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REAGENTS AND METHOD FOR ASSAYING BIOLOGICAL SAMPLES

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ABSTRACT OF THE DISCLOSURE

Substantially anhydrous, solid assay materials for the determination, inter alia, of glucose, adenosine triphosphate, glucose-6-phosphate dehydrogenase, and creatine phosphokinase are rendered storage stable by the presence of certain polyhydric compounds, preferably mannitol, sorbitol, lactose or polyvinyl alcohol.

This invention is a continuation in part of my copending application Serial No. 320,004, filed October 30, 1963, now abandoned.

The present invention relates to processes and compositions for preparing reagent mixtures for detecting and measuring the presence of certain components in a biological sample. It also relates to the novel reagent mixtures.

In the clinical diagnosis of certain pathological conditions, it is frequently valuable to know the amount of activity or the quantity of certain substances present in a specimen of a biological or other fluid, or tissue. One of the more effective means that has been proposed for making assays of such specimens is to provide a liquid reagent which contains one or more biological components. When a given reagent is mixed with the specimen, the components are effective to cause an ezymatic reaction that involves the unknown substance. By observing this reaction, it is possible to determine the quantity or amount of activity of the unknown originally present.

Since such reagents contain one or more biological components such as enzymes, coenzymes and/or substrates, etc., the reagent has inherently been of a very unstable nature and has very little if any shelf life. To insure the reagent being at optimum strength it must be prepared at or immediately prior to the time the assay is made. In adidtion, heretofore the various components such as the enzymes, coenzymes, substrates, etc., included in the reagent have been very unstable. To insure these components being at their optimum it has been necessary for the components to be stabilized in a concentrated form.

When it has been desired to make a biological assay of the present type, a "kit" containing the several different components which may be dry, or in solutions, has been obtained. If the components are in a dry form, aqueous solutions are formed, and maintained separately until just prior to use.

The various components for the reagent are present in separate containers and maintained separated from each other. Some of these solutions and particularly those containing the enzymes are necessarily in a concentrated form in order to preserve their activity.

When employing a "kit" of this type, to assay a specimen, it is necessary to first reconstitute the components to the required strength by adding a specified amount of another liquid such as water to various solutions. After all of the various components have been reconstituted, the appropriate quantities of each are combined to form the reagent. A predetermined quantity of the reagent is then mixed with the specimen to produce the desired assay reactions. The accuracy of the final assay is also dependent upon the accuracy with which components are reconstituted, the accuracy with which the reconstituted components are combined to form the resultant reagent and the accuracy with which the reagent is measured when it is mixed with the specimen. It may thus be seen that the accuracy of the assay is dependent upon the skill of the operator and the accuracy with which he prepares and uses the reagent.

It can be readily appreciated that the foregoing process is very time-consuming particularly when considering the time for using and cleaning the substantial amounts of equipment such as various pieces of glassware, measuring instruments, etc. If any of the equipment has any foreign matter thereon, the reagent may easily be contaminated whereby the results of the assay will be misleading.

It should also be noted that after the reagent is fully prepared, at least one of the components therein and particularly the enzymes are quite unstable and rapidly lose their activity. As a consequence, if the reagent is not used within a matter of a few hours following its preparation, it must be discarded and, therefore, wasted. The percentage of the reagent wasted in this manner becomes very large where only a few assays are made at infrequent intervals.

It may thus be seen that although the foregoing "kits" have been capable of producing the desired reactions and permitting the desired assays to be made they have not been entirely satisfactory for numerous reasons. For example, they have not only been very time-consuming and wasteful, but have also required a person of sufficient skill to insure the accurate preparation of the reagents and their being used in the proper manner. Also, because of the possibility of substantial human errors such reagents have induced a certain degree of unpredictable error in the results of the assay.

It is an object of the present invention to provide means which will be effective to overcome the foregoing difficulties. More particularly, it is proposed to provide new and novel assay materials useful in making biological assays and the method for preparing the materials. All of the assay materials are in a dry, solid state that may be easily handled and used. The assay materials include components such as enzymes, coenzymes and/or substrates which have heretofore been very unstable. Moreover, the combining of such components tends to reduce their stability. However, stabilizers are included that are effective to maintain or preserve the activity of each of the components and of the entire assay material. Each of the components including those containing the enzymes may be stabilized individually and used as such for any desired purpose. Also, the compounds may be combined together to form a new and novel assay material. The resultant assay material contains all of the components except water, for making a liquid reagent that can be used to make a biological assay of the above described type.

Although the assay material contains various components such as enzymes, coenzymes and/or substrates, etc., the material is in a dry, solid form and will be very stable and have a long shelf life. This will permit the assay material to be packaged into containers which are easy to handle and use. Each of the containers may include a quantity of the assay material that is just the right amount for making a particular number of assays, for example a single assay. The assay material may desirably include an agent which, among other things, is effective to increase the volume of the assay material to a standard size whereby the quantity of the assay material required to produce a single assay will always be a standard amount. In order to make an assay, the contents of one of a standard size container may be mixed with a predetermined quantity of water to produce a liquid reagent

that is suitable for making a single assay. In addition this agent can facilitate handling during manufacture and increase shelf life. A preferred agent is mannitol. It will thus be seen that the possibility of human errors is eliminated and a relatively inexperienced person may prepare the liquid reagent and make the desired assay without any time-consuming measurements, using any large quantities of glassware, etc.

The term enzymatic assay is defined as the use of an enzyme as a component of a reagent for the determination of a substance or another enzyme, or for the determination of an enzyme in a biological sample.

These and other objectives and advantages of the present invention will become readily apparent from the following detailed description of a limited number of embodiments of the present invention.

The present invention is particularly adapted to be embodied in a dry assay material for determining in a specimen of fluid, particularly of biological origin, the quantity or the amount of activity of a certain unknown.

In the present instance, the unknowns are in a class which for convenience may be divided into four separate groups. The groups include enzymes such as carboxylases, dehydrogenases, hydrolases, isomerases, oxidases, phosphorylases and transferases. By way of example, this group includes: lactate dehydrogenase, alkaline phosphatase, glucose, oxidase, muscle phosphorylase, glutamate-oxaloacetate transaminase, phosphoenolpyruvate carboxylase, cholinesterase, glutamate-pyruvate transaminase, malate dehydrogenase, acid phosphatase, prostatic acid phosphatase, esterase, diesterase, lipase, amylase, sorbitol dehydrogenase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, alpha-hydroxybutyrate dehydrogenase, aldolase, glutamate decarboxylase, uricase, galactowaldenase, triose phosphate isomerase, carbonic anhydrase, leucine amino-peptidase, 3-phosphoglyceraldehyde dehydrogenase, trypsin and chymotrypsin. Also included are kinases, like creatine kinase.

The second group includes biochemical intermediates or metabolites. By way of example, the second group includes: glucose, lactic acid, pyruvic acid, adenosine triphosphate, phenylpyruvic acid, 3 methoxy-4-hydroxymandelic acid, cholesterol, creatinine, creatine, urea, uric acid, aspartic acid and glycine.

The third group includes chemical constituents of cells or biological fluids which, by way of example, may include dissolved carbon dioxide, triglycerides, protein, starch, glycogen, hemoglobin and insulin.

The fourth group includes drugs and toxins such as antimycin A, diisopropylfluorophosphate, sulfathiazole, ethanol, acetaldehyde and barbiturates.

To assay a specimen for one of the unknowns within a class, a liquid reagent may be mixed with the specimen to produce an enzymatic reaction. The particular reaction that occurs should produce an effect which can be easily measured. By way of example, the optical density of the assay mixture at some predetermined wavelength may change in proportion to the extent of the reaction.

In the present instance, the liquid reagent is prepared by dissolving a dry, solid assay material in water. When this solution is mixed with the specimen an assay mixture will be formed that includes a substrate that will enter into the reaction, an enzyme that will catalyze the reaction, and a co-enzyme that will be oxidized or reduced in the course of the reaction so as to produce a desired change in the assay mixture, for example, its optical density. All of the components that are not present in the specimen are contained in the assay material. In addition, one or more substances are included in the assay material to stabilize the assay material and preserve the activity of the various components. In addition, one or more buffer substances may also be provided that will be effective to maintain the conditions in the assay mixture suitable for the reaction to occur at an optimum rate.

The assay material is in a dry, powdered form and contains all of the components except water, for producing an enzymatic assay when combined with the specimen.

Since the assay material is very stable, the powder may be pre-measured into portions which are of just the right amounts for producing an assay reaction in a single specimen or an integral number of specimens. The pre-measured quantity of the assay material may be dissolved directly into a suitable quantity of water to form a liquid reagent. The liquid reagent may then be mixed with the specimen to induce the assay reaction in the assay mixture.

If the unknown being assayed is an enzyme, the reagent will not necessarily include an enzyme. Accordingly in one embodiment, the assay material is free of any enzymes but includes one or more components such as a substrate that will react at a rate or to the extent that is determined by the amount of activity of the unknnown or enzyme originally present in the specimen.

In a second embodiment of the assay material, a substrate is included which will react with the unknown and an enzyme included in the reagent, that will catalyze the reaction. In order to prepare an assay material of this embodiment, the first step is to select one or more substrates and one or more enzymes that will be effective to produce an enzymatic assay reaction and insure its occurring in the desired manner. The particular enzymes that are chosen will, of course, depend upon the particular unknown to be assayed and the particular reaction which it is desired to create. However, the enzymes will normally be chosen from a class that includes carboxylases, dehydrogenases, hydrolases, isomerases, oxidases, phosphorylases, and transferases. By way of example, this class includes: lactate dehydrogenase, alkaline phosphatase, glucose oxidase, muscle phosphorylase, glutamate oxaloacetate transaminase, phosphoenolpyruvate carboxylase, cholinesterase, glutamate-pyruvate transaminase, malate dehydrogenase, acid phosphatase, prostatic acid phosphatase, esterase, diesterase, lipase, amylase, sorbitol dehydrogenase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, alpha-hydroxybutyrate dehydrogenase, aldolase, glutamate, decarboxylase, uricase, galactowaldenase, triose phosphate isomerase, carbonic anhydrase, leucine aminopeptidase, 3-phosphaoglyceraldehyde dehydrogenase, trypsin, chymotrypsin, alpha-hydroxybutyrate dehydrogenase and beta-hydroxybutyrate dehydrogenase.

