This invention relates to an improved diagnostic composition and to a method for its preparation. Particularly, this invention is concerned with a diagnostic test which is useful for the qualitative detection and quantitative determination of blood in body fluids and products of elimination such as urine, vomitus, gastro-intestinal contents, cerebrospinal fluids and feces. In particular, this invention is concerned with a highly sensitive reagent composition for the detection of occult blood which may be incorporated upon a bibulous carrier.

The detection of occult blood in body fluids and body excreta has become an invaluable aid to the medical practitioner in the diagnosis of a great number of abnormal conditions. Medical science has long recognized a need for and the usefulness of a test for occult blood. Hence, it is considered extremely desirable to provide a simple and economical test for the qualitative detection and quantitative determination of occult blood in body fluids and body excreta.

Blood is found in the gastric contents and in vomitus in conditions associated with erosion of gastric and intestinal mucous membranes and, for example, ulcerous and carcinogenic conditions. In the feces, the regular and frequent occurrence of occult blood is suggestive of, for example, gastro-intestinal cancer, gastric or duodenal ulcers or hemorrhoids. Quite often, hemorrhage is so slight that it is no possible to detect the presence of blood by microscopic examination. Hence, a sensitive and specific diagnostic test for occult blood is highly desirable.

In the urine, the presence of blood cells or blood pigments may be indicative of a number of abnormal conditions, for example, typhus, scurvy, purpura, pyemia, nephritis, third degree burns, carcinogenic conditions of the urinary system, and as a result of the action of various hemolytic toxins and the like. In accordance with this invention, a simple and economical diagnostic test composition is provided which may advantageously be used with ease by hospital and clinical personnel untrained in laboratory technique as well as the skilled laboratory technician and physician.

A variety of techniques, reagents, and devices have been used or proposed in the past for the detection of occult blood in body fluids and body excreta, for example, U.S. Patent 2,290,436 to Jonas Kamlet, issued July 21, 1942; U.S. Patent 2,799,660 to Richard S. Nicholls and Dale E. Fonner, issued July 16, 1957; U.S. Patent 2,838,377 to Dale E. Fonner, issued June 10, 1958; U.S. Patent 3,012,976 to Ernest C. Adams, Jr., and James A. Peterson, issued December 12, 1961; and U.S. Patents 3,092,463 and 3,092,464 to Ernest C. Adams, Jr., and James Peterson, issued June 4, 1963, all assigned to the instant assignees.

The inventive concept of the instant application, as that of the prior art, is based on the catalytic activity of the prosthetic groups present in blood. The catalytically active substances identified in hemoglobin belong to the general class of hemoproteins, conjugate proteins, all of which have the same prosthetic groups, iron protoporphyrin or heme. This prosthetic group has the ability to catalyze the oxidation of certain compounds by peroxides such as hydrogen peroxide, metal peroxides and organic peroxides. If the compound is an indicator or dye precursor, colorless until it becomes oxidized and colored in its oxidized form, then the presence of the catalytic activity and hence the presence of blood is indicated by color formation. The rapidity of the color change and the depth or density of the color when compared to a set of standards is a means of the quantitative determination of the blood present.

In accordance with this invention, we have discovered that the color formation in the presence of blood utilizing reagent compositions comprising an indicator and peroxide is unexpectedly enhanced by the addition of a quinoline derivative to each of the various blood detecting compositions. The quinoline derivatives found to be satisfactory as potentiating agents in the formulation of this invention include quinoline itself and have the general formula

\[
\text{H}_2\text{N}-\text{CH}=\text{CH}-\text{CO}-\text{H}
\]

where \( R_1 \) may be hydrogen, methyl or hydroxy; \( R_2 \) may be hydrogen, methyl or thiophenyl; \( R_3 \) may be hydrogen or methyl or the substituted quinuclidine radical,

\[
\text{H}_2\text{N}-\text{CH}=\text{CH}-\text{CO}-\text{H}
\]

