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[54] **DIAGNOSTIC AGENT FOR THE DETECTION OF BILIRUBIN**

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[56] **References Cited**

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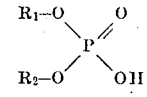
[57] **ABSTRACT**

Bilirubin, particularly as contained in body fluids, is detected by contacting a test sample with a test composition comprising

a. a diazonium salt capable of coupling with bilirubin;

b. an acid in an amount sufficient for the coupling reaction between said salt and bilirubin and;

c. a phosphoric acid diester of the formula:



wherein R_1 and R_2 are individually selected from unsubstituted or substituted aliphatic, cycloaliphatic araliphatic or aromatic radicals, and contain not more than 18 carbon atoms each.

33 Claims, No Drawings

DIAGNOSTIC AGENT FOR THE DETECTION OF BILIRUBIN

The present invention is concerned with an improved diagnostic agent for the rapid and sensitive detection and for the determination of bilirubin in body fluids.

The detection and determination of bilirubin in body fluids are of great importance for the diagnosis of diseases of the liver and gall bladder. Thus, in the case of liver damage and of occlusion of the gall bladder duct, bilirubin occurs in the urine in the early stages, even before the bilirubin content of the serum increases and clinical signs of jaundice appear. On the other hand, this type of jaundice can be distinguished from the so-called haemolytic icterus in which an increased bilirubin level can only be detected in the serum but not in the urine.

Methods for the detection and determination of bilirubin in body fluids have been known for a long time. The methods which are of the greatest importance depend upon the evaluation of the colored compounds formed by the coupling of diazonium salts with bilirubin. Since the discovery of this diazo reaction in **1883,** a large number of such methods has been described.

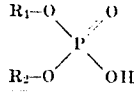
Recently, the so-called rapid tests have been introduced to an ever increasing extent in medical practice and in clinical laboratories. These are simple devices, usually test papers, which enable the detection or semi-quantitative determination of pathological components of the body to be carried out rapidly and with certainty, even by untrained personnel, such as hospital auxiliaries.

Thus, for example, in German Patent No. 1,102,444, there is described a reagent tablet containing a diazonium salt and a strong acid which is placed on a spot plate moistened with the body fluid to be tested and, after moistening with water, makes the bilirubin visible as a violet ring on the substrate.

It is obvious that such a diagnostic agent is relatively laborious to use. For a wide use in medical practice and at the sick bed, it is, however, desirable that the rapid test is of such a nature that, after simply dipping into the body fluid to be investigated, it can easily be read off without difficulty. This purpose is best fulfilled by so-called test papers: these are absorbent carriers which have been impregnated with all the reagents necessary for the detection reaction.

In German Patent No. 2,007,013, test papers have already been described for the detection of bilirubin which contain diazonium salts as the detection reagent. According to our investigations, however, these suffer from certain disadvantages with regard to the speed of the reaction and the sensitivity, which make their practical use questionable.

We have now, surprisingly, found that it is possible not only to increase the speed of the reaction but also the sensitivity of the bilirubin test papers when the coupling of the diazonium salts is carried out in the presence of phosphoric acid diesters of the general formula:



(I)

wherein

R_1 and R_2, which may be the same or different, are unsubstituted or substituted aliphatic, cycloaliphatic, araliphatic or aromatic radicals, of up to 18 carbon atoms.

The substituents R_1 and R_2 are preferably identical because phosphoric acid diesters of this type are especially easy to prepare. The aliphatic radicals R_1 and R_2 can be straight-chained or branched and can contain up to 18 carbon atoms, the effectiveness of the esters initially increasing with the increasing number of carbon atoms and, finally, above a chain length of about 14 carbon atoms, due to the increasingly hydrophobic nature of the esters, again decreases. As cycloalkyl radicals, those containing five to eight carbon atoms have proved to be preferable. Examples of substituents for the aliphatic and cycloaliphatic radicals include halogen atoms, preferably chlorine or bromine atoms, nitro groups and alkoxy radicals containing up to 8 carbon atoms. With regard to the stability of the test strips, those phosphoric acid diesters of general formula (I) are preferred in which R_1 and R_2 are aromatic radicals. As aromatic radicals, there are preferably used mono- or polynuclear, unsubstituted or substituted aryl radicals, for example, phenyl, xylyl, tolyl, chlorophenyl, nitrophenyl or naphthyl radicals. Especially preferred araliphatic radicals include the phenyl and naphthyl radicals connected to the phosphoric acid residue via an alkylene bridge containing up to three carbon atoms.

