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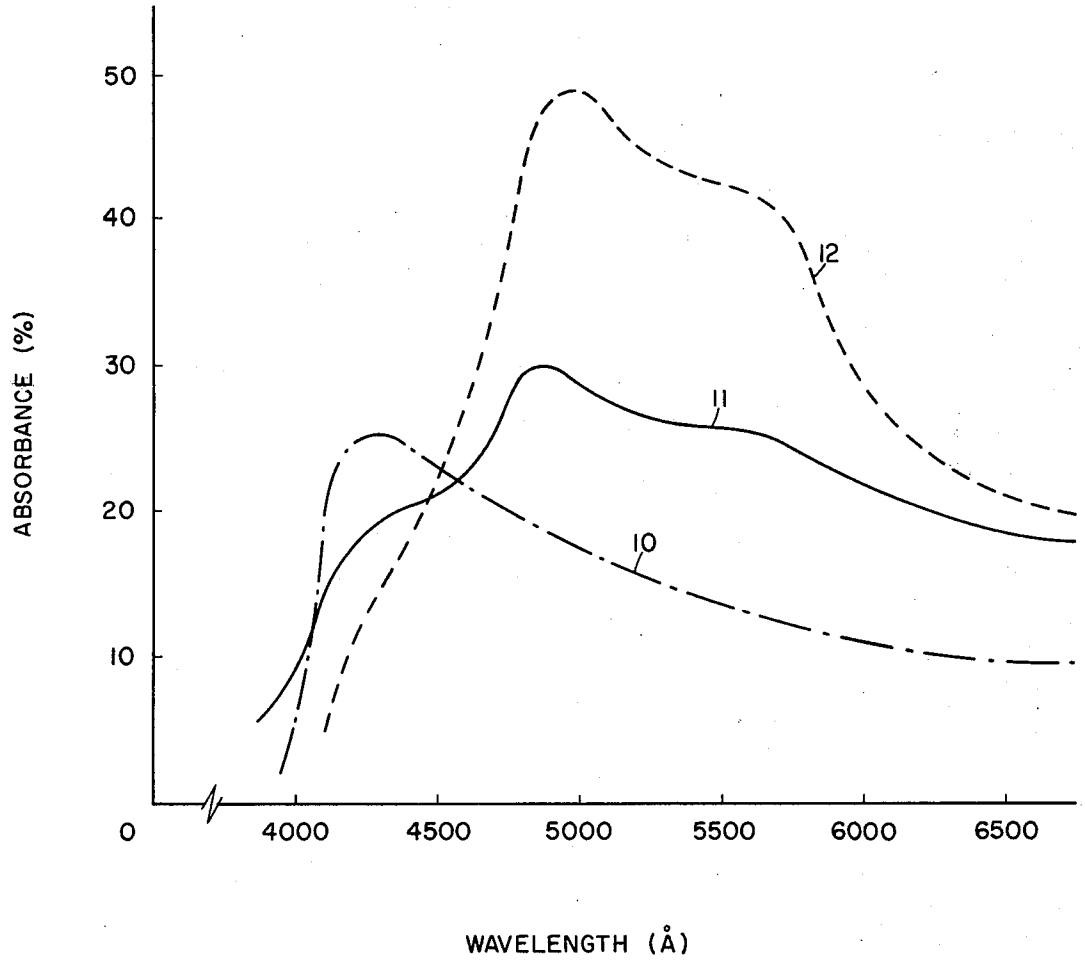

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COMPOSITION, METHOD AND DEVICE FOR DETERMINING BILIRUBIN AND UROBILINOGEN

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ABSTRACT OF THE DISCLOSURE

4,4'-biphenyldiazonium salts in test compositions and devices capable of differential determination of both bilirubin and urobilinogen in an aqueous solution or body fluid such as urine. In addition to a 4,4'-biphenyldiazonium salt, the test compositions preferably include an acid constitutent capable of producing an acid pH upon contacting the aqueous solution to be tested. The test device comprises a bibulous carrier member such as paper incorporated with the test composition in dry form. The method includes contacting the test composition with the aqueous solution to be tested and observing the response either visually or spectrophotometrically.

BACKGROUND OF THE INVENTION

Bilirubin is a degradation product of the heme moiety of hemoglobin molecules. In plasma bilirubin is normally unconjugated and is bound to various proteins. Conjugation results at the liver and possibly at other sites and the conjugated bilirubin is excreted into the duodenum as a constituent of bile. The action of bacteria normally present in the intestinal tract causes the reduction of bilirubin to other compounds, one of which, urobilinogen, is normally found in urine. Normal urine does not contain an appreciable amount (usually less than 0.05 mg./100 ml.) of either conjugated or unconjugated bilirubin.

The diagnostic value of bilirubin and urobilinogen determinations in body fluids is well known. Various diseased conditions cause abnormal levels of both compounds in urine. Such diseased conditions include hemolytic and hepatic diseases, biliary obstruction and other liver and bile duct dysfunctions. However, it is not possible to diagnose differentially a hemolytic condition, a hepatic condition or a biliary obstruction by the performance of a test for only one of these two compounds in urine. Isolated determinations do not indicate particular diseased conditions. For instance, a negative bilirubin determination, i.e. less than 0.05 mg./100 ml. indicates either a normal or a hemolytic condition. On the other hand, in urine urobilinogen determinations, abnormally high levels are indicative of either a hemolytic or hepatic condition while abnormally low levels indicate biliary obstruction.

The performance of both a bilirubin and a urobilinogen determination provides much more specific results due to the interrelationship between the levels of the two compounds in urine and certain diseased conditions. In normal urine, the bilirubin level may be considered as negative (less than 0.05 mg./100 ml.). In a hemolytic condition, more bilirubin is formed than normal due to increased degradation of the heme moiety. This results in an increase in the amount of bilirubin reduction products, including urobilinogen. Therefore, in a hemolytic condition, urine urobilinogen levels become abnormally high while bilirubin remains negative. Levels of bilirubin in the blood stream increase in hepatic conditions and biliary obstruction due to the decrease in the ability of the liver to excrete bile into the intestinal tract. Thus, the amount of bilirubin excreted by the kidneys as a urine constituent

increases resulting in positive urine bilirubin determinations. Urobilinogen levels in urine are found to increase in hepatic conditions but decrease in biliary obstruction. based on a classification of bilirubin levels in urine as positive or negative as hereinbefore defined, and urobilinogen levels as low, normal or high, diagnosis proceeds as follows for each of the following possible bilirubin-urobilinogen levels: negative-normal, normal; negative-high, hemolytic disease; positive-high, hepatic disease; and positive-low, biliary obstruction.