which is common to quinine and cinchonine; \( R_4 \) may be hydrogen, methyl or methoxy; \( R_5 \) may be hydrogen or methyl and \( R_6 \) may be hydrogen, methyl hydroxy or amino. Quinolines substituted in the 4, 6 or 7 position together with quinoline itself and quinoline form a preferred subgroup of potentiating agents. By way of specific examples, suitable potentiating agents include quinoline; quinine; cinchonine; 6-methoxyquinoline; 4,6-dimethylquinoline; 6-methylquinoline; 7-methylquinoline; 2,6-dimethylquinoline; 2-methylquinoline (quinidine); 8-amino-6-methoxyquinoline; 6methoxy-3-phenylisquinoline; 8-methylquinoline; 2,3-dimethylquinoline 2-quinolinol; 2-methyl-8-quinolinol and 8-quinolinol.

As suggested above, each of the occult blood tests aforementioned may be improved by the addition of the quinoline or substituted quinoline potentiating agent. For example the potentiating agent may be mixed with the dry compositions or tablets of U.S. Patent 2,290,436 to produce a diagnostic composition having greater sensitivity than the mixture of indicator peroxide and acidic material disclosed in the patent. In another respect, the tablets disclosed in U.S. Patent 2,799,660 comprising indicator peroxide buffer and effervescent couple and color contrast agent may be similarly improved by use of the potentiating agents of this invention. The blood test of U.S. Patent 2,838,377 comprising an indicator, a peroxide and a buffer incorporated in a closed envelope formed of an electrolyte permeable sheet material may be similarly improved by the utilization of one or more of the various potentiating agents described above.

The occult blood tests described in U.S. Patent 3,012,976 comprise an organic hydroperoxide, an indicator and a buffer and may be improved by the inclusion therein of the potentiating agent of the instant invention. The encapsulated compositions of U.S. Patent 3,092,463 may be likewise improved by use of a potentiating agent of the quinoline type. Likewise the organic hydroperoxides of U.S. 3,092,464 are similarly improved.
In an especially preferred embodiment of this invention, a group of organic hydroperoxides having pronounced sensitivity in an indicator reaction may be encapsulated or entrapped with a colloidal material such as gelatin and the gelatin hardened by fixing with a dialdehyde polysaccharide. The addition of a buffer and a suitable indicator as well as a potentiating agent of the quinoline or substituted quinoline family completes the formulation which results in an extremely sensitive diagnostic test reagent for occult blood.

The compositions of this invention may be prepared in solution or tablet form or may be used to impregnate a bibulous material such as paper, wood, fiber or the like, having any desired size or shape. Such products will undergo a distinct color change when contacted with an occult blood-containing specimen.

The group of organic hydroperoxides mentioned above which have been found to have the pronounced sensitivity which is required for the indicator reaction to be utilized in the diagnostic compositions of this invention comprise a group of materials including cumene hydroperoxide, diisopropylbenzenes hydroperoxide; paramethane hydroperoxide; and 2,5-dimethylhexane, 2,5-dihydroperoxide; to mention a few of the significant members of this group. Other organic hydroperoxides of similar structure may likewise be used if desired.

Organic hydroperoxides, while producing outstanding results in various occult blood diagnostic compositions, have been found to be relatively unstable when mixed with the various other materials which are required in the provision of diagnostic compositions of this nature. In accordance with the co-pending application of Ernest C. Adams, Jr., and Norman R. Novak, S.N., 107,646, filed May 4, 1961, a novel encapsulating material is provided for the organic hydroperoxide. This encapsulating material may be comprised of various protein or polysaccharide materials such as gelatin, algin, carrageenan, casein, albumin or other materials of this nature. These proteinaceous or polysaccharaceous materials are applied to the test compositions and then hardened by means of a fixing process which involves treating with a dialdehyde polysaccharide which is effective for this purpose.

By way of example of buffering systems useful in the composition of this invention are tannate, phosphate, phthalate, citrate and acetate buffer. The preferred range of hydrogen ion concentration to which the composition is buffered is about pH 4 to pH 7.