Thus, the present invention provides test papers for the detection of bilirubin in body fluids which contain a diazonium salt capable of coupling with bilirubin and an amount of an acid which is sufficient for the coupling reaction, as well as a phosphoric acid diester of general formula (I).

The phosphoric acid diesters of general formula (I) are known (see Methoden d. Org. Chem., Houben-Weyl, Vol. XII/2, pp 226 et seq.).

The phosphoric acid diesters of general formula (I) exert their sensitivity-increasing and reaction-promoting action even at concentrations of 2–3 percent in the solution used for impregnating the test papers; only the lower alkyl esters require a somewhat higher concentration. For this reason, the phosphoric acid diesters of general formula (I) usually cannot replace the acid component needed for the coupling reaction so that, for the maintenance of a strongly acidic pH value, additional amounts of acid are necessary. For this purpose, a large number of acids can be used. Those which have proved to be especially useful include oxalic acid, citric acid, potassium bisulfate and, especially with regard to the stability of the diazonium salt, commercially available metaphosphoric acid, which can contain up to about 60 percent of its sodium salt. Test papers which, in addition to the diazonium salts, only contain these acids and no diesters (I) react substantially more slowly with bilirubin and have an insufficient sensitivity for an accurate determination of bilirubin. It is still completely unknown upon what this action of the diesters (I) used according to the present invention depends since there is absolutely no chemical similarity with the accelerators of the diazo reaction otherwise used, for example caffeine or sodium benzoate.

It is surprising that the diesters of phosphoric acid of general formula (I), used according to the present invention, are effective. The good effect of these phosphoric acid diesters (I) is in complete contrast to the

complete ineffectiveness of phosphoric acid, phosphoric acid monoesters, benzenephosphonic acid and phosphoric acid triphenyl ester.

With regard to the use of various diazonium salts, it is necessary to distinguish whether the test papers are intended for the determination of bilirubin in the serum or in urine, i.e. the formulation of the test strips must be appropriately modified.

For a test paper for serum bilirubin, there can be used practically any diazonium salt which, from the chemical standpoint, can be expected to give a rapid and sensitive reaction. These are, in particular, diazonium salts which exclusively or preponderantly contain electron-attracting groups. Thus, for example, in the benzene series, the substituents can be nitro groups, halogen atoms, carboxyl groups, sulfonic acid residues, nitrile groups or quaternary ammonium groups. To a minor extent, electron-donating groups, for example alkoxy radicals, can also be present. Furthermore, diazotized naphthylamine and benzidine derivatives can also be used. Less suitable are benzene-diazonium salts which exclusively contain electron-donating groups, such as alkoxy, alkyl or arylamino radicals, because these only react comparatively slowly with bilirubin. Depending upon the nature of the substituent, the reaction color is red-violet to blue-green and, in addition, as has been found from experience, with increasing acidity of the phosphoric acid diesters, the colors are bathochromically displaced.

Since strong violet shades are preferred, 3-nitro- and 2,4,6-trichlorobenzene-diazonium salts are preferably used.

For measurement in reflection photometers, blue or green color shades are more preferable. In this case, there will then be used, for example, 4-halo- or 4-nitrobenzene-diazonium salts, which give a blue reaction, or 4-(5-methylbenzthiazolyl-2)-benzene-diazonium salts, which give green color reactions.

The diazonium salts are preferably used in the form of fluoborates since the stability thereof is well-known; however, other stable salts, for example, arylsulfonates, especially naphthalene-1,5-disulfonates, can also be employed.

The diazonium salts can be used in the impregnation solutions in amounts of from 0.02 to about 2 percent and preferably of 0.05 to 0.5 percent.

In the case of test papers for the detection of bilirubin in urine, many diazonium salts cannot be employed because they give a yellow-brown to red-brown color reaction with unknown components of the urine and thus can mask the weak bilirubin colorations. Furthermore, disturbing reactions can also take place with urobilinogen, which occurs in the urine of patients with liver and gall bladder diseases in the same way as bilirubin. The reaction color is initially only yellow but, after a short period of time, for example, after only a few seconds, violet to brown color shades occur which can be practically indistinguishable from the actual bilirubin detection reaction.