As is well known, enzymes of this type are generally very unstable. In fact, heretofore, in order to maintain enzymes of this type in a stable condition, it has been necessary to keep them in a concentrated form and at low temperatures. In addition to being concentrated, it has also been usually necessary for the enzyme solution or suspension to include a substantial amount of a salt such as ammonium sulfate to maintain optimal activity.

In the present invention, to prepare the assay material in a dry form, one of the steps in the process is to convert the enzyme from a solution to a dry, solid form such as a powder in which the enzyme is very stable. To accomplish this, one or more stabilizing compounds may be added to the solution containing the enzymes. The particular stabilizers added to the enzyme solution will, in part, vary with the particular enzyme that is to be stabilized. However, for enzymes of this type, at least one stabilizer is chosen from one or more of the following groups. Under some circumstances it has been found to be advantageous to employ a combination of stabilizers which may include a stabilizer from several of the following groups or even a stabilizer from each group.

Group I. Mucilagenous gums or polysaccharides such gum acacia, gum caarageenan, tragacanthin, alginic acid and pectin substances. Gum acacia has been found to be particularly well suited for this purpose. In addition to or as an alternative to the gums, the stabilizer may include other polymers containing hydroxy groups or other hy-

drophilic substitute groups which render the resultant polymer essentially soluble in water such as polyvinylpyrrolidine, carbowax and polyvinyl alcohol. This will also insure all of the assay material dissolving rapidly in the water when the reagent is prepared. However, it is also possible to use any other polymers which because of large chains or hydrophylic substitutent is only partially soluble in water but which equilibriate with the aqueous phase such as ion exchange resins, ion exchange cellulose, carboxymethyl cellulose.

Group II. A buffer consisting of a hydroxyalkylamine including but not limited to primary amines such as tris (hydroxymethyl) aminomethane or a tertiary amine such as triethanolamine.

Group III. A sequestering or complexing agent such as ethylene diamine tetracetic acid or one of its salts which has been found to be particularly well suited.

Group IV. An inert soluble protein such as bovine serum albumin.

Group V. Salts of a polyvalent anion such as ammonium sulfate, or sodium potassium tartrate, which have been found particularly suitable.

Group VI. Sulfhydryl compound such as dithioerythritol, cysteine, or reduced glutathione, which have been found particularly suitable.

After one or more of the stabilizers of the class described above has been completely dissolved or uniformly dispersed throughout the entire mixture, the enzyme or anzymes in the solution are very stable. It has been found that by adding these stabilizers to the solution, the activity of the enzymes is often increased. This is believed to result from the elimination of the effects of certain inhibitors which are usually present with the enzymes. In the event it is desired to decrease the activity of the solution, the solution may be diluted by adding water. Conversely, if it is desired to concentrate or increase the amount of activity of this enzyme solution, a portion of the liquid may be removed. Preferably, the liquid is removed by evaporation while the solution is maintained at a relatively low temperature. It may thus be seen that a very stable enzyme solution is provided at this point and that the stability of the solution is independent of the concentration of the enzyme or the salts therein and exhibits stability over a wider range of temperature.

It is an overall objective to provide an integrated assay material, which is dry, stable, enzymatic, pyridine nucleotide linked, and uniform in results under varying climatic and storage conditions.

The above described stabilized enzyme solution may be used for numerous purposes as a solution. However, under some circumstances such as the preparation of the present assay material, it may be desirable to convert the solution into a dry mixture or powder containing the enzyme. This may be accomplished by lyophilizing or freeze drying the solution. More particularly, the entire solution is frozen to provide a solid mass and placed under a vacuum. The vacuum is of sufficient magnitude to cause the frozen liquid to sublimate. The frozen mass is kept under this vacuum for a period sufficient to insure all of the water, etc., being removed. This will leave a solid residue that contains the enzyme or enzymes in intimate relation with the stabilizers, such as acacia, etc. These stabilizers are effective in preserving the activity of the enzyme for an extended period of time even though the enzyme is in the form of a solid.

The term stabilizer, as used herein, broadly relates to a substance which prevents the change or destruction of a reagent component. It has three major aspects in the present invention, namely: (1) to allow for convenient handling of the components during manufacture; (2) to permit the preparation and storage of a component in dry form; and (3) to provide long-term shelf-life of the finished product.

The residue will normally be in a fluffy or flaky condition. However, if it is desired, the residue may be ground until it is reduced to a finely powdered mixture. The grinding may be accomplished by any suitable means such as a ball mill. The fact that the powder mixture is substantially dry contributes to the chemical stability of the enzymes. It also contributes to the physical stability of the mixture and substantially eliminates the tendency to compact or become lumpy, etc. Since the mixture can be maintained as a loose powder it will be easy to handle and process. Also, it can be easily measured either volumetrically or gravimetrically. At this point a powder is provided that includes one or more enzymes in a very stable form. Since the powder does not include any form of substrate, it can be used as an enzyme for any desired purpose. For example, among other things, the enzyme powder may be employed to complete the preparation of the present assay material.

In order to complete the preparation of the assay material the other components such as the buffers, substrates, coenzymes, and bulking-stabilizing agents, may be prepared for mixing with the stabilized enzymes. It is one of the primary purposes of the buffer materials to maintain the conditions suitable for the assay reaction to occur at an optimum rate. When the assay material is dissolved in water, the buffers will, among other things, be effective to maintain the pH of the liquid reagent. In addition, when the liquid reagent is, in turn, mixed with a specimen containing the unknown, the pH of the resultant specimen mixture will still be suitable for the assay reaction to occur.

The particular buffer material that is employed in any particular assay material will be dependent upon the particular assay reaction to be conducted and the other components in the assay material. However, normally, they will be in a class that includes the salts of polyvalent inorganic anions and organic amines together with the acids and salts thereof. By way of example, the salts of polyvalent inorganic anions may include at least sodium and potassium phosphates and sodium and potassium pyrophosphates. By way of example, organic amines and acids, and their salts may include at least tris (hydroxymethyl) amino-methane and imidazole (and their salts, such as the hydrochloride, succinate, sulfate), succinic, aspartic, and glutamic acids (and their salts such as the sodium, potassium, and lithium), glycylglycine, and glycine.

The buffer materials may be prepared in the form of a dry powder that is mixed directly with the lyophilized powder containing the enzyme and the stabilizer. The resultant powder will contain the enzymes required for the assay reaction. Because of the stabilizers and buffers present in the powder, the enzymes will be very stable. This resultant powder like the enzyme powder first described, will not be hygroscopic, in contrast to corresponding mixtures prepared by lyophilization of the combined buffer and enzyme solutions.

The substrate is effective for reaction with the unknown in the specimen. Accordingly, the particular substrate that is employed in any particular assay material will be dependent upon the nature of the unknown and the particular assay reaction that it is desired to produce. Normally, the substrate will be in a class of biochemicals whose chemical reactions will be specifically catalyzed by the classes of enzymes previously described.

The following are specific examples of some of the substrates that fall within this class: alanine, alpha- or beta-ketoglutaric acid, aspartic acid, fructose-1, 6-diphosphate and glucose.

When 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 and the amount of activity of the enzyme con-

tained in the reagent are 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 coenzyme enters into the reaction and is converted from one form to another form. The extent to which the coenzyme is converted is determined by the extent to which the assay reaction progresses. The coenzyme may be readily converted from one form (such as oxidized) to another form (such as reduced). In addition the coenzyme has a light absorption at some particular wavelength only when it is in one of these forms. 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 coenzyme, being transparent or substantially transparent although some of the various components may absorb limited quantities of light in the region of the selected wavelength 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 coenzyme 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 the pyridine nucleotides are particularly well suited for this purpose. When they are in their reduced form, they show absorption of ultraviolet light with a maximum value at a wavelength of about 340 millimicrons. By employing a coenzyme of this class in all forms of the assay material, the assay reactions may be observed by always measuring the optical density at this wavelength.

Coenzymes of this class have a limited amount of stability in a solid form. They can be stored in a solid form for only short periods of time. The stability of the coenzyme may be increased by preparing a lyophilized powder of the coenzyme and acacia. Further increases in the stability of the coenzyme may be obtained by mixing with mannitol. Accordingly, the coenzyme may be ground into a powder and mixed directly with the lyophilized powder containing the enzyme, stabilizer, and buffer.

Heretofore, substrates, enzymes and coenzymes, and buffers have been dissolved in the solution containing the enzyme before lyophilization. The complete solution may then be lyophilized to provide a dry residue containing all of the components of the assay. However, it has been found, as a practical matter, the resultant assay material is often hygroscopic. As a consequence, the residue sometimes tends to absorb varying or unpredictable quantities of moisture. As a result when prepared in this manner it is desirable for the resultant mixture to be hermetically sealed within a container. However, even when hermetically sealed, the mixture may still be unstable and tend to develop colored specks due to local decomposition and in a fairly short time completely decompose or lose its activity. It has also been found that the mixture tends to form into lumps which makes it difficult to handle and measure into small units of identical amounts on a volumetric or weight basis.

This invention teaches that by preparing the various components of the assay material such as the substrates and coenzymes in a dry or solid state, and in a stabilized form before the mixing thereof with the lyophilized stabilized enzyme, a much more stable and easily handled assay material is now provided.