A pointed out above, the encapsulating material to be used in the diagnostic compositions of this invention is fixed or hardened by treatment with a dialdehyde polysaccharide. Dialdehyde polysaccharides, for example, dialdehyde starch, may be prepared by the well-known oxidation of polysaccharides with periodic acid. This preparation is illustrated by the conversion of starch to dialdehyde starch using periodic acid as the oxidizing agent in accordance with the below set-out equation where in "x" stands for the number of repeating units in a molecule which may range from 20 to several thousand.

$$\begin{align*}
\text{CH}_2\text{OH} & \quad \text{H}_2\text{O} \\
\text{O} & \quad \text{H}_2\text{O} \\
\text{H} & \quad \text{H} \\
\text{OH} & \quad \text{H} \\
\text{H} & \quad \text{OH}
\end{align*}$$

The dialdehyde polysaccharides used in this process may be the dialdehyde derivatives of any polysaccharide such as a corn, wheat, tapioca or potato starches, celluloses, gums, dextrins, algins, inulin or others. Of these polysaccharides, the dialdehyde derivatives of starch, known generally as dialdehyde starch, are the best known and most often used. However, where it is desired to have derivatives of other polysaccharides, these may also be used. In the preferred embodiment, dialdehyde polysaccharides are used which are from about 50% to 100% oxidized, that is, in the case of dialdehyde starch, those wherein 20 to 100 per 100 of the original hydroxyl groups have been converted to dialdehyde units such as by periodate oxidation.

In accordance with this invention, the dialdehyde polysaccharide is used in the form of an aqueous dispersion which may be readily prepared by adding the dialdehyde polysaccharide in the desired concentration, usually from about 0.25% to about 1.5%, to tap water or to a buffer solution and then mixing the aqueous dispersion with warming until a relatively homogenous dispersion is obtained. The dialdehyde polysaccharide may be used in whatever concentration is required to satisfactorily harden the encapsulating material to provide the desired stability features. The precise concentration to be used will be pointed out more particularly hereafter.

In the preparation of the compositions of this invention, it has been found desirable but not essential to mix the organic hydroperoxide being used with an emulsifying agent, for example, acacia mucilage, to form a primary emulsion. Other emulsifying agents which may be used include polyvinyl alcohol, gum arabic, carboxy vinyl polymer, and the like. A surfactant, or wetting agent, may also be used. In the preferred embodiment sodium lauryl sulfate is used as a wetting agent, however, diocetyl sodium sulfosuccinate for example is also satisfactory. An emulsifying agent when used serves to lower the surface tension of the oily organic hydroperoxide and forms a film around each individual oil droplet. The surfactant assists in this regard and, in addition, produces an even diffusion of color on the diagnostic stick. The resulting primary emulsion is buffered by means of an appropriate buffer as discussed hereinabove. Preferably, the buffer in dilute form is first mixed with the material which is to form the capsule for the organic polysaccharide, for example, gelatin, before addition to the hydroperoxide emulsion. If the concentrated buffer is used with the encapsulating agent there is a tendency to precipitate the encapsulating agent prior to completion of the encapsulation. After the organic hydroperoxide has been entrapped or encapsulated, the concentrated buffer is added which has the effect of "setting" the encapsulation while controlling the pH and preventing false positives in testing. An aqueous dispersion of the dialdehyde polysaccharide is next added to serve as a fixing agent for the encapsulating material. The encapsulation material which is present in the film around the oily organic hydroperoxide droplets is thereby fixed by the dialdehyde polysaccharide, resulting in a more stable preparation.

It should be noted that thorough mixing is of extreme importance after each addition. This is particularly so upon the addition of the buffer-encapsulation mixture to the primary emulsion of the organic hydroperoxide. The addition of a suitable surfactant or wetting agent aids in the formation of a stable composition. The indicator, potentiating agent and organic solvent are mixed and then added to the hydroperoxide composition.