As reagents for the detection of bilirubin in the urine, there can be used, for example, 2,6-dihalobenzene-diazonium salts, especially the 2,6-dichloro and/or -dibromo derivatives, since these only possess the above-mentioned disadvantages to a minor extent. For the salts, there can be used the conventional stabilizing anions, for example, the fluoborates and aryl-sulfonates. Test papers are obtained which, depending upon the bilirubin concentration of the urine, change from yellow via orange to red-violet. The concentration of the diazonium salts in the impregnation solutions can be between 0.02 and 0.5 percent and preferably between 0.05 and 0.15 percent. The test papers for urine bilirubin can, of course, also be used for serum bilirubin.

The bilirubin test papers according to the present invention for serum and urine preferably contain stabilizing agents for the diazonium salts, for example, sodium fluoborate, magnesium sulfate, sodium metaphosphate, aryl-sulfonates or the like.

Furthermore, they can contain wetting agents in order to improve the absorptive powers of the test papers. In principle, there can be used all wetting agents which are still surface-active in the strongly acidic medium formed after dipping into the body fluid, namely, cationic agents (for example lauryl-pyridinium chloride), non-ionic agents (for example, polyoxyethylene triglyceride) and anionic agents, especially sulfates and sulfonates (for example, dodecyl-benzene-sulfonate).

The wetting agents can be used in the impregnation solutions in concentrations of 0.1 to 2 percent and preferably of 0.2 to 0.5 percent.

As solvents or solvent mixtures for the impregnation of the test papers, there can be used all those which do not react with the diazonium salts, in which all the components are soluble and which have a low boiling point in order that the diazonium salts do not have to be exposed to too high a temperature during drying. It has also proved to be useful to impregnate with individual components in separate working steps. Thus, for example, an absorbent paper can first be impregnated with a mixture of a diazonium salt and an acid, for example metaphosphoric acid in an aqueous medium and then impregnated with an ester (I), for example phosphoric acid diphenyl ester, in ethyl acetate or chloroform.

As absorbent carrier, it is preferred to use filter paper but other absorbent carriers, for example, glass fiber paper or synthetic fiber fabrics and fleeces made from acid resistant fibers, such as polyesters and polypropylene, can also be used. The above-used term "test paper" is to be understood to include all of these materials.

The test papers can be cut up into long strips and rolled up and, when needed, it is only necessary to cut or tear off a small piece. They can also be cut up into small rectangular pieces and stuck or sealed on to the lower end of narrow synthetic resin strips. It is especially advantageous when the test papers are sealed between two synthetic resin foils in the manner described in German Pat. No. 1,546,307 or between a synthetic resin film and a meshwork in the manner described in German Pat. No. 2,118,455 because there is then no danger that the reagents might be washed out upon dipping into a body fluid.

The following Examples are given for the purpose of illustrating, without limitation, the present invention:

EXAMPLE 1

Filter paper was successively impregnated with the following solutions and then dried at 40°C.:

Solution I:

2,6-dichlorobenzene-diazonium fluoborate		0.08	g.
metaphosphoric acid		10.0	g.
citric acid		3.0	g.
distilled water	ad	100.0	ml.

-Continued

Solution I:

Solution II:

phosphoric acid diphenyl ester		5.0 g.
ethyl acetate	ad	100.0 ml.

Upon dipping into bilirubin-containing urine, there were obtained, depending upon the bilirubin concentration, after about 10 to 20 seconds, a red to violet-red coloration, of the test paper; the limit of sensitivity is about 0.1 to 0.3 mg.% bilirubin. In the case of bilirubin-containing serum, there were obtained red-violet colorations with increasing intensity, the limit of sensitivity being about 0.4 to 0.6 mg.%.

Test papers which contain 2,6-dibromobenzene-diazonium fluoborate reacted in an analogous manner.

Test papers of otherwise the same composition but which did not contain the phosphoric acid diphenyl ester required 2–5 minutes for the reaction and had a limit of sensitivity of about 1 to 2 mg.%. This also applied to test papers which, instead of phosphoric acid diphenyl ester, contained phosphoric acid monophenyl ester, phosphoric acid triphenyl ester or benzene-phosphonic acid.