DESCRIPTION OF THE PRIOR ART

Bilirubin determinations which are known in the art are based on the reaction of bilirubin with diazotized aniline test reagents. These single benzene ring compounds are generally substituted with such constituents as nitro, sulfonic, chloride and methoxy groups. The more commonly employed test reagents are diazotized 2,4-dichloroaniline and diazotized sulfanilic acid. The reaction of bilirubin molecules with the diazo group of the test reagent results in conjugation which shifts the absorption spectrum of the azo reaction complex into the visible spectrum. This colorimetric response provides quantitation in aqueous solutions based on Beer's Law of light energy absorption. The molar extinction coefficient of 2,4-dichloroaniline is such that determinations of bilirubin using this preferred test reagent impregnated in paper for use in a dry test device are sensitive to a minimum concentration of about 1 mg./100 ml. Therefore, the range of bilirubin levels in urine between the normal level of about 0.05 mg./100 ml. and this minimum detectable amount are incapable of being determined using the traditional test reagents incorporated in a dry test device.

The prior art methods for detecting urobilinogen employ benzaldehyde test reagents. The most commonly used benzaldehyde test reagent by far is para-dimethylaminobenzaldehyde which is commonly referred to as Ehrlich's reagent. The reaction of urobilinogen and Ehrlich's reagent yields a conjugated complex with absorption in the visible spectrum. The colorimetric responses exhibited by this reaction are interpreted on the basis of a measurement unit known as an Ehrlich unit, rather than on mg. urobilinogen/100 ml. This is due to the fact that Ehrlich's reagent is known to react with various interferring substances sometimes present in urine such as para-aminosalicyclic acid, porphobilinogen and indole. An Ehrlich unit is defined in the art as the colorimetric response yielded by the reaction of Ehrlich's reagent with 1 mg./100 ml. of urobilinogen. In certain instances the lack of specificity in the detection of urobilinogen, particularly in urine determinations, detracts from the use of such determinations in diagnosis. The use of diazonium salts in the colorimetric detection of urobilinogen is unreported. Urobilinogen determinations therefore are presently based almost exclusively on the use of the non-specific Ehrlich's reagent.

As a result of limitations in the prior art, bilirubin and urobilinogen determinations must be performed separately. Also, known methods employing reagent impregnated paper for the detection of bilirubin are capable of detecting bilirubin only in concentrations greater than 1 mg./100 ml.

It is therefore an object of the present invention to provide a test composition which is capable of detecting both bilirubin and urobilinogen. Such a test composition would exhibit differential colorimetric responses with respect to the two compounds. Such a composition would also allow differential diagnoses to be made between normal, hemolytic and hepatic conditions and biliary obstructions using a single test.

Another object of the present invention is to provide a test composition which is more specific with respect to

the detection of urobilinogen. Such a test composition would eliminate the uncertainty presently encountered in the use of Ehrlich's reagent.

It is a further object of the present invention to provide a test device employing a sensitive and specific test composition for the determination of both bilirubin and urobilinogen. Such a test device would be simple to use and would provide rapid and reliable results which would be easily interpretable. Such a device would find great utility in laboratory and clinical applications as well as in use by physicians.

An even further object of the present invention is to provide a test composition incorporated in a dry test device for the detection of bilirubin which is more sensitive than the prior art dry test devices incorporated with conventional test reagents. Such a test device would provide a means of detecting increased levels of bilirubin at an earlier stage of disease.

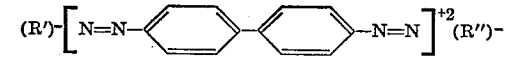
Another object of the present invention is to provide methods of determining both bilirubin and urobilinogen using the disclosed test composition and device.

Further objects will become evident in the specification and claims to follow.

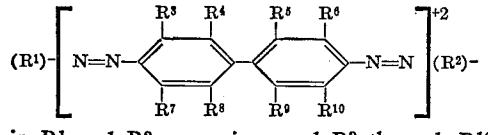
A test composition, method and device which fulfills the above objects would provide a liver function test for use in (1) following the progress of patients with infectious and serum hepatitis by serial testing, (2) monitoring alcoholic patients for the development, progression or improvement of hepatic cirrhosis, (3) testing patients suspected of having an attack of hemolytic disease, (4) testing patients suspected of having biliary obstruction, (5) monitoring workers exposed to hepatotoxins and (6) differentially diagnosing jaundiced conditions.

SUMMARY OF THE INVENTION

It has now been unexpectedly found that a test composition, method and device capable of detecting both bilirubin and urobilinogen in an aqueous solution is provided based on the reaction of these two compounds with a 4,4'-biphenyldiazonium salt. The present invention therefore relates to a new use of 4,4'-biphenyldiazonium salts and test devices which incorporate 4,4'-biphenyldiazonium salts therein. A 4,4'-biphenyldiazonium salt is defined herein as any member of the family of compounds represented by the formula



wherein R' and R'', which may be the same or different, are anions and wherein the benzene rings may contain other substituents. The azo reaction complexes formed by the reaction of 4,4'-biphenyldiazonium salts with bilirubin and urobilinogen exhibit differential responses capable of yielding both qualitative and quantitative determinations. In the context of this disclosure determination implies either qualitative, semi-quantitative or quantitative analysis unless otherwise indicated. The 4,4'-biphenyldiazonium salts which are used are preferably of the formula



wherein R^1 and R^2 are anions and R^3 through R^{10} are selected from the group consisting of —H, —CH_3, —OCH_3, —SO_3H and —Cl. The most preferred 4,4'-biphenyldiazonium salts are the salts of 4,4'-diazobiphenyl, 4,4'-diazo-3,3'-dimethoxybiphenyl and 4,4'-diazo-3,3'-dimethylbiphenyl.

