

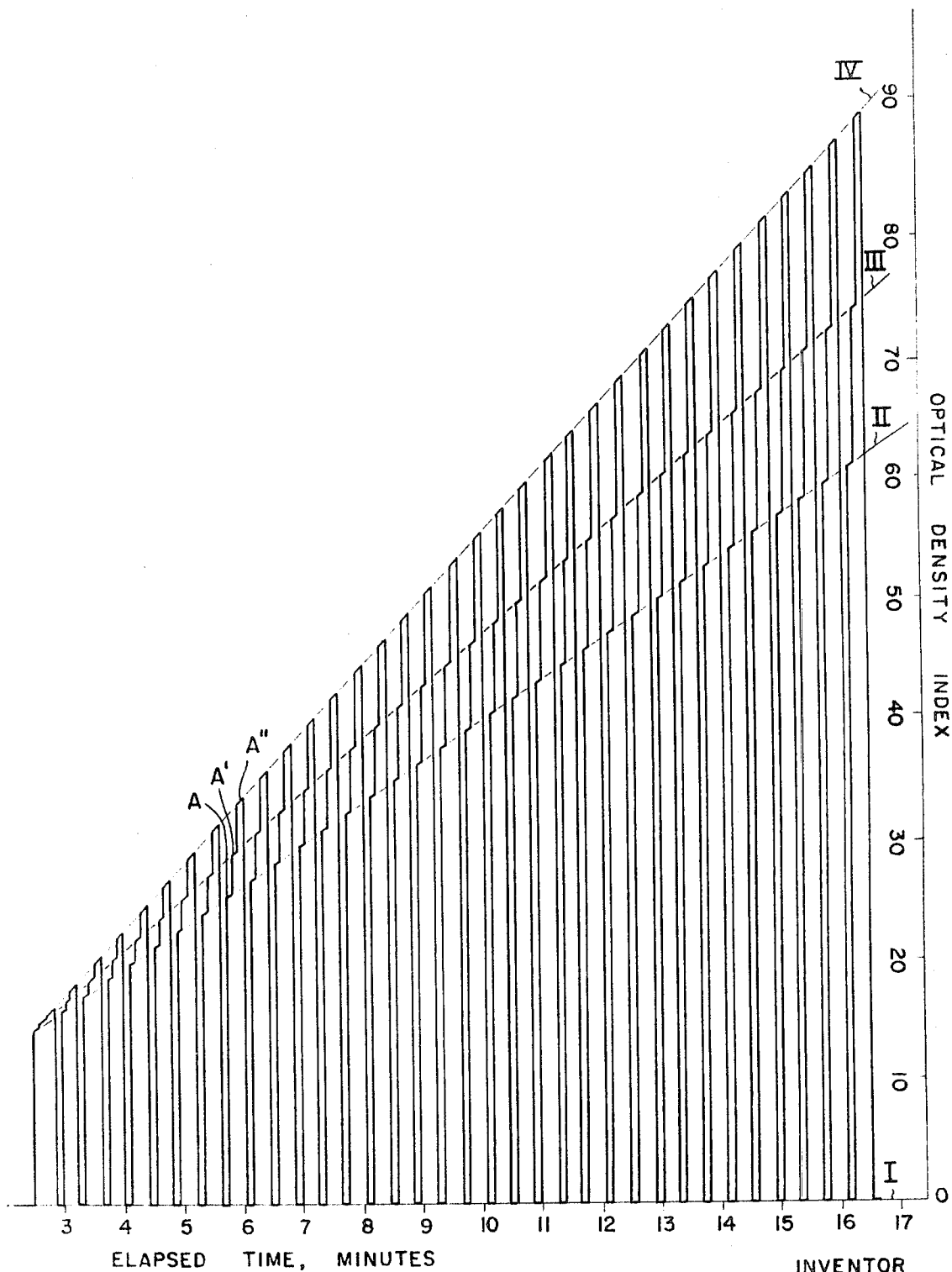
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LABORATORY REAGENT FOR ASSAY OF ALKALINE PHOSPHATASE

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LABORATORY REAGENT FOR ASSAY OF ALKALINE PHOSPHATASE

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10 Claims

ABSTRACT OF THE DISCLOSURE

The sensitivity of the known assay for alkaline and acid phosphatases, employing stable salts of p-nitrophenyl phosphoric acid as the substrate, is significantly enhanced by the incorporation into reagent assay of a polyhydric alcohol, typically mannitol.