In addition to the foregoing components, it has also been found desirable to add a bulking-stabilizing agent to the mixture. This agent may be a polyhydric substance such as mannitol, sorbitol, lactose, polyvinyl alcohol or polymers having from 1 to 5 hydroxyl groups per monomeric unit. The bulking agent is not active in the assay reaction. Accordingly, the quantity of the bulking agent added to the assay material is not critical and may be varied throughout a wide range. However, the bulking agent performs several unexpected and useful functions. First of all, the bulking agent tends to further increase the stability of the assay material for several reasons. Such agents have the ability to absorb and retain limited quantities of moisture whereby the assay material is not materially affected when exposed to reasonable amounts of moisture. This increases the stability of the assay material and prevents its losing its activity. It has also been found that the bulking agent will also be effective in preserving the assay material by increasing the compatibility of its components. It has also been found that bulking agents of this category are also effective in increasing the ability of the assay material to withstand relatively high temperatures, such as 50° C., for longer periods of time. Heretofore, temperatures in this range have caused rapid deterioration of the enzymes, coenzymes, and other components.

Secondly, it has been found that the use of the bulking agent in the assay material results in the assay material dissolving more rapidly into water. This not only reduces the time required for preparing the liquid reagent but also increases the convenience of preparation by reducing the amount of stirring or shaking.

Thirdly, since the bulking agent does not enter into the reaction or affect the components in the assay material, the quantity of the bulking agent added to the assay material may vary over a wide range. Once a batch of the assay material has been prepared, its strength or amount of activity may be determined. The bulking agent may then be added to standardize the assay material to a predetermined level. This will result in the assay material always having a predetermined amount of activity per unit irrespective of the batch in which it is prepared. Of the agents listed above, mannitol is preferred.

After the assay material has had the bulking agent added, it may be divided into units of a standard predetermined size. The size normally will be just large enough to make a single assay or an integral number of assays. Each of these units may then be packaged into a container such as a capsule, glass vial, etc.

It will thus be seen that a plurality of substantially identical packages such as foil containers or capsules may be provided. Each of these capsules will contain just a sufficient quantity of the assay material for making a single assay of a specimen. 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 all 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 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. Every test, irrespective of the particular type of assay, will involve the conversion of a coenzyme from one form to another form wherein one form has an optical absorption at a predetermined wavelength. Accordingly, the optical density of the specimen at that wavelength will vary as a function of the unknown. Thus, by measuring the optical density of the medium at different times, it will be possible to compute the quantity or amount of activity of the unknown in the original specimen.

The invention sought to be patented in a principal process of making aspect, is described as residing in the concept of preparing an assay reagent, useful, inter alia, as

an aid in the clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of an enzyme in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form, comprising a dry coenzyme, a dry buffer, a dry second enzyme capable of catalyzing the conversion of the coenzyme to the other form of the coenzyme, dry substrates, effective to react with the enzyme of which the present quantity or activity is to be determined.

The invention sought to be patented in a second embodiment of a principal process of making aspect, is described as residing in the concept of preparing an assay reagent, useful, inter alia, as an aid in the clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of an unknown, in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form, and comprising a dry coenzyme, a dry buffer, a dry substrate, effective to react with the unknown of which the present quantity of activity is to be determined, a dry first enzyme capable of catalyzing the reaction of the unknown with the substrate to form an intermediate product, a dry second enzyme capable of catalyzing the reaction of the intermediate product and the coenzyme.

The invention sought to be patented in a third embodiment of a principal process of making aspect, is described as residing in the concept of preparing an assay reagent, useful, inter alia, as an aid in the clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of an enzyme in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form, comprising a dry coenzyme, a dry buffer, and a dry substrate effective to react with the coenzyme, of which the quantity or activity present is to be determined to form the other form of the coenzyme.

The invention sought to be patented in a fourth embodiment of a process of making aspect is described as residing in the concept of stabilizing the enzyme malate dehydrogenase, by admixing a stabilizer selected from at least one of tris (hydroxymethyl) aminomethane, its sulfate salt, acacia, ammonium sulfate or ethylenediamine tetraacetic acid.

The invention sought to be patented in a fifth embodiment of a process of making aspect is described as residing in the concept of stabilizing the enzyme lactate dehydrogenase, by admixing a stabilizer selected from at least one of tris (hydroxymethyl) aminomethane, its sulfate salt, acacia, ammonium sulfate or ethylenediamine tetraacetic acid.

The invention sought to be patented in a sixth embodiment of a process of making aspect is described as residing in the concept of stabilizing the enzyme lactate dehydrogenase, by admixing a stabilizer selected from at least one of polyhydric substances and polymers of said polyhydric substances with from 1 to 5 hydroxyl groups per monomeric unit.

The invention sought to be patented in a seventh embodiment of a process of making aspect is described as residing in the concept of stabilizing the enzyme hexokinase, by admixing a stabilizer selected from at least one of acacia, tris (hydroxymethyl) aminomethane, its sulfate salt, ammonium sulfate or ethylenediamine tetraacetic acid.

The invention sought to be patented in an eighth embodiment of a process of making aspect is described as residing in the concept of stabilizing the enzyme triose phosphate isomerase, by admixing a stabilizer selected from at least one of acacia, tris (hydroxymethyl) aminomethane, its sulfate salt, ammonium sulfate or ethylenediamine tetraacetic acid.

The invention sought to be patented in a ninth embodiment of a process of making aspect is described as residing in the concept of stabilizing the enzyme glyceraldehyde phosphate dehydrogenase, by admixing a stabilizer selected from at least one of acacia, tris (hydroxymethyl) aminomethane, its sulfate salt, ammonium sulfate or ethylenediamine tetraacetic acid.

The invention sought to be patented in a tenth embodiment of a process of making aspect is described as residing in the concept of preparing an assay reagent, useful, inter alia, as an aid in the clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of glutamate pyruvate transaminase in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of the dry substrates alanine and alphaketoglutaric acid, the dry enzyme lactate dehydrogenase, at least one dry stabilizer from the class that includes mucilaginous gums, hydroxyalkylamines, ethylene diamine tetraacetic acid and its salts, an inert soluble protein and a sulfate anion, a dry coenzyme consisting of reduced diphosphopyridine nucleotide, a dry buffer from the class that includes the salts of phosphates, organic acids and amines, and the salts of said acids and amines, and at least one dry stabilizing-bulking agent from a class that includes polyhydric substances and polymers of said polyhydric substances with from 1 to 5 hydroxyl groups per monomeric unit; which involves determining the optical density of the solid reagent after forming a liquid reagent therefrom and again following a predetermined incubation period after admixing the specimen.

The invention sought to be patented in an eleventh embodiment of a process of making aspect is described as residing in the concept of preparing an assay reagent, useful, inter alia, as an aid in the clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of glutamate oxaloacetate transaminase in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: the dry substrates of aspartic acid and alpha ketoglutaric acid, the dry enzyme malate dehydrogenase, at least one dry stabilizer from the class that includes mucilaginous gums, hydroxyalkylamines, ethylenediamine tetraacetic acid and its salts, an inert soluble protein and a sulfate anion; a dry coenzyme consisting of reduced diphosphopyridine nucleotide; a buffer from the class that includes the salts of phosphates, organic acids and amines, and the salts of said acids and amines; at least one dry stabilizing-bulking agent from a class that includes polyhydric substances and polymers of said polyhydric substances with from 1 to 5 hydroxyl groups per monomeric unit; which involves determining the optical density of the solid reagent after forming a liquid reagent therefrom, and again following a predetermined incubation period and after admixing the specimen.

The invention sought to be patented in a twelfth embodiment of a process of making aspect is described as residing in the concept of preparing an assay reagent, useful, inter alia, as an aid in the clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of aldolase in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: a dry substrate comprising fructose-1,6-diphosphate; the dry enzymes triose phosphate isomerase and glyceraldehyde phosphate dehydrogenase; a dry coenzyme consisting of reduced diphosphopyridine nucleotide; a dry stabilizer from the class that includes mucilaginous gums, hydroxyalkylamines, ethylene diamine tetracetic acid and its salts, an inert soluble protein and a sulfate anion; a dry buffer from a class that includes the salts of phosphate, organic acids and amines, and the salts of said acids and amines; a dry stabilizing-bulking agent comprising polyhydric substances and polymers of said polyhydric substances with from 1 to 5 hy-

droxyl groups per monomeric unit; which involves determining the optical density of the solid reagent after forming a liquid reagent therefrom, and again following a predetermined incubation period after admixing the specimen.

The invention sought to be patented in a thirteenth embodiment of a process of making aspect is described as residing in the concept of employing preparing an assay reagent, useful, inter alia, as an aid in the clinical diagnosis of pathological conditions to determine the presence and quantity of glucose in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form, including the combination of: the dry enzymes hexokinase and glucose-6-phosphate dehydrogenase; a dry coenzyme consisting of diphosphopyridine nucleotide; a dry stabilizer from the class that includes mucilageous gums, hydroxyalkylamines, ethylenediamine tetraacetic acid and its salts, an inert soluble protein, and a sulfate anion; a dry buffer from the class that includes mucilaginous gums, hydroxyorganic acids and amines, and the salts of said acids and amines; a dry stabilizing-bulking agent comprising polyhydric substances with from 1 to 5 hydroxy groups per monomeric unit; which involves determining the optical density of the solid reagent after forming a liquid reagent therefrom, and again after admixing the specimen and allowing sufficient time for the mixture to react completely.

