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LABORATORY REAGENT FOR ASSAY
OF ALKALINE PHOSPHATASE

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2 Claims

The present invention relates to reagent and reagent mixtures useful for detecting and measuring alkaline and acid phosphatases in biological liquids, such as serum, and to methods of assaying biological liquids using these reagent mixtures.

The disodium salt of para-nitrophenyl phosphoric acid is known in the art as a substrate for this enzymatic reaction. However, its stability in dry form is limited, and it is also more difficult to prepare than the here claimed novel salts.

The stable reagent is an alkyl amine salt of paranitrophenyl phosphoric acid.

This salt is very stable and can be made available as unit amounts in foil containers or capsules. Each of these units will contain just a sufficient quantity of the assay material for making a single assay of a specimen.

In addition, suitable buffers and metal activators may be added to the reagent to give a stable reagent mixture. In order to make an assay, a package containing the assay material for making the particular assay is selected. The assay material contained in the package is pre-measured and of a predetermined activity.

Accordingly, it may be dissolved directly in a standard amount of water so as to form a liquid reagent. This liquid reagent is then mixed with the biological specimen to produce an enzymatic reaction. The extent of or the rate at which the reaction occurs will be a function of the quantity of amount of activity of the original unknown.

In a preferred embodiment, the assay material is dissolved to form a liquid reagent and the reagent is mixed with the specimen, the substrate will react with the unknown. However, in order for the reaction to occur successfully, it is necessary for the enzyme to catalyze the reaction. The quantity of the substrate contained in the reagent is in excess of that required to cause all of the unknown to completely react or to react at a desired rate. As a result the only factor that limits the assay reaction will be the quantity or amount of activity of the unknown.

When the substrates are in a pure solid dry form, they may be ground into a dry powder suitable for mixing with the lyophilized powder.

The substrate enters into the reaction and is converted from one form to another form. The extent to which the substrate is converted is determined by the extent to which the assay reaction progresses. The substrate may be readily converted from one form (p-nitrophenyl phosphoric acid) to another form (p-nitrophenyl). In addition the substrate has a light absorption at some particular wavelength only when it is in the converted form. When it is in the other form, it is transparent at the designated wavelength, although the absorption band may be any desired wavelength that is convenient to use. However, it is desirable that it be distinct from the intense absorption bands of the rest of the components in the assay material and the substances in the specimen. This will insure all of the substances in the reagent and the specimen, except the substrate, being transparent or substantially transparent although some of the various components may absorb limited quantities of light in the region of the selected wave-

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length and they will not vary during the period of assay whereby the only variable will be the coenzyme in the absorbing form. Thus by measuring the optical density at the designated wavelength, the amount of the substrate converted may be determined. More specifically, by measuring the amount of change or rate of change of the optical density at the designated wavelength, the amount or rate of the assay reaction may be measured. It has been found that p-NPP is transparent at 415 millimicrons. When the converted form (p-nitrophenol) shows absorption of ultraviolet light with a maximum value at a wavelength of about 415 millimicrons in alkaline solution. By employing this substrate, the assay reaction may be observed by always measuring the optical density at this wavelength.

Free para nitrophenyl phosphoric acid (p-NPP) may be obtained by any standard procedure, such as the passage of an alkali metal salt of p-NPP thru a cationic exchange resin, for example, Dowex 50 resin, in the acid form. Generally, the amine salts of para-nitrophenyl phosphoric acids may also be obtained by reacting an appropriate amine with an alkali metal salt of para nitrophenyl phosphoric acid. In this manner, the amine salt of para nitrophenyl phosphoric acid may be precipitated by concentration, or by the addition of an organic solvent.

Alternatively, the free para nitrophenyl phosphoric acid is neutralized with the appropriate amine in solution, and precipitated by the addition of an organic solvent, or evaporated until crystallization sets in. The corresponding para nitrophenyl phosphoric acid amine salt is washed, collected, and dried.

The particular amine salts employed will depend upon their availability and their ability to stabilize p-NPPA under test conditions. The salts will normally be chosen from a class that includes aliphatic amines, alicyclic alkylamines, hydroxyalkyl amines, arylamines, and aralkylamines.

Among the alkyl amines are monoalkyl, dialkyl and trialkyl, with from 1 to 18, preferably 1-6, carbon atoms in the alkyl groups. Specific examples are methylamine, ethylamine, tributylamine, and dipropylamine.

Among the alicyclic alkyl amines are mono-cyclohexylamine, dicyclohexylamine, and tricyclohexylamine.

Among the hydroxyalkyl amines are those with from 2 to 12, preferably 2-6, carbon atoms in said alkyl groups, exemplary thereof are monoethanolamine, and 2-amino-2-hydroxymethyl-1,3-propane diol.

The aralkyl amines, with 5 to 15, preferably 7-9, carbon atoms, are exemplified by monobenzyl amine.

The preparation and use of the reagents of this invention are illustrated by the following examples.