This application is continuation-in-part of my presently copending application Ser. No. 627,430, filed Mar. 31, 1967, and now abandoned.

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 (p-NPP) 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 disclosed salts. It has been found that much to be preferred as a stable reagent is an alkyl amine salt of the paranitrophenyl phosphoric acid. Most preferred because of its greater solubility is the tris (hydroxymethyl) amino methane salt of p-NPP. 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.

The assay for alkaline phosphatase is especially plagued by difficulties in reading rate changes. Heretofore, available phosphatase assays have provided only an extremely narrow enzyme unitage range between normals and abnormal values. In the past this analysis has fostered frequent errors in determinations.

I have discovered that by employing polyhydric alcohols, that there is a remarkable enhancement in the sensitivity of the conventional assay for phosphatases, employing a salt of p-NPP as the substrate. This enhancement of rate of change is by a factor of at least 20% or more, with a demonstrable proportionality between the enzymatic reaction velocity and the enzyme concentration.

Representative of the polyhydric linear alcohols useful in this invention are: disaccharides and alcohols derived from the reduction of monosaccharides, for example: the reduced aldoses, such as erythritol, ribitol, and mannitol, the last being preferred; the reduced ketoses, such as sorbitol; the disaccharides such as sucrose, and glycerol.

These adjuvants appear active in the enzymatic reaction; moreover, they appear to increase the stability of the substrate material as well. It has been found that the quantity of adjuvant to be added may vary over a wide range, since the beneficial effect is approximately proportional to the amount of polyhydric substance available in the assay reagent. The upper limit appears to be the solubility of the particular substance in water at the temperature at which determinations are to be made, generally about 37° C. It has been established in actual experiments that appreciable results are achieved from as low as a few mg. of adjuvant for each ml. of water-

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diluted assay reagent to be combined with specimen, up to the known solubility of that adjuvant in water. In the case of mannitol, this ranges from 25 to 180 mg. per ml. of water diluted assay reagent.

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

Accordingly, it may be dissolved directly in a standard amount of water so as to form a liquid reagent. This liquid 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 or 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 amount of enzyme. The quantity of the substrate contained in the reagent is in sufficient quantity so as to allow the substrate to be acted upon at a constant rate. As a result the only factor that limits the assay reaction rate will be the quantity or amount of activity of the unknown enzyme to be measured.

The substrate enters into the reaction and the ester linkage is cleaved. 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 p-nitrophenyl phosphoric acid to p-nitrophenol. The substrate absorbs light at a wavelength that is different from that of the product. 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 salts are transparent in the range of 300 to 415 millimicrons, while the converted form (p-nitrophenol) shows absorption of light in the range of 390 to 420 millimicrons with a maximum value at a wavelength of about 405 millimicrons in alkaline solution. By employing this substrate, the assay reaction may be observed by always measuring the optical density in this latter range of 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.

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

Among the hydroxylalkyl 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 washed 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, 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 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 precipitate 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

Preparation of p-nitrophenyl phosphoric acid, tris (hydroxy methyl) amino methane salt

A solution of 12.5 gms. of disodium para nitrophenyl phosphate of high purity (from CALBIOCHEM) in 50 ml. of water is passed through a column of 125 ml. of Dowex 50 resin, 50-100 mesh, hydrogen form. The column is washed with 200 ml. of water. To the column effluent is added, 3 molar tris (hydroxy methyl) amine methane to a pH of 8.5. The resulting solution was concentrated to 50 ml., and a mixture of 200 ml. of methanol and 200 ml. acetone was added, with stirring and cooling.