The invention sought to be patented in a fourteenth embodiment of a process of making aspect is described as residing in the concept of preparing an assay reagent, useful, inter alia, as an aid in the clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of adenosine triphosphate in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: the dry enzymes hexokinase and glucose-6-phosphate dehydrogenase; a substrate comprising glucose; a dry coenzyme consisting of triphosphopyridine nucleoxide; a dry stabilizer from the class that includes mucilaginous gums, hydroxyalkylamines, ethylene diamine tetraacetic acid and its salts, an inert soluble protein and a sulfate anion; a dry buffer from a class that includes the salts of phosphate anions, organic acids and amines, and the salts of said acids and amines, a dry stabilizing-bulking agent comprising polyhydric substances and polymers of said polyhydric substances with from 1 to 5 hydroxyl groups per monomeric unit; which involves determining the optical density of the solid reagent after forming a liquid reagent therefrom, and again after admixing the specimen and allowing sufficient time for the mixture to react completely.

The invention sought to be patented in a fifteenth embodiment of a process of making aspect is described as residing in the concept of preparing an assay reagent, useful, inter alia, as an aid in the clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of malate dehydrogenase in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: the dry substrate comprising oxaloacetic acid; the dry coenzyme reduced diphosphopyridine nucleotide; a dry stabilizing-bulking agent from the class that includes polyhydric substances and polymers of said polyhydric substances with from 1 to 5 hydroxyl groups per monomeric unit; and a dry buffer from the class that includes the salts of phosphate anions, organic acids and amines, and the salts of said acids and amines; which involves determining the optical density of the solid reagent after forming a liquid reagent therefrom, and again following a predetermined incubation period after admixing the specimen.

The invention sought to be patented in a sixteenth embodiment of a process of making aspect is described as residing in the concept of preparing an essay reagent, useful, inter alia, as an aid in the clinical diagnosis of, pathological conditions to determine the presence of, quantity of, or amount of activity of lactate dehydrogenase in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: the dry substrate comprising sodium pyruvate; a dry coenzyme reduced diphosphopyridine nucleotide; a dry stabilizing-bulking agent from the class that includes polyhydric substances with from 1 to 5 hydroxyl groups per monomeric unit; and a dry buffer from the class that includes the salts of phosphate anions, organic acids and amines, and the salts of said acids and amines; which involves determining the optical density of the solid reagent after forming a liquid reagent therefrom, and again following a predetermined incubation period after admixing the specimen.

The invention sought to be patented in a seventeenth embodiment of a process of making aspect is described as residing in the concept of preparing an assay reagent, useful, inter alia, as an aid in the clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of alpha-hydroxybutyrate dehydrogenase in a biological specimen by mixing therewith a lypholized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: the dry substrate sodium alpha-ketobutyrate; a coenzyme reduced diphosphopyridine nucleotide; a dry stabilizing-bulking agent from the class that includes polyhydric substances and polymers of said polyhydric substances with from 1 to 5 hydroxyl groups per monomeric unit; and a dry buffer consisting of the alkali metal phosphates or tris (hydroxymethyl) aminomethane and succinic acid; which involves determining the optical density of the solid reagent after forming a liquid reagent therefrom, and again following a predetermined incubation period after admixing the specimen.

The invention sought to be patented in an eighteenth embodiment of a process of making aspect is described as residing in the concept of preparing an assay reagent, useful, interalia, as an aid in the clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of glucose-6-phosphate dehydrogenase in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: the dry substrates sodium salt of glucose - 6 - phosphate; a coenzyme triphosphopyridine-nucleotide; a dry stabilizing-bulking agent from the class that includes polyhydric substances and polymers of said polyhydric substances with from 1 to 5 hydroxyl groups per monomeric unit; and a dry buffer from the class that includes alkali metal phosphates or tris (hydroxymethyl) aminomethane and succinic acid; which involves determining the optical density of the solid reagent after forming a liquid reagent therefrom, and again following a predetermined incubation period after admixing the specimen.

The invention sought to be patented in a nineteenth embodiment of a process of making aspect is described as residing in the concept of preparing an assay reagent, useful, inter alia, as an aid in the clinical diagnosis of pathological conditions to determine the presence and quantity of urea in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: a dry substrate comprising alpha-ketoglutaric acid; the dry enzymes comprising urease and glutamic dehydrogenase; a dry coenzyme from a class that includes the pyridine nucleotides; preferably a dry activator; a dry stabilizer comprising dithioerythritol and sodium potassium tartrate; and a dry buffer that includes at least one of sodium or potassium phosphate, tris (hydroxymethyl) aminomethane, sodium or potassium bicarbonate, sodium or potassium carbonate and

glycine; which involves determining the optical density of the solid reagent after forming a liquid reagent therefrom, and again after admixing the specimen and allowing sufficient time for the mixture to react completely.

The invention sought to be patented in a twentieth embodiment of a process of making aspect is described as residing in the concept of preparing an assay reagent, useful, inter alia, as an aid in the clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of creatine phosphokinase in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: a dry substrate comprising creatine phosphate; the dry enzymes hexokinase and glucose-6-phosphate dehydrogenase; a dry coenzyme comprising adenosine triphosphate and triphosphopyridine nucleotide; a dry buffer capable of maintaining the pH between 6.5 and 7.5; at least one dry stabilizer selected from mucilaginous gums, hydroxyalkylamines, ethylenediamine tetraacetic acid and its salts, an inert soluble protein and a sulfate anion; and preferably dry activator; which involves determining the optical density of the solid reagent after forming a liquid reagent therefrom, and again following a predetermined incubation period after admixing the specimen.

The invention sought to be patented in a twenty-first embodiment of a process of making aspect is described as residing in the concept of preparing an assay reagent, useful, inter alia, as an aid in the clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of lactate dehydrogenase in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: the dry substrate comprising lithium lactate; a dry coenzyme diphosphopyridine nucleotide; a dry stabilizing-bulking agent comprising polyhydric substances with from 1 to 5 hydroxyl groups per monomeric unit; and a dry buffer from the class that includes the alkali metal phosphates and glycine-rhodium carbonate which involves determining the optical density of the solid reagent after forming a liquid reagent therefrom, and again following a predetermined incubation period after admixing the specimen.

The invention sought to be patented in a first composition of matter aspect, is described as residing in the concept of a substantially anhydrous solid reagent, useful, inter alia, as an aid in clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of an enzyme in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form and comprising a buffer capable of maintaining a predetermined pH of a mixture of the assay reagent and specimen being assayed; substrate(s) effective to react with the enzyme of which the present quantity of activity is to to be determined to form an intermediate product, a coenzyme capable of conversion from one form to another during use, and a second enzyme capable of catalyzing the reaction of the intermediate product(s) with the coenzyme, to form the other form of the coenzyme.

The invention sought to be patented in a second composition of matter aspect, is described as residing in the concept of a substantially anhydrous solid reagent, useful, inter alia, as an aid in clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of an unknown substance in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form and comprising a buffer capable of maintaining a predetermined pH of a mixture of the assay reagent and specimen being assayed; a substrate effective to react with the unknown substance of which the present quantity or activity is to be determined; an enzyme capable of catalyzing the reaction of the substance with the substrate to form one form to another during use; and, a second enzyme capable of catalyzing the reaction of the intermediate product and the coenzyme to form the other form of the coenzyme.

The invention sought to be patented in a third composition of matter aspect, is described as residing in the concept of a substantially anhydrous solid reagent, useful, inter alia, as an aid in clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of an enzyme in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form, and including a buffer capable of maintaining a predetermined pH of a mixture of the assay reagent and specimen being assayed, a coenzyme capable of conversion from one form to another during use, a substrate effective to react with the coenzyme catalyzed by the enzyme, of which the present quantity or activity is to be determined to form the other form of the coenzyme.

The invention sought to be patented in a fourth composition of matter aspect, is described as residing in the concept of a substantially anhydrous solid reagent, useful, inter alia, as an aid in clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of glutamate pyruvate transaminase in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: the dry substrates of alanine and alpha-ketoglutaric acid, the dry enzyme lactate dehydrogenase, at least one dry stabilizer from the class that includes mucilaginous gums, hydroxyalkylamines, ethylene diamine tetraacetic acid and its salts, an inert soluble protein and a sulfate anion, a dry coenzyme consisting of reduced diphosphopyridine nucleotide, a dry buffer from the class that includes the salts of phosphates, organic acids and amines, and the salts of said acids and amines, and at least one dry stabilizing-bulking agent from a class that includes polyhydric substances with from 1 to 5 hydroxyl groups per monomeric unit.

Each substance above is present in that quantity so as to insure a uniform rate of reaction catalyzed by the unknown being determined, or cause the reaction to go to completion. The above requirement applies to each of the following described composition aspects of the invention.

The invention sought to be patented in a fifth composition of matter aspect is described as residing in the concept of a substantially anhydrous solid reagent, useful, inter alia, as an aid in clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of glutamate oxaloacetate transaminase in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in a substantially anhydrous particulate form including the combination of: the dry substrates of aspartic acid and alpha ketoglutaric acid; the dry enzyme malate dehydrogenase; at least one dry stabilizer from the class that includes mucilaginous gums, hydroxyalkylamines, ethylenediamine tetraacetic acid and its salts, an inert soluble protein and a sulfate anion; a dry coenzyme consisting of reduced diphosphopyridine nucleotide; a dry buffer from the class that includes the salts of phosphates, organic acids and amines, and the salts of said acids and amines; at least one dry stabilizing-bulking agent from a class that includes polyhydric substances and polymers of said polyhydric substances with from 1 to 5 hydroxyl groups per monomeric unit.

The invention sought to be patented in a sixth composition of matter aspect is described as residing in the concept of a substantially anhydrous solid reagent, useful, inter alia, as an aid in clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of aldolase, in a biological speci-

men by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: a dry substrate comprising fructose-1,6-diphosphate; the dry enzymes triose phosphate isomerase and glyceraldehyde phosphate dehydrogenase; a dry coenzyme consisting of diphosphopyridine nucleotide; a dry stabilizer from the class that includes mucilaginous gums, hydroxyalkylamines, ethylene diamine tetraacetic acid and its salts, an inert soluble protein and a sulfate anion; a dry buffer from a class that includes the salts of phosphate, organic acids and amines, and the salts of said acids and amines, a dry stabilizing-bulking agent comprising polyhydric substances and polymers of said polyhydric substances with from 1 to 5 hydroxyl groups per monomeric unit.