The test composition preferably includes an acid constituent which is capable of providing an acidic pH when contacted with the aqueous solution to be tested. The acid constituent is preferably selected from those compounds which yield a pH below 3.0 when in a concentration of 0.1 N. An acidic environment is preferred due to the fact that under acidic conditions the azo reaction complexes are more stable and possess elevated molar extinction coefficients yielding a higher degree of colorimetric response. It is also preferred that the diazonium salt in the test composition be stabilized at its diazo-coupling sites by a diazonium salt stabilizing anion. The test composition may be in the form of a dry solid such as a powder or a tablet having an effervescent couple or may be incorporated in a dry test device carrier member and so forth.

The test devices disclosed herein comprise a bibulous carrier member such as paper incorporated with the above described test composition. Strong acids frequently disintegrate the commonly used bibulous carrier materials. For this reason, where the test composition is to include a strong acid constituent, as is preferred, it is preferred to include a solid adduct of a Friedel-Crafts salt and an organic Lewis base with another acid such as an organic acid. Such a mixture provides a means of maintaining the stability of the bibulous carrier member while employing a strong acid constituent.

The present invention discloses a method for determining both bilirubin and urobilinogen in aqueous solutions. This method basically comprises contacting the above described test composition, in any of its preferred embodiments and in any of its possible forms, with the aqueous solution to be tested and observing the response which results. It is preferred that the response be allowed to develop for a predetermined period of time before observing such response. Semi-quantitative determinations can be made by visually comparing the resultant colorimetric response with a standard color chart. Quantitative determinations can be made by observing the resulting response with spectrophotometric means.

BRIEF DESCRIPTION OF THE DRAWING

The sole Figure in the drawing is a graphical representation of the absorbance spectra yielded by contacting dry test devices comprising paper incorporated with a preferred 4,4' - biphenyldiazonium salt, a salt of 4,4'-diazo - 3,3' - dimethoxybiphenyl, with aqueous solutions of bilirubin, urobilinogen and a mixture of bilirubin and urobilinogen.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

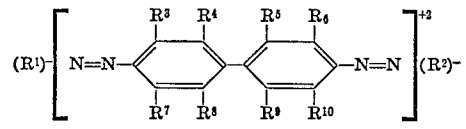
The rapid and reliable determination of bilirubin and urobilinogen in aqueous fluids in particularly desirable in clinical diagnosis. A means of detection which provides easily interpretable results enables nonskilled technicians to perform the procedures involved. Tests which yield color changes have proven to be particularly useful in providing such results. Color changes, or colorimetric responses, depend on the chemical reaction between a test reagent and the substance under determination which results in the formation of a chromophoric reaction complex. The light absorbance properties of this complex determine the usefulness of the test reagent as an indicator for the particular substance being detected. The electronic structure of the complex establishes the absorption spectrum of the complex and therefore determines the sensitivity and readability of a test using that particular test reagent. The search for more useful test reagents must be considered as being primarily empirical since the absorption spectrum of the reaction complex which results from the use of a particular test reagent is unpredictable in most cases.

The coupling reaction which occurs between the bilirubin molecule and the diazonium salt in the present invention is thought to involve the methylene link in the conjugated pyrrole structure of bilirubin. The diazo coupling reaction at the methylene link in the bilirubin molecule causes a splitting of the molecule into two portions forming two azo-bilirubin complexes.

The urobilinogen molecule being a reduction product of bilirubin possesses a conjugated pyrrole structure similar to that found in the bilirubin molecule. A difference is to be found however in the composition of the linkages between the pyrrole rings. Three such linkages are found in both molecules. In bilirubin the center link is a methylene group and the other two links are methane groups, whereas all three links in urobilinogen are methylene groups. The mechanism of the reaction between urobilinogen and the diazonium salts disclosed herein is not known. However, it has been found that 4,4' - biphenyldiazonium salts do react with urobilinogen molecules to yield a reaction complex which unexpectedly exhibits sufficient colorimetric response for qualitative and quantitative determinations.

The 4,4' - biphenyldiazonium salts which are preferably used in the present invention are those which are relatively stable in dry form. Such stability can be enhanced through the use of diazonium salt stabilizing anions as will be discussed hereinafter. The test composition which comprises the above mentioned diazonium salts may be in such forms as dry solids such as powders, tablets having effervescent couples and solids incorporated in a dry test device.

Diazonium salts of the 4,4' - biphenyl class may be represented by the following structure



wherein for the purposes of this disclosure R^1 and R^2 are defined as the anions or anionic portions of the diazonium salt and R^3 through R^{10} are defined as substituents on the benzene rings.

In order to accomplish the diazo-coupling reaction between the diazonium salt and bilirubin or urobilinogen, the 4,4' - biphenyldiazonium salts disclosed herein must dissociate in aqueous solutions to form diazonium ions. Therefore, the choice of a particular anion to occupy the anionic portions of the diazonium salt does not affect the detection of bilirubin or urobilinogen so long as the diazonium salt dissociates in aqueous solution. Due to the inherent affinity of 4,4' - biphenyl diazonium salts with respect to dissociation in aqueous solutions, the choice of a particular anion is usually based on convenience and economy. While in general the anionic portions may comprise any anion, it is preferred that such anionic portions enhance the stability of the diazonium salt in dry form. Such anionic portions which perform this function are defined herein as diazonium salt stabilizing anions. Such diazonium salt stabilizing anions include halogen ions, boron tetrahalogen ions such as $—BF_4$, halo-metallic anions such as the transition metal halogens which include $—ZnCl_4$ and $—CoCl_4$ and sulfonate anions such as $—SO_3H$. Of these diazonium salt stabilizing anions, the arylsulfonate anions which are characterized by two or more sulfonate groups are particularly useful. Such arylsulfonate anions include the naphthylenedisulfonates such as the deprotonized form of naphthylenedisulfonic acid and the biphenyldisulfonates such as the deprotonized form of 4,4' - biphenyldisulfonic acid. The anion portion of arylsulfonate salts may be used as well. The role of the diazonium salt stabilizing anions is to promote the storability and ease of handling of the test composition. They serve to occupy at least one of the reactive diazo-coupling sites of the bivalent diazonium ions, thereby preventing degradation to side reactions at the occupied site or sites. Such chemical bonding between the reactive diazo-coupling sites on the diazonium salt and a diazonium salt stabilizing anion can be accomplished by any known means such as by performing the diazotization reaction in the presence of an acid or salt which includes the diazonium salt stabilizing anion.