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After 24 hours at 4° C. the crystalline product is collected and dried at room temperature in vacuum. The crude product was recrystallized by dissolving 10 grams in 30 ml. of water and adding 100 ml. of methanol and 100 ml. of acetone. After cooling for four hours, the product was collected and dried at room temperature in high vacuum. Yield: 8.5 gms.

EXAMPLE 5

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.

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 (1946), 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 was determined by dissolving the sample (100 mg.) in 100 ml. of 0.01 N NaOH. If insoluble material was present the solution was filtered. A standard solution corresponding to 1% p-nitrophenol was prepared (10 mg. in 1000 ml. of 0.01 N NaOH). The adsorbancy at 405 μ of each solution was determined

$$\text{Percent free p-nitrophenol} = \frac{A_{405} \text{ for the sample}}{A_{405} \text{ for the standard}}$$

ENZYMATIC ACTIVITY OF AMINE SALTS

Salt	Rate	Percent free p-nitrophenol ¹
PNPP, disodium.....	100	0.04
PNPP-methylamine.....	104	0.07
PNPP-dimethylamine.....	107	0.08
PNPP-trimethylamine.....	105	0.09
PNPP-monoethanolamine.....	84	0.10
PNPP-morpholine.....	98	0.05
PNPP-monobenzylamine.....	86	0.12
PNPP-furfurylamine.....	50	0.06
PNPP-aniline.....	66	0.04
PNPP-tris (hydroxymethyl) aminomethane.....	96	0.04
PNPP-tris (hydroxymethyl) aminomethane (dried).....	91	0.03
PNPP-dicyclohexylamine.....	81	0.04
	104	0.04

¹ Before addition of serum.

TEST FOR p-NITROPHENOL RELEASE FROM PNPP SALTS UNDER VARIOUS AMBIENT CON- DITIONS

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 ¹
- (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:

$$\text{Percent free p-nitrophenol} = A_{405} \times .228$$

[Percent free p-nitrophenol]

Salt	Initial	Run 1 room con- dition	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.....	0.035	0.06	0.05	0.05
+PNPP, furfurylamine.....	0.01	0.04	0.04	0.04
+PNPP, aniline.....	0.016	0.05	0.04	0.04
PNPP, tris.....	0.005	0.02	0.02	0.02
PNPP, tris (dried).....	1	0.04	0.04	0.04
+PNPP, cyclohexylamine.....	0.007	0.03	0.02	0.03

¹ Not assayed.

NOTE.—+ = Not completely soluble.

It has been stated earlier that the beneficial effects of the practice of this invention may be obtained with no observable change in zero order kinetics. The following data demonstrates this.

Conditions: Gilford 200 at 37° C. at 415 millimicrons
Serum: Hyland Abnormal control serum, Lot No. 368A16
Alkaline Phosphatase usual units=11. Acceptable range=10–12 (conversion factor to International Units μ mole/min./liter=16.66)

Hyland dilution: Theoretical unitage (I.U.)

- (1) Reconstituted Vial (5 ml.) 183
- (2) 2.5 ml. of (1) 2.5 ml. of H_2O 91.5
- (3) 2.5 ml. of (2) 2.5 ml. of H_2O 46
- (4) 2.5 ml. of (3) 2.5 ml. of H_2O 23

Assay=3.0 ml. H_2O +reagent tablet+20 λ serum
Calculations for 415 $m\mu$:

$$\frac{\text{International Units (I.U.)}}{\text{Liter of Serum}} = \Delta A_{415} \mu\mu/\text{min.}$$

$$\times \frac{T.V.^1}{17.7} \times \frac{1000}{\text{ml. serum}}$$

Hyland Abnormal Serum—415 $m\mu$

EXAMPLE 6

Enzymatic procedure for alkaline phosphatase determination and effect of mannitol thereon

- 5 Solution A: Dissolve following materials in 100 ml. distilled water:

	Mg.
Na_2CO_3	850
$NaHCO_3$	205
10 Magnesium aspartate	50
p-NPP, di(cyclohexylammonium) salt	315

Solution B: Dissolve 1000 mg. mannitol in 10 ml. of the just prepared Solution A.