Although orthotolidine is the preferred indicator for the diagnostic compositions of this invention, various other indicator materials may be used so long as they satisfy the requirements pointed out above, namely, that they undergo a color change in the presence of an oxygen source and the blood for which the unknown material is being tested. Such indicators comprise a variety of organic materials, principally those of aniline and phenol derivation. To name but a few, there may be used in
addition to orthotolidine, orthotoluidine, paratoluidine, orthophenylenediamine, \(\text{N,N}'\)-dimethyl-\(p\)-phenylenediamine, \(\text{N,N}'\)-diethyl-\(p\)-phenylenediamine, benzidine, \(p\)-anisidine, di-\(p\)-anisidine, \(o\)-cresol, \(m\)-cresol, \(p\)-cresol, alpha-naphthol, beta-naphthol, catechol, guaiacol and pyrogallol.

As discussed herebefore, numerous substituted quino- lines may be used as the potentiating agent in the testing compositions of this invention. In the preferred embodiment, however, quinoline itself or quinolines substituted in the 4-, 6- or 7-positions are used. Because of availability, economic reasons, and suitable physical properties, quinoline has been found quite useful.

Among the organic solvents found useful in the composition and methods of this invention are chloroform, ethylene dichloride, benzene and ethyl acetate.

The novel compositions of this invention may be prepared in tablet form, a convenient tablet size being about \(\frac{3}{4}\)" thick and about \(\frac{1}{4}\)" in diameter. In determining the presence or absence of occult blood present in a specimen, a drop of the material to be tested (in the case of feces—an asepous suspension of the specimen) is placed on a piece of dry filter paper and then, the drop has soaked into the paper, a reagent tablet is placed in the center of the drop and activated by the addition of two drops of water to the tablet. With a positive test, a ring of color appears on the filter paper surrounding the tablet, the color intensity varying, dependent upon the concentration of blood in the specimen. The color developed is then compared with a calibrated color chart to determine the concentration.

Alternatively, the dry composition may be added to a specific amount of water and mixed with the material to be tested. In the presence of occult blood, a color will be obtained. An estimation of the amount of occult blood present in the specimen may be made by comparing the color developed with calibrated standards.

It has been found that incorporating the compositions upon discs or strips of paper, small sticks of wood or other biblious materials produces a most useful and satisfactory diagnostic aid. In use, an impregnated strip is simply dipped in the liquid specimen or suspensions of the material to be tested. In the presence of occult blood, the test strip will give a positive color reaction. The color resulting on the strip is then compared with precalibrated color standards for an estimation of the quantitative amount of occult blood contained in the specimen. The color developed in the presence of occult blood varies in intensity according to the amount of blood present. From a commercial point of view, test compositions in the form of bibulous sticks or strips are highly preferred because such provide the diagnostican with a simple "dip and read" test.

The range of compositions of the diagnostic material provided by this invention may vary within fairly wide limits in accordance with the general teachings set forth above.

The following examples will illustrate the improved diagnostic compositions of the present invention and methods of preparing, the scope of the invention not, however, being limited to the specific details of these examples.

**EXAMPLE 1**

*Hydroperoxide solution.*—40 grams of gum arabic were dissolved in 100 ml. of boiling water. To 100 ml. of this solution held at a temperature above \(60^\circ C\) was added 5 ml. of cumene hydroperoxide and the mixture stirred for 5 minutes. 700 mg. gelatin was dissolved in 50 ml. of Buffer I (217.7 g. sodium citrate plus 49.2 g. citric acid made to 2 liters with \(H_2O\)) and added to the cumene hydroperoxide mixture. Both solutions being above \(60^\circ C\). A ml. of 1% diadhyde starch was added and mixed. Ten ml. of 5% sodium lauryl sulfate was added and mixed. The resulting mixture was homogenized and allowed to cool to room temperature.

**EXAMPLE 2**

A hydroperoxide solution was prepared as in Example 1. *Indicator.*—Prepared by dissolving 400 mg. o-tolidine base, 10 mg. quinine alkaloid and 12 mg. polyoxyethyleneenonylophenol in 20 ml. chloroform.

**EXAMPLES 3 and 4**

The testing reagents were prepared in accordance with Example 1, the quinine alkaloid ingredient being increased to 20 mg. and 50 mg. per 20 ml. respectively.