The stabilized diazonium salt can then be dried or incorporated into a test device as described hereinafter.

Substituents on the benzene rings of the 4,4'-biphenyldiazonium salts disclosed herein affect the stability and solubility of the diazonium salt as well as the stability and absorption spectrum of the reaction complex. Such substituents may be chosen from all those radicals known to chemically attach to benzene rings and which will not totally inhibit the diazo coupling reaction between the diazonium ion and bilirubin or urobilinogen. Illustrations of such radicals incldue the following chemical groups: hydrogen, halogens, lower alkyl, i.e. 1–5 carbon atoms, lowei alkoxy, i.e. 1–5 carbon atoms, hydroxyl, carbonyl, amino and so forth. Such substituents may be positioned symmetrically or asymmetrically about the benzene rings. It should be noted that the use of the disclosed diazonium salts in determining bilirubin and urobilinogen is dependent on the diazo coupling reaction which occurs at the diazo groups in the diazonium ion and that one skilled in the art may select substituents in view of desirable stability and solubility of the diazonium salt and desirable stability and absorption spectrum of the reaction complex. On the basis of stability and economy, it is preferred that the substitutions about the biphenyl structure be selected from the group consisting of —H, $—CH_3$, $—OCH_3$, $—SO_3H$ and —Cl. In general, the diazo coupling reaction between the diazonium salt and either bilirubin or urobilinogen will occur independent of any substitutions about the ring structure, however, such substitutions may result in less desirable solubility properties of the diazonium salt and a less desirable absorption spectrum of the reaction complex. The diazonium salt should be sufficiently soluble in water in order to dissociate, thereby releasing the diazonium ion to react with bilirubin and urobilinogen in aqueous solution.

It is also desirable to use a diazonium salt which is essentially colorless in order that the colorimetric response of the azo reaction complex be dramatic. Of course, background dyes may also be used to enhance the colorimetric response. On the additional basis of solubility and absorption characteristics of the reaction complex, specific compounds which have been shown to be especially preferred are the salts of 4,4'-diazobiphenyl, 4,4'-diazo-3,3'-dimethoxybiphenyl and 4,4'-diazo-3,3'-dimethylbiphenyl. The most useful of these compounds have been found to be the salts of 4,4'-diazo-3,3'-dimethoxybiphenyl. In general, these compounds are soluble and exhibit dramatic differential colorimetric response upon reaction with bilirubin and urobilinogen as illustrated by the Figure of the drawing. The colorimetric response exhibited by such compounds in aqueous solutions in the presence of bilirubin is green while in the presence of urobilinogen it is red. Such a dramatic distinction between the colors developed due to reaction with bilirubin and urobilinogen provides sufficient differential colorimetric response to yield a highly useful means of detecting both bilirubin and urobilinogen in aqueous solutions.

Due to the chemistry involved, it has been found that the colorimetric response of the azobilirubin reaction complex is highly specific for bilirubin. None of the primary interferring substances present in aqueous solutions such as urine are capable of reacting with the disclosed diazonium salts to form a reaction complex sufficiently conjugated to give a colorimetric response near that produced by the azobilirubin complex. It has also been found that the effect of the primary interferring substances, such as porphobilinogen, indole and ascorbic acid, can be minimized relative to urobilinogen determinations. The colorimetric response of the azo-urobilinogen complex can potentially be interfered with by ascorbic acid; however, other substances which interfere with conventional urobilinogen determinations using Ehrlich's reagent, do not interfere with the present system. Moreover, the interference from ascorbic acid can be almost competely eliminated by adjusting the pH of the environment through the addition of a suffi-

ciently strong acid constituent. The present invention, therefore, is not limited by the possibility of inaccurate results as found in conventional techniques which use benzaldehydes.

The test composition preferably includes an acid constituent. Acid constituent means a compound or mixture of compounds which are capable of producing an acidic pH when contacted with the aqueous solution to be tested. In addition to eliminating ascorbic acid interferences, an acidic environment aids in stabilizing the azo reaction complexes and elevates the molar extinction coefficient of the complex so that a higher degree of colorimetric response is obtained. A preferred strongly acidic environment is obtained when the acid constituent yields a pH of less than 3.0 at a concentration of 0.1 N. Where the acid constituent consists of a single compound it is also preferred that the compound have a pK_a value of less than about 4.0. It has usually been found that increasing the acidity of the evironment of the azo reaction complexes produces a more intense colorimetric response. It has been shown that the addition of the acid constituent may be accomplished subsequent to the formation of the azo-reaction complexes. This supports the fact that an acidic environment is not required for the diazo coupling reaction to proceed, but rather acts to stabilize the reaction complex and to raise the molar extinction coefficient thereof.

Acid constituents which may be added to the aqeuous solution to be tested subsequent to the diazo coupling reaction include all organic and inorganic compounds which when contacted with the aqueous solution will produce an acidic pH. Such compounds include gases such as hydrogen chloride gas, liquids such as aqueous hydrogen chloride or aqueous acetic acid and solids such as citric acid. These are but instances of the numerous compounds which can comprise the acid constituent without limiting the present invention. The acid constituent may also be in admixture with the diazonium salt in dry form. In this case, any solid compound, organic or inorganic, capable of producing an acidic pH upon contacting the aqueous solution may be used such as citric acid, sulfosalicylic acid, tartaric acid, succinic acid, cyclohexanesulfamic acid and maleic acid. Preferred proportions between the diazonium salt and the acid constituent where the acid constituent is included in the test composition are: diazonium salt, 1 to 4 parts by weight, acid constituent, 4 to 8 parts by weight. In all cases wherein an acid constituent is included in the test composition, none, one or both of the anionic portions of the diazoium salt may comprise a diazoium salt stabilizing anion as hereinbefore discussed. The proportions of the anionic portion of the diazonium salt and the acid constituent, if included in the test composition, should be based on individual requirements of reaction time, stability and extent of increased colorimetric response desired. The reaction time of the diazo coupling between the diazonium salt and bilirubin or urobilinogen can be controlled by appropriate selection of diazonium salt stabilizing anions, concentration of reactants and pH. Increasing the latter two factors reduces the reaction time.