The invention sought to be patented in a seventh composition of matter aspect is described as residing in the concept of a substantially anhydrous solid reagent, useful, inter alia, as an aid in clinical diagnosis of pathological conditions to determine the presence and quantity of glucose, in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: the enzymes hexokinase and glucose-6-phosphate dehydrogenase; the dry coenzymes consisting of triphosphopyridine nucleotide and adenosine triphosphate; a dry stabilizer from the class that includes mucilaginous gums, hydroxyalkylamines, ethylenediamine tetraacetic acid and its salts, an inter soluble protein and a sulfate anion; a dry buffer from a class that includes the salts of phosphate anions, organic acids and amines, and the salts of said acids and amines; and a dry stabilizing-bulking agent comprising polyhydric substances and polymers of said polyhydric substances with from 1 to 5 hydroxyl groups per monomeric unit.

The invention sought to be patented in an eighth composition of matter aspect is described as residing in the concept of a substantially anhydrous solid reagent, useful, inter alia, as an aid in clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of adenosine triphosphate, in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combinations of: the dry enzymes hexokinase and glucose-6-phosphate dehydrogenase; a dry substrate comprising glucose; a dry coenzyme consisting of triphosphopyridine nucleotide; a dry stabilizer from the class that includes mucilagenous gums, hydroxyalkylamines, ethylene diamine tetracetic acid and its salts, an inert soluble protein and a sulfate anion; a dry buffer from a class that includes the salts of phosphate anions, organic acids and amines, and the salts of said acids and amines; a dry stabilizing-bulking agent comprising polyhydric substances and polymers of said polyhydric substances with from 1 to 5 hydroxy groups per monomeric unit.

The invention sought to be patented in a ninth composition of matter aspect is described as residing in the concept of a substantially anhydrous solid reagent, useful, inter alia, as an aid in clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of malate dehydrogenase in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: the dry substrate comprising oxaloacetic acid; the dry coenzyme reduced diphosphopyridine nucleotide; a dry stabilizing-bulking agent from the class that includes polyhydric substances and polymers of said polyhydric substances with from 1 to 5 hydroxyl groups per monomeric unit; and a dry buffer from the class that includes the salts of phosphate anions, organic acids and amines, and the salts of said acids and amines.

The invention sought to be patented in a tenth composition of matter aspect is described as residing in the concept of a substantially anhydrous solid reagent, useful, inter alia, as an aid in clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of lactate dehydrogenase in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: the dry substrate comprising sodium pyruvate; the dry coenzyme reduced diphosphopyridine nucleotide; a dry stabilizing-bulking agent from the class that includes polyhydric substances with from 1 to 5 hydroxyl groups per monomeric unit; and a dry buffer from the class that includes the salts of phosphate anions, organic acids and amines, and the salts of said acids and amines.

The invention sought to be patented in an eleventh composition of matter aspect is described as residing in the concept of a substantially anhydrous solid reagent useful, inter alia, as an aid in clinicial diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of alpha-hydroxybutyrate dehydrogenase in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: the dry substrate sodium alphaketobutyrate; the dry coenzyme reduced diphosphopyridine nucleotide; a dry stabilizing bulking agent from the class that includes polyhydric substances and polymers of said polyhydric substances with from 1 to 5 hydroxyl groups per monomeric unit; and a dry buffer consisting of the alkali metal phosphates or tris(hydroxymethyl) aminomethane and succinic acid.

The invention sought to be patented in a twelfth composition of matter aspect is described as residing in the concept of a substantially anhydrous solid reagent useful, inter alia, as an aid in clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of glucose-6-phosphate dehydrogenase in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: the dry substrate sodium salt of glucose-6-phosphate; the dry coenzyme triphosphopyridine nucleotide; a dry stabilizing-bulking agent from the class that includes polyhydric substances and polymers of said polyhydric substances with from 1 to 5 hydroxyl groups per monomeric unit; and a dry buffer from the class that includes alkali metal phosphates or tris(hydroxymethyl) aminomethane and succinic acid.

The invention sought to be patented in a thirteenth composition of matter aspect is described as residing in the concept of a substantially anhydrous solid reagent useful, interalia, as an aid in clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of urea in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: the dry substrate alpha-ketoglutaric acid; the dry enzymes urease and glutamic dehydrogenase; a dry coenzyme from a class that includes the pyridine nucleotides; a dry stabilizer comprising dithioerythritol and sodium potassium tartrate; and a dry buffer that includes at least one of sodium or potassium phosphate, tris-(hydroxymethyl) aminomethane, sodium or potassium bicarbonate, sodium or potassium carbonate and glycine, and preferably, a dry activator.

The invention sought to be patented in a fourteenth composition of matter aspect is described as residing in the concept of a substantially anhydrous solid reagent useful, interalia, as an aid in clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of creatine phosphokinase in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of:

a dry substrate creatine phosphate; the dry enzymes hexokinase and glucose-6-phosphate dehydrogenase; and dry coenzymes adenosine triphosphate and triphosphopyridine-nucleotide; a dry buffer capable of maintaining the pH between 6.5 and 7.6; at least one dry stabilizer selected from mucilagenous gums, hydroxyalkylamines, ethylenediamine tetracetic acid and its salts, an inert soluble protein and a sulfate anion; and preferably a dry activator.

The invention sought to be patened in a fifteenth composition of matter aspect is described as residing in the concept of a substantially anhydrous solid reagent useful, inter alia, as an aid in clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of lactate dehydrogenase in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: the dry substrate lithium lactate; the dry coenzyme diphosphopyridine nucleotide; a dry stabilizing-bulking agent comprising polyhydric substances with from 1 to 5 hydroxyl groups per monomeric unit; and a dry buffer from the class that includes the alkali metal phosphates and glycine-sodium carbonate.

The invention sought to be patented in a sixteenth composition of matter aspect is described as residing in the concept of a substantially anhydrous solid reagent useful, inter alia, as an aid in clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of a substance in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: hexokinase and at least one dry stabilizer from the class consisting of acacia, ammonium sulfate, tris(hydroxymethyl) aminomethane, and its sulfate salt.

The invention sought to be patented in a seventeenth composition of matter aspect is described as residing in the concept of a substantially anhydrous solid reagent useful, inter alia, as an aid in clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of a substance in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particulate form including the combination of: triose phosphate isomerase and a stabilizer that includes one of ammonium sulfate and acacia.

The invention sought to be patented in a eighteenth composition of matter aspect is described as residing in the concept of a substantially anhydrous solid reagent useful, inter alia, as an aid in clinical diagnosis of pathological conditions to determine the presence of, quantity of, or amount of activity of an unknown in a biological specimen by mixing therewith a lyophilized, stabilized, catalytic reagent while in substantially anhydrous particlate form including the combination of: glyceraldehyde phosphate dehydrogenase and a stabilizer that includes one or more sulfate and acacia.

EXAMPLE A

The first example of the first embodiment is a solid reagent or assay material that is particularly adapted to be employed for measuring the amount of activity of glutamatepyruvate transaminase (GPT) present in a serum. This reagent when fully prepared will consist of the dry mixture of the following substances:

Enzyme: lactate dehydrogenase (LDH)
Buffer: phosphate buffer (NaH_2PO_4+Na_2HPO_4)
Stabilizer: tris-(hydroxymethyl)-aminomethane and its sulfate salt, ammonium sulfate, ethylenediamine tetraacetic acid, acacia and albumin
Substrate: alanine and alpha-ketoglutaric acid
Coenzyme: reduced diphosphopyridine nucleotide (DPNH)
Bulking agent: mannitol

In order to prepare a large number of units of this assay material or reagent, the following procedure may be employed to produce a batch of a dry assay material or reagent that may then be divided into small quantities and packaged in containers such as capsules. Wherever quantities are specified, they are suitable for preparing a batch that will yield about 10,000 capsules. However, it should be understood that these values may be varied if it is desired to produce larger or smaller batches.

The first step in the procedure is to prepare a tris-EDTA stabilizer solution. This may be accomplished by mixing the chemicals together in approximately the indicated quantities;

	G.
Tris (hydroxymethyl) aminomethane	500–1,000
Ammonium sulfate	250–500
Ethylenediamine tetraacetic acid, tetrasodium salt	150–300

In order to mix these chemicals together, the tris (hydroxymethyl) aminomethane is dissolved in a volume of water that is somewhat less than 1 liter to form a first solution. If it is necessary to assist in this dissolving, the solution may be heated in a water bath. After the tris is completely dissolved, the solution is allowed to cool to about 25° C. and adjusted to about pH 7.5. This adjustment may be accomplished by adding sulfuric acid in the necessary quantities to convert part of the tris (hydroxymethyl) aminomethane to its sulfate salt. After the solution is brought to pH 7.5, sufficient water is added to bring the total volume to 1 liter.

Following this, a second solution is prepared by dissolving the ammonium sulfate in 850 to 900 ml. of water. This solution is adjusted to pH 7.5 by the addition of ammonium hydroxide. The first and second solutions may then be mixed with each other to form a resultant solution. The ethylenediamine tetraacetic acid, tetrasodium salt, may then be added to the resultant solution and dissolved therein to form a buffer solution which is sometimes hereinafter referred to as the tris-EDTA buffer.

A dry, lyophilized powder containing the lactate dehydrogenase (LDH) enzyme is then prepared. However, before this powder can be prepared, it is necessary to determine first the amount of activity in the lactate dehydrogenase to be added.