EXAMPLE 1

Preparation of diethanolamine salt of p-nitrophenyl phosphate

A solution of 12.5 gms. of disodium para nitrophenyl phosphate of high purity (such as obtained from Calbiochem) in 50 ml. of water, is passed thru a column of 125 ml. of Dowex 50 resin, 50-100 mesh, hydrogen ion form. The column is washer with 200 ml. of water. To the column effluent is added 6 gms. of ethanolamine. The solution was evaporated under reduced pressure to approximately 25 ml. and 600 ml. of acetone added. The mixture was then cooled to 0° C. for 2 hours and the precipitate collected on a filter and washed with acetone. The product was dried in vacuum to constant weight. Yield: 7.0 gms. of p-nitrophenyl phosphoric acid, di-diethanolamine salt.

EXAMPLE 2

Preparation of dimorpholine salt of p-nitrophenyl-phosphate

A solution of 12.5 gms. of disodium para nitrophenyl phosphate of high purity (from Calbiochem) in 50 ml. of water is passed thru a column of 125 ml. of a cationic exchange resin such as Dowex 50 resin, 50-100 mesh, hydrogen ion form. The column is washed with 200 ml. of water. To the column of effluent is added 13 gms. of morpholine. The solution is then evaporated under reduced pressure to a volume of approximately 25 ml., and 700 ml. of acetone added. The precipitant was collected, washed with cold acetone, and dried in vacuum to constant weight. Yield: 7.75 gms. of p-nitrophenyl phosphoric acid, dimorpholine salt.

EXAMPLE 3

Preparation of di(cyclohexylammonium-p-nitrophenyl phosphate

A solution of 12.5 gms. of disodium para nitrophenyl phosphate of high purity (from Calbiochem) in 50 ml. of water, was passed thru a column of 125 ml. of a cationic exchange resin, such as Dowex 50 resin, 50-100 mesh, hydrogen ion form. The column was washed with 200 ml. of water. To the column effluent is added 10 gms. of cyclohexylamine. The solution is evaporated under reduced pressure to approximately 25 ml., and 400 ml. of acetone is added. The mixture is cooled to 0° for 2 hours and the precipitate collected on a filter and washed with acetone. The product is dried in vacuum to constant weight. Yield: 8 gms. of p-nitrophenyl phosphoric acid, dicyclohexylamine salt.

EXAMPLE 4

When the following amines are substituted for cyclohexylamine in the procedure of Example 3, the corresponding listed products are obtained.

Amines	Stabilized p-NPP
Methylamine.....	p-NPPA-methylamine salt.
Ethylamine.....	p-NPPA-ethylamine salt.
Dimethylamine.....	p-NPPA-dimethylamine salt.
Trimethylamine.....	p-NPPA-trimethylamine salt.
Monobenzylamine.....	p-NPPA-monobenzylamine salt.
Furfurylamine.....	p-NPPA-furfurylamine salt.
Aniline.....	p-NPPA-aniline salt.
Tris(hydroxymethyl) amino-methane.	p-Tris(hydroxymethyl) amino-methane salt.

The amino salts of p-NPP, prepared as described above, are next tested to determine their suitability as substrates in an assay for alkaline phosphatase activity, by being compared to a standard salt form, such as disodium p-nitrophenyl phosphoric acid. This is measured by determination of rate of hydrolysis and the percent of free p-nitrophenol formed from equimolar aqueous solutions of the listed amine salts.

The rate of p-nitrophenolate release is conducted according to the method of O. A. Bessey, O. H. Loury, and M. J. Brock, J. Biol. Chem. 164, 321 (1964), and O. A. Bessey and R. H. Love, J. Biol. Chem 196, 175 (1952). Reagent components:

(a) Buffer-pH 10.2

Dissolve 8 g. Na_2CO_3 +2 g. NaHCO_3 +300 mg. Mg. glutamate in 1 liter H_2O .

(b) Hyland Abnormal Clinical Chemistry Control Serum

(c) PNPP-amine salt solution-.03 M each, or

(d) PNPP- Na_2 salt-.03 M (as the control)

Calculations:

(a) The rate percent of a PNPP-amine salt is equal to

$$\frac{\Delta A_{405}/\text{min. (amine)} \times 100}{\Delta A_{405}/\text{min. (Na}_2\text{)}}$$

(b) The weight percent of free p-nitrophenol present initially in each solution is equal to:

$$A_{405} \times 97.5$$

Mol. wt. PNPP salt

ENZYMATIC ACTIVITY OF AMINE SALTS

Salt	Rate	Percent free-p-nitrophenol*
PNPP, disodium.....	100	0.04
Do.....	104	0.07
PNPP-methylamine.....	107	0.08
PNPP-dimethylamine.....	105	0.09
PNPP-trimethylamine.....	84	0.10
PNPP-monoethanolamine.....	98	0.05
PNPP-morpholine.....	86	0.12
PNPP-monobenzylamine.....	50	0.06
PNPP-furfurylamine.....	66	0.04
PNPP-aniline.....	96	0.04
PNPP-tris (hydroxymethyl) amino-methane.....	91	0.03
PNPP-tris (hydroxymethyl) amino-thane (dried).....	81	0.04
PNPP-cyclohexylamine.....	104	0.04

*Before addition of serum.