As stated hereinbefore, the test composition of the present invention may be in the form of a dry solid such as a powder or a tablet having an effervescent couple or may be incorporated in a dry test device, and so forth. As a dry solid, the test composition comprises a 4,4'-biphenyldiazonium salt and may include a diazonium salt stabilizing anion and/or an acid constituent. Where the test composition is in the form of a tablet, an effervescent couple may be included to facilitate dissolution of the test composition. Inert filler ingredients and other diluents may also be employed to facilitate the tableting operation. An effervescent couple refers to the combination of an acid component and a carbonate or bicarbonate component which produces effervescence upon contacting an aqueous solution. The use of an acid constituent capable of producing an acidic pH as referred to hereinbefore may be included as the acid component of the effervescent couple which acid component is in sufficient excess of the carbon or bicarbonate component so as to produce an acidic pH when contacted with the aqueous solution to be tested.

The test device disclosed herein comprises a bibulous carrier member incorporated with the test composition in dry form. The incorporated test composition may include an acid constituent and/or the use of a diazonium salt stabilizing anion in the structure of the diazonium salt. Any material which is capable of absorbing and retaining moisture may be used in the composition of the bibulous carrier member. Such material include paper, cotton, polymeric pads, porous plastics, and so forth. The dry test composition may be impregnated in the carrier member, coated on the carrier member, chemically bonded to the carrier member or any combination thereof, and so forth. The bibulous carrier member may also be attached or otherwise associated with a holder or support. Such a holder or support may be adapted to provide a means of contacting the aqueous solution such as by dipping the bibulous carrier member into the solution while holding the holder or support by means of fingers.

Where the test composition incorporated with the bibulous carrier member includes an acid constituent, problems with regard to stability of the carrier material may be encountered, especially where the acid constituent is strongly acidic as is preferred. For instance, certain paper carrier materials have been found to degrade when incorporated with a strong acid constituent. In order to employ a strong acid constituent, as is preferred, it is desirable to include a combination of an acidic compound which is not strong enough to degrade the carrier material and a non-acidic or slightly acidic mixture of compounds capable of reacting upon contacting the aqueous solution to produce additional acidity. Such a combination which has been found to be useful comprises an organic acid and a solid adduct which consists of a Friedel-Crafts salt and an organic Lewis base. The solid adduct hydrolyzes upon contacting the aqueous solution releasing a mineral acid to produce a desirable acidic environment in combination with the organic acid. The dry solid adduct as incorporated with the bibulous carrier member is non-acidic or only slightly acidic and thereby avoids degradation of the carrier material. Such organic acids which have been found to be useful in this mixture include citric acid, sulfosalicylic acid, tartaric acid, succinic acid, cyclohexanesulfamic acid and maleic acid. The Friedel-Crafts salts used may be those halogen salts employed as catalysts in the classical Friedel-Crafts alkylation reaction such as stannic chloride, boron trifluoride, zinc chloride, ferric chloride, and so forth. Organic Lewis bases which may be used include pyridine and substituted pyridines such as the picolines, N,N-dialkylalkolamides such as dimethylformamide and dimethyl acetamide, other amides and imides such as benzamide and succinimide, ureas such as sym-dimethylurea, ethers such as dioxane and tetrahydrofuran, nitrites such as acetonitrite and amines such as morpholine.

The preparation of the test device basically comprises the two steps of incorporating the test composition in the device and drying the device. Such incorporation of the test composition may be accomplished by such means as impregnating the bibulous carrier member with a solution or solutions containing the constituents of the test composition, casting a film or films of the constituents in solution on the carrier member, and so forth. The device may be dried by any means which will not deleteriously affect the incorporated test composition. It should be noted that where a solid adduct of a Friedel-Crafts salt and an organic Lewis base are to be included in the incorporated test composition, such solid adduct must not be incorporated with the carrier member through the use of an aqueous solution since the adduct will hydrolyze.

Where a solution is required to accomplish the incorporation of the solid adduct with the carrier member, non-aqueous solvents should be used such as chloroform, carbontetrachloride, dimethylsulfoxide, anhydrous alcohol, acetone, and so forth. The incorporation of the test device with the test composition may include the use of one or more solutions of ingredients resulting in the incorporation of the test composition as a whole. For example, one or more aqueous solutions may be used to impregnate the carrier member with the diazonium salt and the acid constituent followed by drying and subsequent impregnation of the carrier member with the solid adduct in a non-aqueous solution.

In its most fundamental sense, the disclosed method of detecting both bilirubin and urolilinogen comprises the contacting of a 4,4'-biphenyldiazonium salt with the aqueous solution to be tested resulting in the formation of azo-reaction complexes which provide differential responses. A preferred method includes contacting the aqueous test solution with an acid constituent. The contacting of the acid constituent with the test solution may be before or after the contacting of the diazonium salt or may be simultaneous in the sense that the test composition itself may include the acid constituent. Where the test composition in any of its embodiments is in the form of a solid, contact with the aqueous test solution may be accomplished by adding it to the solution, or by placing a drop of solution to be tested on a slide on which the test composition in solid form has already been placed, or by dissolving the test composition and mixing the two solutions, and so forth. Also, where the tablet form of the test composition is used, the tablet may be dissolved in the aqueous solution to be tested, or dissolved in a second solution followed by mixing the two solutions, or placed on a bibulous mat and the aqueous solution added, and so forth.

The test composition is most conveniently contacted with the aqueous solution to be tested when it is incorporated in a dry test device. The dry test device may be dipped into the aqueous solution to be tested or the aqueous solution may be placed on to the test device by means of a dropper, and so forth. Where the solution to be tested is urine the test device may be brought into contact with the stream of urine.

It is preferred to allow the diazo coupling reaction to go to substantial completion before reading or measuring the resulting colorimetric response. As stated hereinbefore, an appropriate selection of diazonium salt stabilizing anions, concentration of reactants and pH reduces the reaction time of the diazo coupling mechanism. Where the test composition is incorporated in a dry carrier and contacted with the aqueous solution to be tested, the concentration of a portion of the reactants, namely bilirubin and urobilinogen, are fixed by their concentration levels in the aqueous solution. The use of a 4,4'-biphenyldiazonium salt in the test composition provides a test device capable of detecting bilirubin concentrations down to 0.2 mg./100 ml. This test device, therefore, is more sensitive than those in the prior art which are capable of detecting bilirubin only in concentrations of 1 mg./100 ml. or more.