In order to insure that the capsules produced from successive batches will be of identical activity, the activity of the LDH should be standardized at a predetermined level. By way of example, in a batch of 10,000 capsules, there may be about 20×10^6 Wroblewski units [Wroblewski, F., and LaDue, T. S., Proc. Soc. Exp. Biol. Med. 90, 210 (1955)] for the entire batch, or about 2,000 units per finished capsule. The assay may be made by employing the following chemicals in about the indicated amounts:

	Ml.
Phosphate buffer, 0.1 M, pH 7.5	1.75
Sodium pyruvate (3.3 mg./10 ml. of phosphate buffer: reagent 1)	1.00
DPNH solution, 2.5 mg./ml. of 1% $NaHCO_3$ sol'n	0.15

To begin the assay, the enzyme lactate dehydrogenase is diluted one part to 10,000 by mixing it with the phosphate buffer. A small quantity of the diluted enzyme, such as 0.1 ml. is mixed into a suitable quantity of the buffer solution containing the pyruvate and DPNH. The optical density of the mixture at a constant temperature such as 32° C. is then measured at suitable intervals such as one minute for an extended period such as ten minutes. By determining the average rate of change per minute of the optical density and multiplying by ten (if 0.1 ml. is used), the units of enzyme per milliliter of the diluted enzyme can be determined. Then, dividing by the number of milligrams of enzyme per milliliter, it will be possible to find the number of units of enzyme per milligram.

From this, the number of milligrams of the enzyme required to produce the necessary units can be calculated.

Following this, the dry, lyophilized powder containing the lactate dehydrogenase is prepared by mixing the following chemicals in the indicated ranges:

Lactate dehydrogenase (the exact amount is determined by the above assay) ____mg__	90–100
Acacia (gum arabic, white powder, U.S.P.)__g__	2–6
Albumin ____mg__	50–500
Tris-EDTA solution ____ml__	10–30
Ammonium sulfate ____g__	15–40

These chemicals may be mixed as follows. First, dissolve the acacia in a convenient quantity of distilled water, for example 80 to 160 milliliters. The albumin is then dissolved in a second quantity of water (for example, 10 milliliters). When fully dissolved, the two solutions are mixed. The tris-EDTA prepared in the beginning is then added to the solution. The solution is now placed under a vacuum for a period of time that will assure all of the trapped air in the solution being removed. The amount of the enzyme lactate dehydrogenase determined by the assay is then added to the solution. The resultant solution is then completely mixed to insure a uniform dispersion of the enzyme throughout the entire solution. The solution is then frozen and a vacuum applied to remove all of the water. The resultant lyophilized mixture will now be in a dry solid state in a quantity in the general range of about 15 to 20 grams. This dry mixture may then be mixed with the dry crystals of ammonium sulfate. The dry mixture of solids may then be pulverized into a fine powder by any suitable means such as placing it in a ball mill for several hours.

After the powder is completely mixed and pulverized to a sufficient degree, an assay may be made of the mixture to determine the units of activity of the enzyme lactate dehydrogenase in each milligram of the lyophilized powder. The next step is to distribute the lyophilized powder into the capsules. However, if it is desired to delay this step, the lyophilized powder may be stored for extended periods of time in a cold place such as a refrigerator or freezer. If this is done, it is desirable to include a drying agent near the powder to prevent the absorption of any moisture.

In order to make the capsules, the following are mixed together. Normally, the quantities will be in about the indicated ranges:

Sodium carbonate (to give pH 7.4 to 7.6) ____g__	10
Sodium phosphate, dibasic ____g__	350–1,050
D,L-alanine ____g__	1,250±10%
Alpha-ketoglutaric acid ____g__	60±10%
DPNH ____g__	6 to 8
Lactate dehydrogenase, lyophilized__units__	20×10^6

The first three compounds in the above list are first ground together to form a dry mixture. It has been found preferable to use D,L-alanine recrystallized from water only. L-alanine may be substituted for D,L-alanine, in which case, the amount used is one half that for the D,L compound. The mixture is then placed under a vacuum at a temperature of about 50° C. for a sufficient number of hours to insure all of the moisture being removed. In addition, a drying material such as phosphorous pentoxide may be included in the oven to insure a complete drying. After the foregoing drying step, the alpha-ketoglutaric acid is then added to the mixture formed by the first three compounds. The alanine and alpha-ketoglutaric acid form a substrate for the reaction that takes place when assaying the serum for GPT. Accordingly, the exact amounts of these compounds to be used are determined by the optimum amounts needed to produce a satisfactory reaction. Normally, these will be within the range indicated in the above table.

The resultant mixture of the four compounds is then pulverized into a fine powder, for example, by placing in a ball mill for several hours. When completely mixed into a homogeneous mixture, a small sample may be dissolved in water and the pH determined. If it is necessary, the pH should be adjusted so as to fall within the range of 7.4 to 7.6. If the pH is too low, additional sodium carbonate is added. If the pH is too high, a new mixture similar to the above is prepared in the same manner. However, this mixture is made definitely deficient in sodium carbonate. The second mixture is then blended with the first mixture to bring the pH into the range of 7.4 to 7.6. When the mixture is finished, it is dried again as described above. From this point forward it is important that the powder not be exposed to moisture. Generally, it is best that if the powder is to be exposed to the atmosphere, the relative humidity should be below 15%.

The pulverized DPNH and the lyophilized powder containing the lactate dehydrogenase may then be added to the powder mixture just prepared above. Before the DPNH is added, it is dried under vacuum in the presence of phosphorous pentoxide to remove moisture. At the time that the lyophilized powder is prepared, the amount of activity of the lactate dehydrogenase is assayed and a quantity of LDH is computed that would produce a total of 20×10^6 Wroblewski units for the entire batch (if 10,000 capsules are to be prepared), or 2,000 units per capsule or single assay portion. Accordingly, the quantity of the LDH to be blended into the mixture will be determined from that assay. The quantity of DPNH to be employed is chosen to produce on optical density that is compatible with spectrometers that are suitable for measuring the optical density during the reaction. Normally, the quantity of DPNH will be such as to produce an optical density of the order of 0.8 at 340 millimicrons. This may be checked by dissolving an appropriate quantity of the powder in water (for example, about 4 mg. per 5 ml.) and measuring the optical density.

At this point, a powder is provided that contains the enzyme lactate dehydrogenase, the coenzyme DPNH, and all of the buffers, substrates and stabilizers, to insure the powder being in a stable state that will have a very long shelf life. A sample of this powder is withdrawn (being careful not to introduce moisture) and tested. The tests are for the appropriate optical density, homogeneity with respect to the DPNH, and the effectiveness of the powder to form an assay for glutamate-pyruvate transaminase. Accordingly, this powder may be divided into a plurality of small parts that are just large enough to contain the desired 2,000 Wroblewski units and appropriate quantities of buffer, DPNH, L-alanine and alpha-ketoglutarate to perform a GPT determination in 3 ml. Each part may then be enclosed in a suitable package such as a capsule that will protect the mixture from any moisture.

If a pharmaceutical gelatin capsule is used, a desiccant such as an alumina pellet should be placed with the capsule within the package.

Normally, between successive batches, there will be some variations in the size of the parts into which the mixture is divided as a result of variations in the activity of the LDH. In order to make all of the parts of identical size in all batches, a standard package size in excess of the largest size that will be required is chosen. A suitable quantity of a bulking and stabilizing agent such as mannitol may then be added to the mixture to bring its volume up to an amount that will insure each of the parts having a volume that will just fill the package.

In order to make an assay of a serum for glutamate-pyruvate transaminase or GPT using a capsule of this example, a specimen of the serum is first obtained in a quantity such as 0.1 milliliter. Following this, the contents of a capsule of this type are dissolved. The resultant solution will form a liquid reagent that is of just the right size to make a single assay and may be mixed directly with the specimen. As soon as the reagent

and specimen serum are mixed together, the following reactions will occur:

$$\text{alpha-ketoglutarate} + \text{alanine} \xrightarrow{\text{GPT}} \text{glutamic acid} + \text{pyruvate}$$

$$\text{pyruvate} + \text{DPNH} \xrightarrow{\text{LDH}} \text{lactate} + \text{DPN}$$

Since the alpha-ketoglutarate, alanine and DPNH are supplied in the capsule in excess of that required for the reactions, the only limiting factor is the activity of GPT. The pyruvate formed will thus be proportional to the GPT. When the pyruvate reacts with DPNH, it will be converted to DPN in direct proportion of the pyruvate and, therefore, to the GPT. By placing the assay mixture in a suitable spectrophotometer and measuring the optical density at 340 millimicrons, the rate at which DPNH is converted can be measured. This, in turn, will permit the amount of GPT originally present to be determined.

EXAMPLE B

The second example of the first embodiment is a solid reagent that is particularly adapted to be employed for measuring the amount of activity of glutamate-oxaloacetic transaminase, or GOT, present in the serum. This reagent when fully prepared will consist of a dry mixture containing the following substances:

Enzyme—malate dehydrogenase (MDH)
Buffer—disodium hydrogen phosphate
Stabilizer—acacia, and tris (hydroxymethyl)-amino methane and its sulfate salt, ammonium sulfate and ethylenediamine tetraacetic acid
Substrate—aspartic acid and alpha-ketoglutaric acid
Coenzyme—reduced diphosphopyridine nucleotide (DPNH)
Bulking agent—mannitol

In order to prepare a large number of units of this reagent, the following procedure may be employed to produce a batch of dry reagent that may then be divided into a large number of small quantities and packaged into capsules. Wherever quantities for a substance are specified, the quantities are suitable for preparing a batch of reagent that will yield about 10,000 capsules. It should be understood, however, that these values are for illustrative purposes only and may be varied to satisfy the demands of the particular application to which the reagent is to be used.

