroxyde d'hydrogène in situ dans l'échantillon, à partir d'un substrat pour



cette seconde enzyme présent dans l'échantillon et en proportion de la concentration de ce substrat.

11. Composition de test conforme à la revendication 10, dans laquelle ladite seconde enzyme est une oxydase.

12. Composition de test conforme à la revendication 11, dans laquelle ladite oxydase est l'une des oxydases énumérées dans la revendication 4.

5

10

15

20

25

30

35

40

45

50

55