Semi-quantitative results can be obtained through a visual comparison of the resultant colorimetric response and a color chart illustrating colors generated using known concentrations of bilirubin and urobilinogen. Where both compounds are present in the aqueous solution to be tested, the resultant color is a mixture of the two colors respectively generated. The standard color chart, therefore, takes the form of a two dimensional grid with bilirubin colorimetric color responses on one axis, urobilinogen colorimetric responses on the other axis, and the colorimetric responses of their mixtures in the quadrant formed by the axes. Quantitative determinations may be obtained by observing the resulting response by spectrophotometric means such as by instruments which measure transmission or reflectance and which are capable of yielding output in terms of absorbance. For instance, where the test composition is added directly to the aqueous solution, the mixture can be placed in a cuvette and absorbance measured at an appropriate wave length. On the other hand, where the test composition is incorporated in an opaque test device, the reflectance of the bibulous carrier member may be measured at an appropriate wave length.

Aqueous solutions which may be tested using the test composition, device and method of the present invention include urine, tissue fluid, cerebral and spinal fluid, fecal extracts, and so forth, as well as other body fluids and artificially prepared aqueous solutions containing bilirubin or urobilinogen.

The present invention will now be illustrated by the following examples, but is not intended to be limited thereby.

EXAMPLE 1

100 mg. of Fast Blue Salt B, a 4,4'-diazo-3,3'-dimethoxybiphenyl salt, was dissolved in 3 ml. of H_2O. Three aqueous test solutions were prepared having the following concentrations of bilirubin and urobilinogen: 1.8 mg./100 ml. bilirubin; 8.0 Ehrlich units urobilinogen and 0.9 mg./100 ml. bilirubin; and, 4.0 Ehrlich units urobilinogen. One drop of each of the three test solutions was placed in separate 0.5 ml. wells in spot plates. One drop of the diazonium salt solution was then added to each of the three wells with the following results:

Test solution:	Color developed
bilirubin	brown-green
bilirubin-urobilinogen	brown-red
urobilinogen	red

When one drop of 1 N HCl was added, the colors present were intensified. One drop of each of the same test solutions was placed in separate wells and one drop of 1 N HCl was added to each followed by the addition of one drop of the diazonium salt solution. The results were as follows:

Test solution:	Color developed
bilirubin	green
bilirubin-urobilinogen	orange
urobilinogen	orange-red

EXAMPLE 2

A. 100 mg. of 4,4'-diaminobiphenyl was mixed with 2 ml. 1 N HBF_4 and 1 ml. 1 M $NaNO_2$. One drop of each of three test solutions of Example 1 was placed in separate wells in a spot plate and one drop of the diazonium salt solution was then added to each of the wells with the following results:

Test solution:	Color developed
bilirubin	green
bilirubin-urobilinogen	green-orange
urobilinogen	orange

B. 100 mg. of 4,4'-diaminobiphenyl was mixed with 2 ml. 1 N HCl and 1 ml. 1 M $NaNO_2$ and this diazonium salt solution was added to the three test solutions in the same manner as in A, with the following results:

Test solution:	Color developed
bilirubin	green
bilirubin-urobilinogen	green-orange
urobilinogen	orange

C. 100 mg. of 4,4'-diaminobiphenyl was mixed with 2 ml. 1 N H_2SO_4 and 1 ml. 1 M $NaNO_2$ and this diazonium salt solution was added to the three test solutions in the same manner as in A, with the following results:

Test solution:	Color developed
bilirubin	green
bilirubin-urobilinogen	green-orange
urobilinogen	orange

D. 100 mg. of 4,4'-diamino-3,3'-dimethylbiphenyl was mixed with 2 ml. 1 N HBF_4 and 1 ml. 1 M $NaNO_2$ and this diazonium salt solution was added to the three test solutions in the same manner as in A, with the following results:

Test solution:	Color developed
bilirubin	green (clumps)
bilirubin-urobilinogen	pale cream-green (clumps)
urobilinogen	cream (clumps)

E. 100 mg. of 4,4'-diamino-3,3'-dimethylbiphenyl was mixed with 2 ml. 1 N HCl and 1 ml. 1 M $NaNO_2$ and this diazonium salt solution was added to the three test solutions in the same manner as in A, with the following results:

Test solution:	Color developed
bilirubin	dark orange (turbid)
bilirubin-urobilinogen	red-dark orange (turbid)
urobilinogen	orange

F. 100 mg. of 4,4'-diamino-3,3'-dimethylbiphenyl was mixed with 2 ml. 1 N H_2SO_4 and 1 ml. 1 M $NaNO_2$ and this diazonium salt solution was added to the three test solutions in the same manner as in A with the following results:

Test solution:	Color developed
bilirubin	beige-purple
bilirubin-urobilinogen	beige-purple
urobilinogen	pink-purple

EXAMPLE 3

A. 2–5 mg. of Fast Blue Salt B, a 4,4'-diazo-3,3'-dimethoxybiphenyl salt, was placed in each of three 9.5 ml. wells in a spot plate, 50–100 mg. of an organic acid followed by four drops of H_2O were added to each of the three wells. One drop of each of the test solutions of Example 1 was added to the wells. Six different organic acids were used with the following results:

	Color developed		
Organic acid	Bilirubin	Bilirubin-urobilinogen	Urobilinogen
Citric	Green-gray	Grey-orange	Orange.
Tartaric	Green-orange	do	Do.
Maleic	Green	Red-grey	Red.
Succinic	Pale yellow	Pale yellow-orange	Orange.
Sulfosalicylic	Deep green	Grey-pink	Deep red.
Cyclohexanesulfamic	Dusty green	Dusty pink	Orange-pink.

B. 2–5 mg. of 4,4'-diaminobiphenyl was mixed with 1 drop 1 N HCl and 1 drop 1 M $NaNO_2$ in each of three 0.5 ml. wells in a spot plate and treated as in A, with the following results:

	Color developed		
Organic acid	Bilirubin	Bilirubin-urobilinogen	Urobilinogen
Citric	Light grey	Grey-green	Light red.
Tartaric	Green	Amber	Red-orange.
Maleic	Grey-green	Muddy orange	Orange.
Succinic	do	do	Do.
Sulfosalicylic	Green	Grey-orange	Red-orange.
Cyclohexanesulfamic	do	do	Do.