(II) TEST FOR p-NITROPHENOL RELEASE FROM PNPP SALTS UNDER VARIOUS AMBIENT CONDITIONS

Procedure:

(a) Weigh out three 100 mg. samples of each salt.

(b) Put one sample of each PNPP salt in a dry desiccator; Put the second sample in a desiccator over saturated ammonium sulfate solution to maintain constant humidity; leave the third sample exposed to room conditions.

(c) Allow all the samples to be so exposed for at least 48 hours.

(d) Dissolve each sample in 5 ml. H_2O (remove solid if not completely soluble).

(e) Pipette 0.05 ml of sample into a cuvette and add 2.5 ml. of buffer (pH 10.2).

(f) Read at 405 $m\mu$ with any suitable spectrophotometer.

Calculations: percent free p-Nitrophenol= $A_{405} \times .228$

PERCENT FREE p-NITROPHENOL

Salt	Initial	Run 1, room cond.	Run 2, dry	Run 3, humid
PNPP, disodium.....	0.01	0.04	0.28	0.04
PNPP, ethylamine.....	0.009	0.07	0.07	0.07
PNPP, methylamine.....	0.014	0.09	0.10	0.09
PNPP, dimethylamine.....	0.006	0.07	0.11	0.20
PNPP, trimethylamine.....	0.004	0.15	0.14	0.11
PNPP, monoethanolamine.....	0.007	0.05	0.04	0.05
PNPP, morpholine.....	0.016	0.13	0.11	0.15
PNPP, monobenzylamine ¹	0.035	0.06	0.05	0.05
PNPP, furfurylamine ¹	0.01	0.04	0.04	0.04
PNPP, aniline ¹	0.016	0.05	0.04	0.04
PNPP, tris.....	0.005	0.02	0.02	0.02
PNPP, tris (dried).....	(?)	0.04	0.04	0.04
+PNPP, cyclohexylamine.....	0.007	0.03	0.02	0.03

¹ Not completely soluble.

² Not assayed.

According to this invention, a dry reagent powder is provided that contains p-nitrophenyl phosphoric in stabilized form as one of its amine salts (as an example). Preferably it contains a buffer from the class that includes alkali metal salts of carbonates, such as disodium and sodium hydrogen carbonates. Also, an metal activator, such as a magnesium salt is included, preferably magnesium aspartate.

Generally, dependent upon whether an acid or alkaline phosphatase measurement is involved, the pH is maintained within the broad range of between 4 and 11, by appropriate selection of one or more of the buffers well known in the art for this purpose.

As long as this powder is maintained dry, it is very stable and will have a long shelf life. Accordingly, it may then be divided into unit amounts to form a liquid reagent with water suitable for making a single assay of a serum.

Each of these parts may then be packaged into a suitable container such as a capsule for subsequent use.

Additionally, an aqueous solution of the amine may be frozen and lyophilized, without as great a formation of p-nitrophenol as in the case of the sodium salt.

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In order to use one of the capsules to make an assay of the amount of activity of the alkaline phosphatase present in a serum, a specimen of the biological fluid such as serum is first obtained. Following this, the assay material in one of the capsules of this example is dissolved in a suitable quantity of water. This will form a liquid reagent having the right size for making a single assay of the serum. This liquid reagent is thus mixed with the specimen. As soon, as the reagent and the specimen are mixed together, the following reaction will occur:

Amine salt of p-NNP $\xrightarrow[\text{ph 8-10 (buffer)}]{\text{alkaline phosphatase}}$ nitrophenol + salt of phosphoric acid

This reaction is dependent upon being catalyzed by the enzyme present in the serum.

What is claimed is:

1. A stable assay material for dissolving in water to create a liquid reagent for assaying a specimen for the enzymes, alkaline or acid phosphatases, including the combination of:

the substrate para-nitrophenyl phosphoric acid amine salt;

a dry buffer capable of maintaining the pH between 4 and 11;

a metal activator comprising a magnesium salt;

each substance above being present in that quantity so as to insure a uniform rate of reaction catalyzed

by the alkaline phosphatase being determined and so as to cause said rate to be optimal or maximal.

2. The method of assaying a specimen for the enzymes, acid or alkaline phosphatases, including the steps of dissolving into water, a substantially anhydrous solid reagent material comprising:

the substrate para-nitrophenyl phosphoric acid amine salt;

said substrate being present in that quantity to insure a uniform rate of reaction catalyzed by the enzyme being determined, thereby to produce a liquid reagent

having a measurable optical density;

mixing said liquid with said specimen to form a specimen-reagent assay mixture, and

measuring the rate of change of optical density of the reacting specimen-assay mixture.

References Cited

UNITED STATES PATENTS

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