The first step in preparing such a batch is normally to prepare a tris-EDTA stabilizer. This buffer is a mixture of tris (hydroxymethyl) aminomethane and its sulfate salt, ammonium sulfate, and ethylenediamine tetraacetic acid, tetrasodium salt, and may be prepared in the same manner described in Example A.

Following this, a dry, lyophilized powder containing the malate dehydrogenase or MDH is prepared. This may be accomplished by employing the following chemicals in the indicated ranges:

Malate dehydrogenase	units	$3–6\times10^6$
Gum acacia	gm	2–10
Tris-EDTA buffer	ml	1–4

The acacia may first be dissolved in a suitable quantity of water such as about 50 milliliters. This solution may then be mixed with the malate dehydrogenase solution and the tris-EDTA solution. After the solution has been thoroughly mixed, it may be placed under a vacuum for a sufficient interval of time to remove any entrapped air. Following this, the solution may be frozen and placed under a vacuum until all of the moisture is removed therefrom. This will produce a dry lyophilized powder which contains the enzyme malate dehydrogenase together with stabilizers which will be effective to maintain the activity of the malate dehydrogenase at its desired level.

After the lyophilized powder containing the malate dehydrogenase has been prepared, it may be assayed to determine the amount of activity of enzyme malate dehydrogenase present in the powder. This may be accomplished by employing a reagent containing the following chemicals in the indicated quantities:

	Ml.
Oxaloacetic acid, 1 mg./ml.	0.1
DPNH, 2 mg./ml.	0.1
Phosphate buffer, 0.1 M, pH 7.5	2.7

After the foregoing solutions have been prepared in the indicated concentrations, they may be all mixed together to form a liquid reagent suitable for performing the present assay. A suitable quantity of the lyophilized powder containing the enzyme malate dehydrogenase may be dissolved in water. This may be accomplished by dissolving about 20 milligrams of powder in about 20 milliliters of water. About 0.02 milliliter of this solution may then be combined with the reagent. When this mixture is made, the DPNH will immediately begin to be converted into DPN. While this reaction is occurring, the solution may be placed in a suitable spectrophotometer and the optical density in the reagent measured at suitable increments of time, for example, every minute. After several readings have been taken, an average may be provided which will indicate the amount of activity of the malate dehydrogenase in the lyophilized powder. A change in the optical density of 0.001 per minute indicates an activity of 1 unit. From this, it will be possible to calculate the amount of the lyophilized powder containing the malate dehydrogenase that is required to produce an activity in the ranges of about 8 to 20×10^6 units.

Following this, the dry lyophilized powder containing the malate dehydrogenase may be prepared for packaging in a capsule. This may be accomplished by employing the following chemicals in the indicated ranges:

L-aspartic acid	gm	220–260
Alpha-ketoglutaric acid	gm	50–60
Disodium hydrogen phosphate	gm	200–600
Sodium carbonate	gm	140–280
Mannitol	gm	800–2000
DPNH	gm	6–8
Lyophilized powder	units	$8–20\times10^6$

The alpha-aspartic acid, alpha-ketoglutaric acid, disodium hydrogen phosphate and sodium carbonate may all be mixed together and ground into a fine powder by any suitable means such as placing in a ball mill for about 6 to 10 hours. Following the mixing, a sample such as about 100 milligrams of the mixture may be dissolved in a suitable quantity of water such as 5 milliliters. The pH of this solution should be between 7.4 and 7.6. If the pH is too low, a suitable quantity of sodium carbonate may be added to raise the pH to the desired level. If the pH is too high, additional quantities of alpha-aspartic acid and alpha-ketoglutaric acid may be added. The ball milling may be repeated—the powders are mixed with mannitol by ball milling or the like, and then dried at 40° C. under vacuum in the presence of phosphorous pentoxide.

When the foregoing powders have been blended to provide a pH in the desired range from this point forward it is important that the powder not be exposed to moisture. Generally, it is best that if the powder is to be exposed to the atmosphere, the relative humidity should be below 15%. The DPNH and the calculated quantity of lyophilized powder containing malate dehydrogenase may be added to the mixture. The amount of DPNH added is such that one ten-thousandth of the resulting mixture when dissolved in 3 milliliters of water will have, for convenience of measurement, an optical density at 340 millimicrons of 0.8 to 1.0. This mixture may then be ground in a ball mill.

At this point, a powder is provided that contains the enzyme malate dehydrogenase, together with the stabilizers acacia, tris-EDTA, ammonium sulfate and ethylenediamine tetra-acetic acid so as to insure the powder be-

ing very stable and having a very long shelf life wherein the enzyme activity will not deteriorate. In addition, the powder includes a buffer-disodium hydrogen phosphate and substrates alpha-aspartic acid and alpha-ketoglutaric acid that will insure the desired reaction occurring when an assay is made. A sample of this powder is withdrawn (being careful not to introduce moisture) and tested. The tests are for the appropriate optical density, mixing of the DPNH, and the effectiveness of the powder to form an assay for glutamate-pyruvate transaminase. Accordingly, this powder may be divided into a plurality of small parts that are just large enough to form a liquid reagent suitable for making one assay of a specimen. The entire bulk of the powder may then be divided into units or parts which contain the desired amount of activity. Each of these parts may then be packaged in a suitable container such as a capsule. If a pharmaceutical gelatin capsule is used, a desiccant such as an alumina pellet should be placed with the capsule within the package. It is desirable that the same size capsule be employed at all times irrespective of which batch the capsule is produced from. Accordingly, the capsule may be of a sufficiently large size to insure packaging of all variations. A bulking agent such as mannitol may then be added to each of the parts so as to bring its volume up to an amount that will just fill the capsule.

In order to use one of the capsules to make an assay of a serum for the glutamic oxaloacetate transaminase (GOT), a specimen of a serum or other biological fluid is first obtained in a suitable quantity. Following this, a capsule of this example is dissolved in a suitable quantity of water. The resultant solution will form a liquid reagent that is of just the right size and strength to make a single assay of the serum. Accordingly, this liquid reagent may then be mixed with the specimen. As soon as the reagent and specimen are mixed together, the following reactions will occur:

$$\text{alpha-ketoglutarate+aspartate} \xrightarrow{\text{GOT}} \text{glutamate+oxaloacetate}$$

$$\text{oxaloacetate+DPNH} \xrightarrow{\text{MDH}} \text{malate+DPN}$$

Since the alpha-ketoglutarate, aspartic acid and malate dehydrogenase are supplied in the capsule in sufficient amounts, the rate of the foregoing reactions will only be limited by the amount of activity of glutamic oxaloacetate transaminase or GOT originally present. This, in turn, will cause the DPNH to be converted to DPN in direct proportion to the amount of activity of the GOT originally present. By placing the specimen in a suitable spectrophotometer and measuring the optical density at 340 millimicrons, the rate at which the DPNH is being converted can be determined. This, in turn, will permit the amount of activity of the GOT to be calculated.

EXAMPLE C

The third example of the first embodiment is a solid reagent that is particularly adapted to be employed for measuring or assaying the amount of activity of aldolase present in a serum. This reagent when fully prepared will consist of a dry mixture of the following substances:

Enzyme: triosephosphate isomerase and glyceraldehyde-phosphate dehydrogenase
Buffer: glycine and sodium pyrophosphate
Stabilizer: acacia, albumin, ethylene diamine tetra-acetic acid
Substrate: fructose-1,6-diphosphate
Coenzyme: DPN
Decoupler: sodium arsenate
Bulking agent: mannitol

In order to prepare large numbers of units of this reagent, the following procedure may be employed to produce a batch of a dry reagent or assay powder that may then be divided into small quantities and packaged into capsules. Wherever quantities are specified in this example, they are suitable for preparing a batch that will yield on the order of about 10,000 capsules. It should be understood, however, that these values are for illustrative purposes only and may be varied to satisfy any particular requirement.

Normally, the first step is to prepare a standard liquid assay system which may be used at several different points in the procedure. The assay liquid includes the following:

Glycine-pyrophosphate buffer, containing 998 mg. of glycine and 181 mg. of sodium pyrophosphate decahydrate per 100 ml.	ml. 1.0
Sodium acid arsenate, containing 1.63 g. per 100 ml.	ml. 1.0
Fructose-1,6-diphosphate, sodium salt, containing 30 mg. per 5 ml.	ml. 0.5
Ethylene diamine tetra-acetic acid, tetrasodium, 100 mg. per 10 ml.	ml. 0.1
DPN, 12 mg. per ml.	ml. 0.1
Triosephosphate isomerase (Boheringer), 2 mg. per ml.	μl. 5
Glyceraldehydephosphate dehydrogenase (Boheringer), 10 mg. per ml.	μl. 15
Distilled water to bring volume to 3 ml.	

All of the foregoing liquids are mixed together to form the standard liquid assay. This solution may then be allowed to stand for a relatively short time such as several minutes. After the standard liquid assay reagent has been completed, a 1 to 400 dilution of the enzyme aldolase is prepared by diluting 5 lambdas of the concentrated aldolase Boheringer, 10 mg. per ml., to 2 milliliters with a 2 molar ammonium sulfate solution. 5 lambdas of the diluted enzyme are then added to a part of the standard liquid. In order to obtain an accurate indication, it is necessary the enzyme be at its maximum activity at the time of the test. Accordingly, it is desirable that this solution be prepared immediately prior to the making of the test.

After the solution containing the enzyme has been added to the standard assay system, the resultant mixture is allowed to incubate for some interval of time such as 5 minutes. The optical density of the solution is then measured at 340 millimicrons by means of a suitable spectrophotometer. Thereafter, the optical density is repeatedly measured at this wavelength periodically for some predetermined interval of time such as 3 minutes. By recording these readings, a reference indicating the effectiveness or amount of activity in the standard assay liquid will be provided. Future assays in the present process may then be compared with this reference to determine whether they are within acceptable ranges.