EXAMPLE 4

A. 100 mg. of 4,4'-diaminobiphenyl was mixed with 2 ml. 1 N HCl and 1 ml. 1 M $NaNO_2$ and diluted to 65 ml. with H_2O. Three strips of Whatman 3MM paper were immersed in this diazonium salt solution, removed immediately and dried in a forced air oven at 70° C. for 10 minutes. These three strips were respectively dipped into the test solutions of Example 1 and removed immediately, with the following results:

Test solution:	Color developed
bilirubin	grey-beige purple
bilirubin-urobilinogen	beige-purple
urobilinogen	pink-purple

B. 100 mg. of 4,4'-diamino-3,3'-dimethylbiphenyl was mixed with 2 ml. 1 N H_2SO_4 and 1 ml. 1 M $NaNO_2$ and diluted to 65 ml. with H_2O. Strips of paper were treated with this diazonium salt solution and used as in A, with the following results:

Test solution:	Color developed
bilirubin	brown-purple
bilirubin-urobilinogen	beige-purple
urobilinogen	pink-purple

C. 250 mg. of Fast Blue Salt B, a 4,4'-diazo-3,3'-dimethoxybiphenyl salt, was dissolved in 65 ml. of H_2O. Strips of paper were treated with this diazonium salt solution and used as in A, with the following results:

Test solution:	Color developed
bilirubin	grey-beige purple
bilirubin-urobilinogen	grey-purple
urobilinogen	pink-purple

EXAMPLE 5

0.5 g. of Fast Blue Salt B, a 4,4'-diazo-3,3'-dimethoxybiphneyl salt, and 1 gm. of an organic acid was mixed in 50 ml. of H_2O and 50 ml. methanol was added. Three strips of E & D 204 paper were immersed in this diazonium salt solution, immediately removed and dried in a forced air oven at 70° C. for 10 minutes. These strips were respectively dipped in the test solutions of Example 1 and immedliately removed with the following results:

	Color developed		
Organic acid	Bilirubin	Bilirubin-urobilinogen	Urobilinogen
Citric	Green	Raspberry	Red.
Tartaric	do	do	Red.
Maleic	do	do	Red.
Succinic	do	do	Red.
Sulfosalicylic	do	do	Red.
Cyclohexanesulfamic	do	do	Red.

EXAMPLE 6

A solution was prepared by mixing the following ingredients at room temperature:

Fast Blue Salt B	gm.	0.48
Sulfosalicylic acid	gm.	2.0
Methanol	ml.	50
Water	ml.	50

Strips of Mead #469 filter paper were immersed in this solution, immediately removed and dried in a forced air oven at about 70–75° C. for 15 minutes. The dried strips were cream white in appearance. Urines containing bilirubin, urobilinogen or both were subjected to known quantitative assay techniques to determine actual bilirubin and urobilinogen concentrations. Urobilinogen was determined by the method of Watson-Schwartz *Am. J. Clin. Path.*, 14:605 (1944) and bilirubin was deter-

mined by the method of Golden-Snavely *J. Lab. Clin. Med.*, 33:890 (1848). Treated strips were dipped into the various urines and immediately removed. A red color was observed within 15 seconds in the presence of urobilinogen. A green color was observed within 45 seconds in the presence of bilirubin or a brown color was observed if urobilinogen was also present. Urobilinogen was able to be detected at 0.3 Ehrlich units and billirubin was able to be detected at 0.2 mg./100 ml. Ascorbic acid was found to interfere by developing an orange color in 25 seconds. Protein did not interfere with the color development.

EXAMPLE 7

A solution was prepared as in Example 6 with the addition of one of the following compounds: $ZnCl_2$, $CoCl_2$, NaCl, NH_4BF_4, 1,5-naphthylenedisulfonic acid and 4,4'-biphenyldisulfonic acid. The procedure of Example 6 was then followed and the results were substantially the same. In general, the addition of one of these compounds increased the stability of the solid diazonium salt powders. Dried powders that did not include these compounds degraded within 24 hours.

EXAMPLE 8

A. 250 mg. of Fast Blue Salt B, a 4,4'-diazo-3,3'-dimethoxybiphenyl salt, was dissolved in 65 ml. of H_2O. Three strips of Whatman 3MM paper were immersed in this diazonium salt solution, immediately removed and dried in a forced air oven at 70° C. for 10 minutes. They were then dipped in a second solution of 1 gm. $SnCl_4$-N,N-dimethylformamide complex in 25 ml. N,N-dimethylformamide. The treated strips were immediately removed and dried in a forced air oven at 70° C. for 15 minutes. The three strips were respectively dipped into the test solutions of Example 1 and immediately removed with the following results:

Test solution:	Color developed
bilirubin	beige-green
bilirubin-urobilinogen	beige-light purple
urobilinogen	dark rose violet

B. The same procedure as in A was applied to three other strips except that the second solution was 1 gm. $SnCl_4$-N,N-dimethylacetamide complex in 25 ml. of methanol. The results were as follows:

Test solution:	Color developed
bilirubin	beige-green
bilirubin-unrobilinogen	beige-light purple
	dark violet

C. The same procedure as in A was applied to three other strips except that the second solution was 1 gm. $SnCl_4$-dioxane complex in 25 ml. of methanol. The results were the same as in B.

EXAMPLE 9

A. A solution was prepared by mixing the following ingredients at room temperature:

4,4'-diamino-3,3'-dimethoxybiphenyl	mg.	1
Sodium nitrite	gm.	0.1
Citric acid	gm.	8
4,4'-biphenyldisulfonic	gm.	0.5
Methanol	ml.	50
Water	ml.	50

This solution was mixed thoroughly for 5 minutes. Three strips of Watman #17 filter paper were immersed in this diazonium salt solution, immediately removed and dried in a forced air oven at 70° C. for 10 minutes. The dried strips were then immersed momentarily in a solution containing the following ingredients:

$SnCl_4$-N,N-dimethylformamide complex	gm.	3.2
N,N-dimethylformamide	ml.	25
Acetone	ml.	75

The strips were then dried in a forced air oven at 70° C. for 15 minutes. These strips were respectively dipped in the test solutions of Example 1 and immediately removed yielding results substantially the same as in Example 6; however, there was no significant color interference from ascorbic acid in concentrations below 400 mg./liter.

