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COMPOSITION FOR DETECTING GUANASE AND PROCESS FOR DIAGNOSING VIRAL HEPATITIS THEREWITH

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This invention relates to novel diagnostic compositions and methods for the detection and estimation of purine deaminases. More particularly this invention relates to diagnostic compositions which are useful in the determination and estimation of guanase in body fluids. Still more particularly this invention relates to a novel means for the detection and diagnosis of viral hepatitis based on the presence of guanase.

In recent years, considerable interest has been shown in the association and relationship of specific diseases with particular enzymes thereby making the determination of these various enzymes a potentially useful tool in clinical and research areas. For example, there was reported in Medical World News, December 20, 1963, evidence linking the presence of the enzyme guanase in blood serum with patients displaying symptoms of viral hepatitis. This report also states that in "normal" individuals with cirrhosis or obstructive jaundice little or no guanase activity was detected. This difference is significant because the symptoms for viral hepatitis, cirrhosis and obstructive jaundice are, for the most part, similar and may be distinguished only by lengthy and cumbersome laboratory tests. Therefore, diagnostic compositions which may assist in distinguishing between viral hepatitis and cirrhosis, for example would be most useful to the medical and biochemical sciences.

Prior to this invention purine deaminases were determined chemically by employing complex and time consuming techniques which required the attention of specially skilled technicians. One such technique which is presently in use for the determination of guanase involves the use of the spectrophotometer. This procedure not only involves expensive optical apparatus and highly trained technicians, but also requires lengthy periods of incubation which are necessary in readying the sample for analysis. Constant surveillance by skilled technicians is required and only a limited number of samples may be run each day.

By this invention, both the time spent on analysis as well as the initial cost for equipment is drastically reduced. In addition this invention provides a diagnostic tool which may be used in a physican's office by personnel not specially trained in complex analytical devices.

It is therefore a primary object of this invention to provide novel diagnostic compositions which will determine and estimate purine deaminases in fluids economically and in a minimum length of time.

Another object of this invention is to provide novel diagnostic compositions and methods for detecting and determining guanase in body fluids.

A further object of this invention is to provide a means whereby a diagnosis for viral hepatitis or confirmation thereof may be made in a matter of minutes.

These objects may be accomplished and the disadvantages of the prior art overcome by the diagnostic compositions and techniques of this invention. Generally stated, this invention comprises (1) a purine containing reaction system which is capable of producing and sustaining peroxides in the presence of a purine deaminase, and (2) a chromogenic system for detecting the peroxides thus produced. More specifically this invention comprises an enzymatic type "reaction system" coupled with

a peroxide type "detection system" for the determination and estimation of purine deaminases in fluids.

To assist in understanding the probable transformations occurring in this invention, the following illustrative reaction sequence is presented:

$$\text{Reaction System}\begin{cases}(1)\ \text{Guanine} + H_2O \xrightarrow{\text{Guanase}} \text{Xanthine} + NH_3 \\ (2)\ \text{Xanthine} + O_2 \xrightarrow{\text{Xanthine Oxidase}} \text{Uric Acid} + H_2O_2\end{cases}$$

$$\text{Detection System}\begin{cases}(3)\ H_2O_2 + \text{Orthotolidine (reduced)} \xrightarrow{\text{peroxidase}} \\ \text{Orthotolidine (oxidized)} + 2H_2O\end{cases}$$

Although the above illustrative reaction sequence may be incorporated in or may explain in part the chemistry of the instant invention, such incorporation or explanation does not in itself detract from the novelty of the resulting inventive compositions. This is so because in this invention the reaction system is not merely designed for producing hydrogen peroxide but for producing peroxides in such quantity and rate so as to provide both a quantitative and qualitative method for determining a purine deaminase. In addition, this invention is designed to protect the hydrogen peroxide produced from forming undesirable complex formations which would adversely affect the purine deaminase determination.