**EXAMPLES 5 and 6**

The hydroperoxide solution was prepared in accordance with that of Example 2, the indicator solution was prepared containing 2 mg. polyoxyethyleneenonylophenol and 20 mg. and 50 mg. quinine alkaloid per 20 ml. respectively.

**EXAMPLE 7**

The hydroperoxide solution was prepared in accordance with that of Example 1. The indicator solution was prepared to contain 80 mg. o-tolidine, 10 mg. quinine alkaloid and 2 mg. polyoxyethyleneenonylophenol per 20 ml. chloroform.

**EXAMPLE 8**

A hydroperoxide solution was prepared in accordance with that of Example 1. The indicator solution was prepared to contain 80 mg. o-tolidine, 5 mg. 6-methylquinoline and 2 mg. polyoxyethyleneenonylophenol per 20 ml.

**EXAMPLE 9**

A hydroperoxide solution was prepared in accordance with that of Example 1. The indicator solution was prepared to contain 80 mg. o-tolidine, 5 mg. 6-methylquinoline and 2 mg. polyoxyethyleneenonylophenol per 20 mg.

**EXAMPLE 10**

A hydroperoxide solution was prepared in accordance with that of Example 1. The indicator solution was prepared to contain 80 mg. o-tolidine, 5 mg. 7-methylquinoline and 2 mg. polyoxyethyleneenonylophenol per 20 ml.

**EXAMPLE 11**

A hydroperoxide solution was prepared in accordance with that of Example 1. The indicator solution was prepared to contain 80 mg. o-tolidine, 5 mg. 4,6-dimethylquinoline and 2 mg. polyoxyethyleneenonylophenol per 20 mg.

**EXAMPLE 12**

A hydroperoxide solution was prepared in accordance with that of Example 1. The indicator solution was prepared to contain 80 mg. o-tolidine, 4 mg. quinoline and 2 mg. polyoxyethyleneenonylophenol per 20 ml.

**EXAMPLE 13**

A hydroperoxide solution was prepared in accordance with that of Example 1. Indicator solution was prepared to contain 80 mg. o-tolidine, 4 mg. quinoline and 2 mg. polyoxyethyleneenonylophenol per 20 ml.

Further reagent solutions were prepared in accordance in Examples 1 and 2 utilizing as solvents ethylene dichloride and benzene. Where benzene is used as the solvent, it was found to be advantageous to decrease the
amount of o-tolidine in the formulation for the reason that o-tolidine is less soluble in benzene than in chloroform or ethylene dichloride. While it is not essential, it was unexpectedly discovered by observation under ultraviolet light that the addition of a surface-active agent, for example, poloxymethyleneenonphenol, to the indicator solution resulted in a more even application of the color indicator and quinine alkaloid when prepared in a strip or stick form. It was found that the amount of surface-active agent could be varied over a range between 1 and 20 mg. per 20 ml., the preferred amount being in the lower end of the range.

**Preparation of reagent strips**

Bibulous “sticks,” that is, absorbent paper cut into narrow strips having dimensions of about 3” x ½” x 0.029”, were dipped into the hydroperoxide solution followed by drying in a drying tunnel at a temperature of about 100° C. The dried strips were subsequently heated in an oven at 70° C. for 8 to 16 hours to remove excess cumene hydroperoxide. The strips were similarly dipped into the indicator solution and dried in a 70° C. oven or a 50° C. drying tunnel. The finished impregnated strips were white to cream in color.

By way of illustration of the increased sensitivity of the test reagents of this invention, Table 1 sets out the results of 114 urine examinations from hospitalized patients, comparing the improved diagnostic test of this invention with prior known formulations.