- 15 Solutions A and B were then combined to produce four assay reagents as described below:

Reagent No. 1: 3 ml. of distilled water

Reagent No. 2: 3 ml. of solution A+0 ml. Solution B (Mannitol concentration of 0 mg./ml. of reagent)

- 20 Reagent No. 3: 2 ml. of Solution A+1 ml. Solution B (Mannitol concentration of 33 mg./ml. of reagent)

Reagent No. 4: 0 ml. of Solution A+3 ml. Solution B (Mannitol concentration of 100 mg./ml. reagent)

The reagents are transferred to appropriate cuvettes.

- 25 The above reagents are preincubated at 37° C. for 10 minutes; after which the O.D. readings at 415 $m\mu$ are observed and recorded.

The enzymatic reaction is initiated by the addition of 20 λ of Hyland Abnormal Serum (Lot #368A16)³. Readings are taken 10 minutes after the reagent is offered to serum ($A_{10'}$), and again at 15 minutes ($A_{15'}$).

Thus

$$\Delta O.D. = \frac{A_{15'} - A_{10'}}{5 \text{ min.}}$$

Reagent No.:	$A_{0'}$	$A_{10'}$	$A_{15'}$	minute	Δ / minute	Calcu- lated I. U.	Percent of rate increase over No. 2
1.....							
2.....	.051	.480	.645	.033	.279		
3.....	.051	.560	.772	.042	.355		27
4.....	.051	.652	.932	.056	.473		70

- 45 Referring now to the drawing in detail, which demonstrates the practice of a preferred embodiment of the invention, there is given plot of optical density versus elapsed

³ This lot of serum has been previously calibrated by well known methods to determine the corresponding number of International Units (I.U.) as follows:

One micromole of (p-Nitrophenol/ml.) gives $A_{415} m\mu$ of 17.7
I.U./Liter of serum= $\Delta A_{415} m\mu/\text{min.}$

$$\times \frac{\text{Total volume}}{17.7} \times \frac{1000}{\text{ml. serum}}$$

- 55 Wherein

$$\Delta A_{415} m\mu/\text{min.} = \frac{A_{415} m\mu \text{ at } 10 \text{ min.} - A_{415} m\mu \text{ at } 5 \text{ min.}}{5 \text{ minutes}}$$

Total volume=3.0 ml.

ml. serum=0.020 ml. (20 λ ml.).

- 60 Calculate I.U./liter serum= $\Delta A_{415} m\mu/\text{min.}$

$$\times \frac{3.0}{17.7} \times \frac{1000}{0.020}$$

Calculate I.U./liter serum=8450.

Hyland abnormal serum—415 $m\mu$						International units, Δ / minutes		
Tablet O.D. on dissolution	O.D. at 10'	O.D. at 20'	Δ /min- utes 10'	O.D. at 30'	Δ /min- utes 20'	10'	20'	Mean
(1) .167.....	.595	.960	.037	1.350	.038	313	322	318
(2) .168.....	.380	.600	.022	.795	.021	186	177	181
(3) .160.....	.225	.340	.011	.450	.011	93	93	93
(4) .159.....	.200	.250	.005	.300	.005	42	42	42

¹ After serum addition and start of incubation.

² The free p-nitrophenol will dissolve in 5 ml. H_2O .

time for the four filled cuvettes, which are scanned sequentially. The pen recordings are made by the operation of a Gilford Recording Spectrophotometer Model 2000, using all four sample wells.

This series of plotted curves demonstrates the rate of change of optical density at 415 millimicrons of the above described alkaline phosphatase containing specimen-liquid reagent mixtures at 37° C. These rates vary only as a function of the amount of mannitol incorporated into the assay reagent.