For purposes of this invention, the reaction system generally involves the hydrolytic deamination of a purine, exemplified above by guanine, and the subsequent oxidation of the deaminated purine, exemplified above by the oxidation of xanthine to uric acid with the concomitant formation of hydrogen peroxide. The detection system generally includes the peroxidation of a chromogenic substance exemplified above by orthotolidine to produce a color change.

More specifically the reaction system generally comprises, for example guanine, a peroxide-complex inhibitor, xanthine oxidase and a buffer to maintain the reaction system within its optimal pH range. Xanthine oxidase may be found in milk, liver, and other tissues of animals.

Although the relationship of concentrations of reactants in the reaction system to the reactants in the detection system is not particularly critical, it is advantageous, due to the high cost of enzymes, to use only minimum amounts of the enzymes. Obviously the reactants of the two systems should be present in quantities sufficiently complementary to each other to sustain a complete reaction. The quantitative relationship of the reactants within the reaction system and particularly the relationship of substrate to the enzyme being detected is, on the other hand, more critical. Generally the substrate should be present in the reaction mixture in quantities well in excess of the amount required for reaction with the enzyme being detected. An excess amount of substrate insures both the reliability of the qualitative and the quantitative determinations. In other words, a reaction system exhibiting a zero order reaction rate rather than a first order reaction rate is preferred.

In many cases these quantitative relationships may be further refined by experimentation. For example, in preparing a composition for use in the diagnosis or confirmation of viral hepatitis, the minimum amount of xanthine oxidase which may be used is that amount which will give a meaningful color reaction for all cases and during all stages of viral hepatitis. By experimentation it has been found that a composition containing approximately 0.1 ml. of 5 u./ml. of xanthine oxidase and 150 to 200 micrograms of guanine will satisfy these minimums requirements when analyzing a 0.1 ml. sample of blood serum. The unit of xanthine oxidase activity is defined as that amount of xanthine oxidase which will produce 2.46×10^{-4} micromols of uric acid per minute at a tem-

perature of 20° C. and a pH of 7.5 in an excess of a anthine substrate.

It has been further found, particularly where guanase s analyzed in blood samples normally containing catalase, hat a catalase-peroxide complex inhibitor can be added o the reaction system to insure complete color formation. f an inhibitor is not added, substantial portions of the peroxide may be lost and color development inhibited by he competitive reaction and formation of a catalase-peroxide complex. Although azides in general have been found to be most useful as catalase-peroxide complex inhibitors, alkali metal azides such as sodium azide, potassium azide, or lithium azide are preferred. The use of sodium azide has given excellent results and is still further preferred over the other alkali azides.

The amount of the inhibiting azide added is generally minor; however, excess amounts may be used. Generally, concentrations of sodium azide of between about 10^{-4} M to about 10^{-2} M are used. The desirable amounts of sodium azide can be conveniently added to the composition by incorporating the sodium azide into the buffer system. In many instances where the catalase is present in very small quantities, concentrations of sodium azide as low as 10^{-6} M in the buffer solution have been used and good results obtained. Generally the buffer solution, which contains the azide inhibitor, comprises about one-half of the total composition volume.

The detection system of this invention, as previously stated, comprises a combination of ingredients capable of responding to the hydrogen peroxide produced in the reaction system, by producing a visual color change upon combination of the reaction and detection systems. More specifically the detection system comprises a suitable chromogen, a catalyst having peroxidative activity and a buffer capable of maintaining an optimum pH range during color development.

Chromogenic substances which have been found to be effective in producing color reactions, as described above, include various derivatives of aniline and phenol. Examples of such chromogens include orthotolidine, benzidine, catechol, guaiacol, pyrogallol, ortho-toluidine, para-toluidine, ortho-dianisidine, ortho-phenylenediamine and the like.

The catalyst employed in the detection system, as pointed out above, is one having peroxidative activity. Generally, specific enzymes are employed; however, various inorganic or organic compounds may be used. Suitable inorganic compounds which may be used to catalyze the color reaction include compounds such as a mixture of sodium molybdate and potassium iodide. Suitable organic materials which are known to have peroxidative activity including urohemin, normal whole blood, red blood cells, hemin, lyophilized whole blood and various metalloporphyrin materials can also be used. In the case of metalloporphyrins it is preferred that the metalloporphyrins be combined with other organic substances in order to produce an effective catalytic system. Such organic substances include 2-aminobenzothiazole, pyridine, bipyridyl, 2,2'-bipyridylpyridine, nicotinic acid and the like.