EXAMPLE 2

Test papers which were analogous to those described in Example 1 were obtained when the phosphoric acid diphenyl ester was replaced by one of the following phosphoric acid esters:

phosphoric acid di-o-tolyl ester;
phosphoric acid di-p-tolyl ester;
phosphoric acid bis-3,5-xylyl-ester;
phosphoric acid bis-o-chlorophenyl ester;
phosphoric acid bis-p-chlorophenyl ester;
phosphoric acid bis-p-nitrophenyl ester;
phosphoric acid dibenzyl ester;
phosphoric acid dicyclohexyl ester; or
phosphoric acid dipentyl ester.

EXAMPLE 3

Filter paper was impregnated with the following solutions and then dried at 40°C.:

Solution I:

diazonium salt (see Table 1)		0.3 g.
metaphosphoric acid		10.0 g.
water-methanol (4:1)	ad	100.0 ml.

Solution II:

phosphoric acid diphenyl ester		5.0 g.
dodecyl-benzene-sulfonic acid sodium salt		0.5 g.
ethyl acetate	ad	100.0 ml.

Test papers were obtained which indicated a bilirubin content in serum by the reaction colors given in table 1, the sensitivity limit being between about 0.3 and 0.6 mg.%, depending upon the diazonium salt:

TABLE 1

diazonium salt	reaction color
4-nitrobenzene-diazonium fluoborate	blue
3-nitrobenzene-diazonium-naphthalene-2-sulfonate	violet
2-chlorobenzene-diazonium-naphthalene-1,5-disulfonate	blue-violet
4-fluorobenzene-diazonium fluoborate	blue
2,5-dichlorobenzene-diazonium fluoborate	violet
2,4-dibromobenzene-diazonium fluoborate	blue
2,4,6-trichlorobenzene-diazonium fluoborate	violet
4-carboxybenzene-diazonium-p-toluene-sulfonate	blue
diazosulfanilic acid	blue
benzidine-bis-diazonium fluoborate	green
4-(5-methyl-benzthiazolyl-2)-benzene-diazonium fluoborate	green

EXAMPLE 4

Filter papers were impregnated with solutions of the following composition and then dried at 40°C.:

Solution I:

2-chlorobenzene-diazonium fluoborate		0.05 g.
oxalic acid		10.0 g.
sodium fluoborate		5.0 g.
water-methanol	ad	100.0 ml.

Solution II:

phosphoric acid di(A)-and monoalkyl ester (B) mixture		x g. (See Table 2)
ethyl acetate or chloroform	ad	100.0 ml.

Test papers were obtained which reacted with bilirubin-containing serum to give red-violet to blue-violet colors when there were used phosphoric acid esters with the alkyl radicals indicated in the following Table 2:

TABLE 2

alkyl radical	x	%A	%B
methyl	40	50	40
ethyl	20	40	50
2-chloroethyl	10	50	45
2-butoxyethyl	10	50	45
n-propyl	10	45	50
isopropyl	20	25	55
n-butyl	10	50	50
2-ethylhexyl	5	92	3
isooctyl +	10	55	45
isononyl +	10	60	40
isodecyl +	10	60	40
dodecyl	10	55	45
isotridecyl +	10	60	40

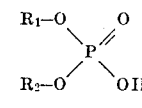
+ isomeric mixture

In the above-mentioned ester mixtures, the phosphoric acid monoesters, together with the oxalic acid in Solution I, merely served as acid component for the coupling reaction.

It will be understood that the specification and examples are illustrative but not limitative of the present invention and that other embodiments within the spirit and scope of the invention will suggest themselves to those skilled in the art.

What is claimed is:

1. Test paper for the detection of bilirubin in body fluids, comprising an absorbent carrier and, impregnated thereinto a diazonium salt capable of coupling with bilirubin, an amount of an acid which is sufficient for the coupling reaction, and at least one phosphoric acid diester of the general formula:



wherein R_1 and R_2 are individually selected from unsubstituted or substituted aliphatic, cycloaliphatic, araliphatic or aromatic radicals, and contain not more than 18 carbon atoms each.