The operation of the Gilford 2000 is well known in the art, but for the sake of convenience, the significance of the pen recordings depicted will be briefly described for one of the intermediate sequence of determinations. The recording pen proceeds along the base line I, which represents the relative optical density (O) of the water blank, Reagent No. 1, when scanned by the visible beam.

Next the U.V. beam impinges on the cell bearing Reagent No. 2, with the instantaneous O.D. being recorded by the pen joggle denoted as A. The beam then impinges on the cell bearing Reagent No. 3, with the measured O.D. being recorded by the joggle A'. Lastly, the beam finally impinges on the cell bearing Reagent No. 4, the observed O.D. being indicated by joggle A." This sequence of scans is repeated, approximately every 20 seconds, starting with the water blank, causing the pen to drop back to the base line I before proceeding in its three-step upward travel.

Smooth curves can now be plotted through the plurality of determinations of instantaneous O.D. for each of the four reagents. It becomes readily apparent how the rate of enzymatic conversion of p-NPP to p-nitrophenol is accelerated by following the teaching of this invention, and importantly without sacrifice of the linearity of ΔO.D relationship. This makes for greater ease and accuracy in distinguishing between normal and abnormal enzyme levels.

The other practical advantage of increased sensitivity has been broadly set forth previously. For example, curve II indicates that Reagent No. 2 (O mannitol) took almost 16 minutes to reach an optical density, relative to the base line, of 60 chart units. Curve III shows that Reagent No. 3 (33 mg. mannitol/ml.) accomplished this in under 13 minutes, while curve IV shows that Reagent No. 4 accomplished this in under 11 minutes. Thus, a reliable ΔO.D. can be discerned much sooner. This permits a greater number of determinations to be made in a given period of time, according to the novel teaching of the present invention.

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

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 suitable container such as a tablet or capsule for subsequent use.

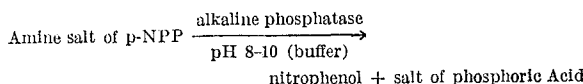
Alternatively, an aqueous solution of the amine may be frozen and lyophilized, without as great a formation of p-nitrophenol as in the case of the sodium salt.

Additional data on exemplary activators disclosed above, are provided below. Again employing a Gilford 2000 at 37° C. at 415 millimicrons, 50λ of pooled human

serum was mixed with 3.0 ml. of water, previously inoculated with 0.5 millimole of the candidate activators, one specimen and cuvette is required for each activator.

Activator	Mg./Ml. reagent	O.D. in 10'	Percent of rate increase
Omitted.....		.118	
Mannitol.....	91	.188	59
Sorbitol.....	91	.214	81
Sucrose.....	171	.142	20
Glycerol.....	46	.184	56

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 is one of the capsules of this example is dissolved in a suitable quantity of water. This will form a liquid reagent having the proper concentration of all reagents 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:



This reaction is dependent upon being catalyzed by the enzyme present in the serum, and its salt is determined as described above.

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 in the form of a water soluble salt;
 - a polyhydric alcohol selected from one of glycerol, disaccharides, and alcohols derived from the reduction of monosaccharides;
 - a dry buffer capable of maintaining the pH between 4 and 11; and
 - 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 in the form of a water-soluble salt;
 - a polyhydric alcohol selected from one of glycerol, disaccharides, and alcohols derived from the reduction of monosaccharides;
 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;
 - said polyhydric substances being present in the range of 25 to 180 mg. per ml. of said liquid reagent;
 - mixing said reagent 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.
3. The material of claim 1 wherein said polyhydric alcohol is mannitol.
4. The material of claim 1 wherein said polyhydric alcohol is sucrose.
5. The material of claim 1 wherein said polyhydric alcohol is sorbitol.

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6. The material of claim 1 wherein said polyhydric alcohol is glycerol.
7. The method of claim 2 wherein said polyhydric alcohol is mannitol.
8. The method of claim 2 wherein said polyhydric alcohol is sucrose.
9. The method of claim 2 wherein said polyhydric alcohol is sorbitol.
10. The method of claim 2 wherein said polyhydric alcohol is glycerol.

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