Although there are many materials which can be used effectively to catalyze the detection system, the enzyme peroxidase is definitely preferred. Peroxidase may be obtained from horseradish, fig leaves or potatoes.

The range of concentrations of the various materials comprising the detection system may be varied quite widely. In general for the production of a consistent, reproducible color change it is advantageous that there be present in the detection system sufficient quantities of reagents so that a complete reaction occurs. In other words it is preferred that the concentrations of the chromogen and catalyst be in slight excess to the concentrations of peroxides produced in the reaction system thereby insuring complete and accurate color formation. These concentrations, in most instances, have to be determined experimentally.

As to the pH limits, however, the requirements are more specific since it is the pH of the detection system which eventualy determines the pH of the combined systems which in turn controls color development. Preferably the pH range for the detection system is maintained at a pH between about 4.0 to about 5.0. However, a pH as high as 8.0 and as low as 3.5 may be used. If the pH of the detection system is varied too extensively on either side of these limits, the reaction may be inhibited and color formation may be sluggish.

As previously noted the pH range for both the reaction system and the detection system may be varied slightly from the preferred ranges and good results obtained. When the reaction and detection systems are combined the resulting pH must be such that optimum color development results. The pH range of the combined systems is normally between a pH of about 4.5 to 5.5 and preferably between a pH of about 4.8 to 5.2. When the pH of the combined systems is below 4.5 or above 5.5, color development becomes sluggish and erratic. The pH of the combined systems, as previously mentioned, is controlled almost entirely by varying the pH of the detection system.

Before the ingredients of the reaction system (containing the fluid to be analyzed) are combined with the detection system, the hydrogen-peroxide formation if any is to occur, must be quantitatively complete. To insure a quantitative reaction within the shortest period of time, the reaction system is incubated with the fluid being tested for a certain period of time within a particular temperature range. If during the incubation period, the temperature is too low, such as room temperature, the reaction will be incomplete and sluggish and after about one hour will give only a faded, non-reproducible color indication when combined with the detection system. If the temperature, on the other hand, is too high, such as about 70° C., the enezymes are generally deactivated and little if any color change occurs when combined with the detection system. Generally incubation temperatures of between about 30° C. and 50° C. will give good results. However, incubation temperatures of between about 35° C. and 40° C. for periods of about 5 to 10 minutes are preferred. Although the normal incubation period will be less than 10 minutes, longer periods of incubation may be used and good results obtained. The choice of temperature and length of incubation will vary somewhat with each purine deaminase being analyzed.

The reaction system and the detection system as above described can be provided in various usable forms. For example, the systems can be prepared in either a wet or dry form. If the systems are prepared in dry form, the materials comprising each of these systems may be mixed and used as a powder or in the form of a tablet or pellet. Obviously these powders, tablets or pellets may also be dissolved in suitable solvents and used as solutions. If desired, combinations of wet and dry forms can also be employed. Another form in which the reaction and detection systems can be utilized is as sticks or strips of bibulous material upon which have been impregnated the formulations of the two systems. Any desired form in which the two systems may be maintained as separate systems can be used and excellent results obtained.

The following examples will illustrate in greater detail the formulations and uses of the diagnostic compositions of this invention. These examples, however, are not intended to be interpreted as limitations of this invention.

Example I

The diagnostic composition for this example was formulated by preparing separate vials containing in one the reaction system and in the other the detection system.

The reaction system was prepared by combining 0.8 ml. of an aqueous solution containing a 0.2 M concentration of sodium phosphate buffer and a 9.4×10^{-3} M concentration of sodium azide in a 5 ml. vial. To this phosphate-azide solution, there was added 0.5 ml. of an

aqueous solution containing 300 micrograms of guanine per ml. and 0.1 ml. of a solution containing 5 u./ml. of xanthine oxidase from milk. The resulting solution had a pH of 8.0.