B. A solution was prepared as in A using 4,4-diaminobenzidine in place of 4,4'-diamino-3,3'-dimethoxybiphenyl. The same procedure as in A was then followed with substantially the same results.

EXAMPLE 10

The absorbance spectra of the reacted strips of Example 9A were measured over the visible spectrum with a Beckman DK2 spectrophotometer set to yield output in terms of percent absorbed based on measured reflectance. The results are given in the sole Figure in the drawings which shows an illustration of the differential colorimetric responses yielded by the 4,4'-biphenyldiazonium salts disclosed herein. Curve **10** is the absorbance spectrum of a reacted strip which was dipped in the test solution containing 8.0 Ehrlich units urobilinogen. Curve **11** resulted from the dipping of a strip into the test solution containing 0.9 mg./100 ml. bilirubin and 4.0 Ehrlich units urobilinogen. The dipping of a strip into the test solution containing 1.8 mg./100 ml. bilirubin resulted in curve **12.**

EXAMPLE 11

Three tablets were formed having the following ingredients:

	G.
Fast Blue Salt B	0.2
Sulfosalicylic acid	2.0
Sodium bicarbonate	0.5

These tablets were then placed separate 0.5 ml. wells in a spot plate and one drop of each of the test solutions of Example 1 were respectfully added with the following results:

Test solution:	Color developed
bilirubin	green
bilirubin-urobilinogen	brown
urobilinogen	red

EXAMPLE 12

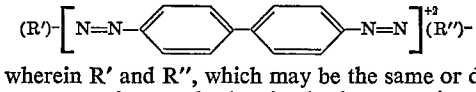
Various other 4,4'-biphenyldiazonium salts may be prepared in the conventional manner and added to test solutions containing bilirubin, urobilinogen or a mixture of both with the following results using 4,4'-diazo-3,3'-biphenyldisulfonic acid being typically expected.

Color developed		
Bilirubin	Bilirubin-urobilinogen	Urobilinogen
Green	Brown	Red.

What is claimed is:

1. A method for the differential determinaton of both bilirubin and urobilinogen in an aqueous solution which comprises the steps of:

(a) contacting said aqueous solution and a test composition which comprises a compound of the formula



wherein R' and R", which may be the same or different, are anions and wherein the benzene rings may contain other substituents; and

(b) observing the colorimetric response which results.

2. A method as in claim **1** wherein at least one of said anions is a diazonium salt stabilizing anion.

3. A method as in claim **1** wherein said test composition additionally comprises an acid constituent capable of producing an acidic pH when contacted with said aqueous solution.

4. A method as in claim 3 wherein at least one of said anions is a diazonium salt stabilizing anion.

5. A method as in claim 1 wherein said test composition additionally comprises an effervescent couple whose acid component is in sufficient excess to be capable of producing an acidic pH when said test composition is contacted with said aqueous solution.

6. A method as in claim 1 wherein at least one of said benzene rings contains at least one $-CH_3$ substituent.

7. A method as in claim 1 wherein at least one of said benzene rings contains at least one $-OCH_3$ substituent.

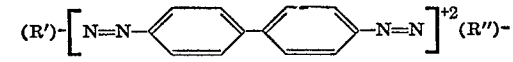
8. A method as in claim 1 wherein said compound is selected from the group consisting of the salts of 4,4'-diazo-3,3'-di-methylbiphenyl, 4,4'-diazo-3,3'-dimethoxybiphenyl, and 4,4'-diazobiphenyl.

9. A method as in claim 1 which comprises the additional step of allowing the resulting colorimetric response to develop for a predetermined period of time between steps (a) and (b).

10. A method as in claim 1 wherein said colorimetric desponse is observed visually and which comprises the additional step of comparing the observed colorimetric response to a standard color chart.

11. A method as in claim 1 wherein said observation of the colorimetric response which results is performed by spectrophotometric means.

12. A test device capable of detecting both bilirubin and urobilinogen in an aqueous solution which comprises a bibulous carrier member incorporated with a test composition which comprises a compound of the formula



wherein R' and R'', which may be the same or different, are anions and wherein the benzene rings may contain other substituents.

13. A test device as in claim 12 wherein said substituents are selected from the group consisting of —H, $-CH_3$, $-OCH_3$, SO_3H and —Cl.

14. A test device as in claim 12 wherein said compound is selected from the group consisting of 4,4'-diazobiphenyl, 4,4'-diazo-3,3'-dimethylbiphenyl, and 4,4'-diazo-3,3'-dimethoxybiphenyl.

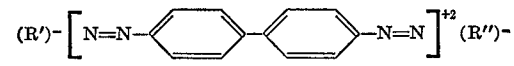
15. A test device as in claim 12 wherein at least one of said anions is a diazonium salt stabilizing anion.

16. A test device as in claim 12 wherein said test composition additionally comprises an acid constituent capable of producing an acidic pH when contacted with said aqueous solution.

17. A test device as in claim 16 wherein at least one of said anions is a diazonium salt stabilizing anion.

18. A test device as in claim 16 wherein said acid constituent includes a solid adduct consisting essentially of a Friedel-Crafts salt and an organic Lewis base, which adduct is hydrolyzable to release a mineral acid.

19. A test composition capable of determining both bilirubin and urobilinogen in an aqueous solution which comprises a compound of the formula



wherein R' and R'', whcih may be the same or different, are anions and wherein the benzene rings may contain other substituents, and an acid constituent capable of producing an acidic pH when contacted with said aqueous solution.

20. A test composition as in claim 19 wherein at least one of said anions is a diazonium salt stabilizing anion.

21. A test composition as in claim 20 wherein said diazonium salt stabilizing anion is selected from the group consisting of boron tetrafluoride ion, the halogen ions, the sulfonate ions and the halo-metallic anions.

22. A test composition as in claim 19 wherein said substituents are selected from the group consisting of —H, $-CH_3$, $-OCH_3$, $-SO_3H$ and Cl.

23. A test composition as in claim 19 wherein said compound is selected from the group consisting of 4,4'-diazobiphenyl, 4,4'-diazo-3,3'-dimethylbiphenyl and 4,4'-diazo-3,3'-dimethoxybiphenyl.

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