2. Test paper as claimed in claim **1**, wherein R_1 and R_2 in the formula are identical.

3. Test paper as claimed in claim **1**, wherein R_1 in the formula is straight or branch-chained alkyl of up to 18 carbon atoms.

4. Test paper as claimed in claim **1**, wherein R_1 is cycloalkyl of from five to eight carbon atoms.

5. Test paper as claimed in claim **1**, wherein R_1 is substituted alkyl or cycloalkyl and the substituent is at least one of halogen, nitro and alkoxy of up to eight carbon atoms.

6. Test paper as claimed in claim **1**, wherein R_1 is aryl of up to 10 carbon atoms.

7. Test paper as claimed in claim **1**, wherein R_1 is aryl of up to 10 carbon atoms, substituted with halogen, nitro or alkyl of from one to three carbon atoms.

8. Test paper as claimed in claim **1**, wherein R_1 is arylalkyl of up to 10 carbon atoms in the aryl moiety and up to three carbon atoms in the alkyl moiety.

9. Test paper as claimed in claim **1**, wherein R_2 in the formula is straight or branch-chained alkyl of up to 18 carbon atoms.

10. Test paper as claimed in claim **1**, wherein R_2 is cycloalkyl of from five to eight carbon atoms.

11. Test paper as claimed in claim **1**, wherein R_2 is substituted alkyl or cycloalkyl and the substituent is at least one halogen, nitro and alkoxy of up to eight carbon atoms.

12. Test paper as claimed in claim **1**, wherein R_2 is aryl of up to 10 carbon atoms.

13. Test paper as claimed in claim **1**, wherein R_2 is aryl of up to 10 carbon atoms, substituted with halogen, nitro or alkyl of from one to three carbon atoms.

14. Test paper as claimed in claim **1**, wherein R_2 is arylalkyl of up to 10 carbon atoms in the aryl moiety and up to three carbon atoms in the alkyl moiety.

15. Test paper as claimed in claim **1**, wherein said phosphoric acid diester is phosphoric diphenyl ester.

16. Test paper as claimed in claim **1**, wherein said phosphoric acid diester is phorphoric acid di-o-tolyl ester.

17. Test paper as claimed in claim **1**, wherein said phosphoric acid diester is phosphoric acid di-p-tolyl ester.

18. Test paper as claimed in claim **1**, wherein said phosphoric acid diester is phosphoric acid bis-3,5-xylyl-ester.

19. Test paper as claimed in claim **1**, wherein said phosphoric acid diester is phosphoric acid bis-o-chlorophenyl ester.

20. Test paper as claimed in claim **1**, wherein said phosphoric acid diester is phosphoric acid bis-p-chlorophenyl ester.

21. Test paper as claimed in claim **1**, wherein said phosphoric acid diester is phosphoric acid bis-p-nitrophenyl ester.

22. Test paper as claimed in claim **1**, wherein said phosphoric acid diester is phosphoric acid dibenzyl ester.

23. Test paper as claimed in claim **1**, wherein said phosphoric acid diester is phosphoric acid dicyclohexyl ester.

24. Test paper as claimed in claim **1**, wherein said phosphoric acid diester is phosphoric acid dipentyl ester.

25. Test paper as claimed in claim **1**, wherein said diazonium salt is a fluoborate or an aryl sulfonate.

26. Test paper as claimed in claim **1**, also containing a stabilizing agent.

27. Test paper as claimed in claim **26**, wherein the stabilizing agent is sodium fluoborate, magnesium sulfate, sodium metaphosphate or an aryl-sulfonate.

28. Test paper as claimed in claim **1**, also containing a wetting agent.

29. Test paper as claimed in claim **1**, wherein said acid is metaphosphoric acid, oxalic acid, citric acid or potassium bisulfate.

30. Test paper as claimed in claim **1**, wherein said absorbent carrier is adhered to one end of a narrow strip of synthetic resin.

31. Test paper as claimed in claim **1**, wherein said absorbent carrier is sealed between two synthetic resin films.

32. Test paper as claimed in claim **1**, wherein said absorbent carrier is sealed between a synthetic resin film and a meshwork.

33. Method for detecting bilirubin in body fluids, which method comprises contacting a test sample suspected of containing bilirubin with a test strip as claimed in claim **1**.

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