The detection system was formulated by combining in a 5 ml. vial, 1.5 ml. of a 0.3 M aqueous solution of sodium citrate buffer, 0.5 ml. of a solution containing mg./ml. peroxidase from horseradish and 1.0 ml. of a 1% ortho-tolidine·2HCl aqueous solution. The resulting mixture had a pH of about 4.3.

A trace amount of guanase derived from liver (approximately 10 mg. dry weight of liver) was placed in the 5 ml. vial containing the above prepared reaction system. The vial was then plugged with gauze and incubated at a temperature of 37° C. After an incubation period of 10 minutes the contents of the 5 ml. vial were transferred into the vial containing the detection system. Immediately after the two solutions had been thoroughly mixed, a sharp blue-green color developed indicating the presence of guanase.

The color variation and intensity will vary depending on the concentration of enzyme present and the type of dye used.

Example II

Example I was repeated with the exception that 0.1 ml. of blood serum taken from an individual with viral hepatitis was added to the reaction system instead of liver guanase. After incubation, the two systems were combined as they were in the above example. A sharp blue-green color change was detected.

The above procedure was again repeated except that serum from an individual having cirrhosis rather than viral hepatitis was used. In this case no color change was detected.

Example III

Example I was repeated with the exception that an incubation temperature of 25° C. instead of 37° C. was used. As in Example I the two systems were combined and thoroughly mixed. There was no visible color change detected.

Example IV

The compositions as set out in Example I were reformulated except that in the reaction system a 0.2 M concentration of tris(hydroxymethyl)aminomethane-HCl buffer (pH 8.0) was used instead of the phosphate buffer. In addition a 0.3 M concentration of sodium succinate replaced the citrate buffer used in the detection system. The results obtained were comparable to those obtained in Example I.

Example V

The compositions as set out in Example I were reformulated with the exception that the sodium azide used in Example I was replaced with potassium azide. After incubating the mixture at a temperature of 37° C. for about 10 minutes, it was noted that the results obtained were similar to those obtained in Example I.

Example VI

The procedure of Example V was repeated except that an incubation temperature of 25° C. instead of 37° C. was used. There was no visible color change observed.

In summary this invention provides novel and most useful compositions for the detection of purine deaminases and particularly new and useful compositions for the detection of guanase and the diagnosis of viral hepatitis. The compositions of this invention comprise a reaction system and a detection system. The reaction system comprises a mixture of ingredients such as guanine, xanthine oxidase, a catalase inhibitor and a buffer effective in maintaining optimal activity. The detection system comprises a mixture of ingredients such as a chromogenic substance, a material having peroxidative activity to effectively catalyze a color reaction and a buffer to maintain an optimal pH. In order to detect guanase or other purine deaminases or in diagnosing viral hepatitis, a sample of unknown is introduced into the reaction system, incubated at an elevated temperature and then combined with the detection system. If guanase or other purine deaminase is present or if the patient has contacted viral hepatitis a color change will develop indicating same.

The foregoing description is intended to illustrate and explain in a non-limiting manner the compositions of this invention. Although certain obvious variations and modifications of the above disclosure may be made by those skilled in the art, such variations are to be considered within the intent and scope of the instant invention.

What is claimed is:

1. A composition for the detection of the enzyme guanase in fluids which comprises
- a reaction system comprising
  - guanine,
  - xanthine oxidase,
  - an azide for inhibiting the formation of interfering catalase-peroxide complexes,
  - a buffer for maintaining the reaction system at a pH of between about 7.5 to 8.5 during the hydrolytic deamination and oxidation of guanine with the concomitant formation of peroxides, and
- a detection system comprising
  - a chromogen responsive to the presence of peroxides,
  - a catalyst having peroxidative activity in the presence of the azide for catalyzing the color producing reaction resulting from contact of said chromogen with peroxide, and
  - a buffer for maintaining the combined reaction system and detection system during color development at a pH of between 4.5 and 5.5.