<table>
<thead>
<tr>
<th>Number of Uries in Clasification</th>
<th>Cells per High Power Field</th>
<th>Composition Without Potentiating Agent</th>
<th>Applicants Improved Testing Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>62</td>
<td>Occasional 1</td>
<td>35 negatives 4 trace 3 small</td>
<td>48 negatives 5 trace 7 small 2 moderate</td>
</tr>
<tr>
<td>26</td>
<td>1-6</td>
<td>19 negatives 2 trace 4 small 1 moderate</td>
<td>10 negatives 1 trace 8 small 6 moderate 1 large</td>
</tr>
<tr>
<td>11</td>
<td>2-10</td>
<td>5 negatives 0 trace 2 small 4 moderate</td>
<td>1 negative 1 trace 3 small 4 moderate 2 large</td>
</tr>
<tr>
<td>7</td>
<td>10-20</td>
<td>3 negatives 1 trace 2 small 1 moderate</td>
<td>0 negative 0 trace 0 trace 0 trace 0 trace</td>
</tr>
<tr>
<td>8</td>
<td>20-50</td>
<td>2 negatives 0 trace 3 small 2 moderate</td>
<td>0 negative 0 trace 3 small 3 moderate 2 large</td>
</tr>
</tbody>
</table>

**Example 14**

**Benzidine method**

To 2 ml. of urine add 3 ml. of saturated benzidine in glacial acetic acid (4 g.-50 ml.) and 1 ml. of 3% hydrogen peroxide mix. A green to blue color indicates a positive reaction.

2 mg. of quinine were added to the above described method. A faster and more intense color was obtained. 2 mg. of 6-methoxyquinoline were added. This also made the color more intense but not as much as did the quinine.

Similar results have been obtained utilizing each of the potentiating agents described above.

It will be clearly understood by those skilled in the art that certain changes may be made in the above compositions and methods without departing from the spirit and scope of the invention and it is intended that all matter contained in the foregoing description shall be interpreted as illustrative and not in a limiting sense. It is also understood that other modifications may be made without departing from the spirit and scope of the appended claims.

What is claimed is:

1. A composition for detecting occult blood in body fluids and excreta comprising an organic hydroperoxide, a buffer capable of maintaining the pH of the material being tested within the range of about 4 to 7, gelatin and as a fixative for said gelatin a dialdehyde polysaccharide, an indicator capable of being oxidized in the presence of the prosthetic group of hemoglobin with an accompanying color change and a potentiating agent of the formula

\[ \text{H} \]  
\[ \text{CH}-\text{N} \]  
\[ \text{CH}_2\text{CH}-\text{CH} \]  
\[ \text{CH}_3\text{CH} \]  
\[ \text{CH}_3\text{CH} \]

wherein \( R_1 \) is a member selected from the group consisting of hydrogen, methyl and hydroxyl; \( R_2 \) is a member selected from the group consisting of hydrogen, methyl and thiolphenyl; \( R_3 \) is a member selected from the group consisting of hydrogen, methyl and the radical
R₄ is a member selected from the group consisting of hydrogen, methyl and methoxy; R₅ is a member selected from the group consisting of hydrogen and methyl; and R₆ is a member selected from the group consisting of hydrogen, hydroxy, methyl and amino.

10. A composition according to claim 9 wherein the potentiating agent is a member selected from the group consisting of quinine, 6-methoxyquinoline, 6-methyl quinoline, 7-methyl quinoline, 4,6-dimethyl quinoline and quinoline.

11. A test indicator for detecting occult blood in body fluids and excreta comprising a fibrous material containing therein the dried residue resulting from the deposition on said material of a mixture comprising a first aqueous solution of an organic hydroperoxide, a buffer capable of maintaining the pH of the material being tested within the range of about 4 to 7, gelatin and as a fixative for said gelatin a dialdehyde polysaccharide, and a second solution of an indicator capable of being oxidized by the hydroperoxide in the presence of the prosthetic group of hemoglobin with an accompanying color change, a potentiating agent of the formula

wherein R₄ is a member selected from the group consisting of hydrogen, methyl and hydroxy; R₅ is a member selected from the group consisting of hydrogen, methyl and thiophenyl; R₆ is a member selected from the group consisting of hydrogen, methyl and the radical

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JOSEPH SCOVRONEK, Acting Primary Examiner.
Z. PAROCZAY, Assistant Examiner.