2. A composition according to claim **1** wherein the azide is an alkali metal azide.

3. A composition according to claim **1** wherein the catalyst having peroxidative activity is peroxidase.

4. A composition according to claim **1** wherein the chromogen is orthotolidine.

5. A composition for the detection of the enzyme guanase in blood which comprises
- a reaction system comprising
  - guanine,
  - xanthine oxidase,
  - sodium azide,
  - a phosphate buffer for maintaining the reaction system at a pH of between 7.0 and 9.0 during the hydrolytic deamination of guanine and the subsequent oxidation of xanthine whereby substantially quantitative amounts of hydrogen peroxide are produced,
- a detection system comprising
  - ortho-tolidine,
  - horseradish peroxidase, and
  - a citrate buffer capable of maintaining the combined reaction system and detection system at a pH of between about 4.5 and 5.5 during color development.

6. A process for the detection of guanase in blood which comprises contacting blood serum with a reaction system comprising guanine, xanthine oxidase, an azide and a buffer for maintaining the reaction system at a pH of between about 7.5 and 8.5 during the deamination of guanine to xanthine and the subsequent oxidation of xanthine; contacting the resulting mixture with a detection system comprising a chromogen responsive to the presence of peroxides, a catalyst having peroxidative activity in the presence of the azide and a buffer for maintaining said combined systems at a pH of between about 4.5 and 5.5 during color development; and observing a chromogenic response if guanase is present in the blood serum.

7. A process according to claim **6** wherein the azide is an alkali metal azide.

8. A process according to claim 6 wherein the catalyst aving peroxidative activity is peroxidase.

9. A process according to claim 6 wherein the hromogen is orthotolidine.

10. A process for detecting guanase in blood serum /hich comprises contacting the blood serum with a eaction system containing guanine, xanthine oxidase, odium azide and a phosphate buffer for maintaining the eaction system at a pH between about 7.5 to 8.5, in- ubating the resulting mixture at a temperature of between bout 30 C. and 50° C. for a period sufficient for any uanase present to convert quantitatively guanine to anthine, and thereafter contacting said resulting mixture /ith a chromogenic detection system comprising or- hotolidine, horseradish peroxidase and a buffer to main- ain the pH of the combined systems between about 4.5 o 5.5 during the color development which occurs if uanase is present in said blood serum.

11. A process for the diagnosis of viral hepatitis com- rising contacting blood serum from an individual sus- ected of having viral hepatitis with a guanase detecting omposition comprising a guanine containing reaction ystem capable of hydrolytic deamination and oxidation vith the concomitant formation of hydrogen peroxide in he presence of guanase and a chromogenic detection ystem for detecting said hydrogen peroxide and there- ıfter diagnosing hepatitis if a positive response is obtained.

12. A process for the diagnosis of viral hepatitis com- rising incubating blood serum from an individual sus- ected of having viral hepatitis with a guanase detecting omposition containing a reaction system comprising

guanine,

xanthine oxidase,

an azide catalase inhibitor, and

a buffer for maintaining a pH of between about 7.5 to 8.5 at a temperature of between 30° C. and 50° C., and adding a detection system comprising

a chromogen responsive to the presence of peroxides

a catalyst having peroxidative activity in the presence of the azide for catalyzing the chromogenic response, and

a buffer for maintaining optimal pH for color development and thereafter diagnosing hepatitis if a positive response is obtained.

13. A process as in claim 12 wherein the blood serum is incubated for about 5 to about 10 minutes at about 30° C. to about 50° C.

14. A process as in claim 12 wherein the detection system comprises:

orthotolidine,

horseradish peroxidase, and

a buffer for maintaining the combined reaction system and detection system at a pH between 4.5 and 5.5.

References Cited by the Examiner

UNITED STATES PATENTS

3,099,605 7/1963 Free ---------- 195—103.5

OTHER REFERENCES

Colowick et al.: Methods In Enzymology, vol. II, pages 480 to 482, 763 and 764.

Medical World News, December 20, 1963, pages 84 and 85.

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