Following this, an assay powder may be prepared by mixing the following chemicals in about the indicated ranges:

Glycine	g.	90–180
Sodium pyrophosphate	g.	15–35
Sodium arsenate	g.	125–250
Fructose-1,6-diphosphate	g.	27–35
Ethylene diamine tetra-acetic acid	g.	8–20
DPN	g.	10–18
Triose phosphate isomerase	mg.	80–200
Glyceraldehyde phosphate dehydrogenase	g.	1.2–2.0
Albumin	g.	30–80
Acacia USP	g.	40–90

The first step in preparing this powder is to mix the sodium pyrophosphate and glycine together to form a buffer powder. This mix is first ground into a fine powder in any suitable means such as a mortar and pestle. The buffer powder may then be placed under a vacuum in the presence of a moisture absorbing agent for a sufficient period of time to insure any moisture being removed. Following the drying, the buffer powder may be further pulverized by placing in a ball mill for an extended period of time. A small sample of the buffer may be

dissolved in water and the pH of the solution measured. The pH of this solution should be about 8. If it is not sufficiently close to this, the required quantities of either salt may be added to the buffer to adjust the pH to the required level and the grinding repeated.

The next step is to grind the sodium arsenate into a fine powder and add it to the glycine-sodium pyrophosphate buffer. The resultant combination is then ball milled to form a fine powder and dried under a vacuum for an extended period of time. After this powder has been prepared, it is desirable to test the powder to make sure that there are no inhibitors which may impair the operation of the assays. This determination may be made by preparing a solution similar to the standard liquid assay. However, the just prepared powder is substituted for the first pair of chemicals in the list for the standard assay.

The same dilution of the aldolase is then mixed with the new essay liquid and the resultant changes in the optical density at 340 millimicrons measured as described in connection with the standard. If the optical density varies, the same as the reference, the preparation of the lyophilized enzymes triose phosphate isomerase and glyceraldehyde phosphate dehydrogenase may proceed.

The next step will then be to dissolve the acacia into about one liter of distilled water. The ethylene diamine tetra-acetic acid and the albumin are then added to the solution and the solution thoroughly mixed. It should be noted that during the mixing a certain amount of foaming may occur. Steps should be taken to keep the foaming to a minimum. After the solution is completed, it should be placed under a vacuum for a sufficient period of time to remove any air that may still be trapped in the solution.

When the solution has been cleared of all air, the enzymes triose phosphate isomerase and glyceraldehyde phosphate dehydrogenase are added to the solution. The solution should be very thoroughly mixed to insure a uniform dispersion of the enzymes throughout all portions of the solution. Since these enzymes in this solution tend to be somewhat unstable and lose their activity, the solution should be frozen as soon as they are properly suspended in the liquid.

The frozen solution is then placed under a vacuum and all of the water removed therefrom.

After the enzyme mixture has been lyophilized in the foregoing manner, a sample should be assayed to determine if they still have adequate activity. This assay is made by comparing the activity of the powder with the activity of the standard liquid assay described in the beginning of this example. To accomplish this, a new assay solution is prepared by substituting a solution prepared from the lyophilized powder for the enzyme solutions. The optical density should vary in the same manner as the reference. It is desirable that this powder be stored under refrigeration and kept as dry as possible until the process is to be continued.

If the results of the foregoing assay are within acceptable limits, the buffer arsenate mixture and the lyophilized powder containing the enzymes may be mixed together. Following this, the fructose-1,6-diphosphate and the DPN are added to the mixture and the entire mixture pulverized to a very fine powder by any suitable means such as ball milling. The resultant powder will constitute, in bulk, a dry powdered reagent containing the enzymes triosephosphate isomerase and glyceraldehydephosphate dehydrogenase and the coenzyme DPN together with stabilizers that will maintain the activity of the enzymes for extended periods of time. In order to make this solid reagent into a form that is more readily usable, it may be divided into units that are of a size suitable for assaying a single specimen. These units may then be enclosed in a suitable package for preventing the absorption of any moisture. Since there may be some variations in the volumes of the units between successive batches, it may be desired to add a bulking agent such as mannitol to each batch that will bring the volume of each unit up to some predetermined volume. The mannitol likewise aids in conferring additional stability to the reagent. The bulked powder may then be packaged in suitable containers such as capsules.

In order to make an assay of a serum for aldolase using one of the foregoing capsules of this example, a suitable sample of the serum or biological fluid is first obtained. Following this, the contents of one of the capsules is dissolved into a standard amount of water such as 3 to 5 milliliters. This will produce an active liquid reagent that is of just the right size to make a single assay of one specimen of the foregoing size. Furthermore, the activity of this reagent will be of a predetermined level. The liquid reagent may then be mixed directly with the specimen in a suitable test tube. As soon as the reagent and specimen or serum are mixed together, the following reactions will occur:

$$\text{fructose-1,6-diphosphate} \xrightarrow{\text{aldolase}} \text{3-hydroxyacetone phosphate} + \text{3-glyceraldehydephosphate}$$

$$\text{3-hydroxacetonephosphate} \xrightarrow{\text{triosephosphate isomerase}} \text{3-glyceraldehydephosphate}$$

$$\text{3-glyceraldehydephosphate} + \text{DPN} \xrightarrow[\text{dehydrogenase}]{\text{glyceraldehydephosphate}} \text{3-phosphoglycerate} + \text{DPNH}$$

$$\xrightarrow[\text{AsO}_4]{}$$

Since the fructose-1,6-diphosphate is supplied by the capsule, it will be present in an abundant quantity. As a result, the rate at which it is converted directly into 3-glyceraldehyde phosphate will be limited only by the amount of activity of the aldolase. In addition, since the triosephosphate isomerase is supplied in abundant quantities, the rate at which the 3-hydroxyacetonephosphate is produced and converted into the 3-glyceraldehyde phosphate will be limited only by the amount of activity of the aldolase. Since the rate at which the 3-glyceraldehydephosphate is produced is directly related to the amount of activity of the aldolase originally present in the specimen, the rate at which the DPN is converted to DPNH will also be determined by the aldolase. By placing the assay-reagent-specimen solution in a spectrophotometer while the foregoing reactions are progressing, the rate at which the DPNH is being produced may be determined by measuring the rate of change of the optical density of the specimen at 340 millimicrons. Knowing this rate of change, by employing well-known equations, the amount of aldolase originally present in the specimen may be computed.

EXAMPLE D

The fourth example of the first embodiment in this group is a solid reagent that is particularly adapted to be employed for measuring the quantity of glucose present in a serum. This reagent when fully prepared will have the following components that include the indicated chemicals:

Enzyme: hexokinase and glucose - 6 - phosphate dehydrogenase
Buffer: tris(hydroxy)methylaminomethane succinate
Stabilizer: acacia, tris(hydroxy)methylaminomethane sulfate and ammonium sulfate
Substrate: none
Accelerator: insulin and magnesium sulfate
Coenzymes: adenoaine triphosphate and triphospho-pyridine nucleotide
Bulking agent: mannitol

In order to prepare a large number of capsules or individual assay portions of this reagent, the following procedure may be employed to produce a batch of dry reagent that may then be divided into small quantities and

packaged into capsules. Wherever quantities are specified in this example, they are suitable for preparing a batch that will yield about 10,000 capsules. It should be understood, however, that these values are for illustrative purposes only and may be varied to satisfy any particular requirement.

The first step in this procedure is to prepare a suitable buffer solution. This buffer solution may be prepared by mixing the following chemicals together in about the indicated quantities:

	Grams
Tris (hydroxymethyl) aminomethane	100–200
Succinic acid	30–70

Both of these chemicals are mixed together and then ground into a fine powder by any suitable means such as placing in a ball mill for a period of about eight hours. The pH of the resultant mixture may then be checked by disolving a small quantity in distilled water and measuring the pH. If the pH is not in a range of about 7.4 to 7.6, more succinic acid may be added to lower it and more tris (hydroxymethyl) aminomethane added to raise it. It may be seen that the exact proportions of the constituents of this buffer are determined by the pH. When the buffer is properly powdered and the pH is within the indicated range, the mixture may be dried under vacuum at a temperature of about 50° C. in presence of phosphorous pentoxide (P_2O_5) for a period of 24 hours or until the moisture is removed.

A dry lyophilized powder containing the enzymes hexokinase and glucose-6-phosphate dehydrogenase may then be prepared by mixing the following chemicals in the indicated ranges:

Gum acacia	gms.	50–100
Tris-sulfate buffer 0.2 M, pH 7.4 to 7.6		
ammonium sulfate, 1 M, pH 7.5	ml.	75–150
(Adjusted with ammonia)	ml.	75–150
Insulin	mg.	50–750
Hexokinase	mg.	100
Glucose-6-phosphate dehydrogenase	mg.	100

The tris-buffer is first prepared by making a solution containing tris (hydroxymethyl) aminomethane and adding a sufficient quantity of sulfuric acid to make a 0.2 molar solution with a pH of 7.4 to 7.6. The acacia, ammonium sulfate and insulin are then mixed with the tris-sulfate solution to form a homogenous solution. The resultant solution is then placed under a vacuum for a sufficient period of time to remove all of the air entrapped therein. The hexokinase and glucose-6-phosphate dehydrogenase are then mixed into the solution to obtain a homogeneous mixture. The solution is then frozen and placed under a vacuum until all of the water is removed. This will result in a dry or lyophilized homogeneous powder containing the enzymes.

Following the drying thereof, the lyophilized powder may be assayed to determine the amount of activity of the hexokinase and glucose-6-phosphate dehydrogenase enzymes by employing the following procedure and using the indicated quantities of the following chemicals:

Tris-succinate buffer solution (1.7 g./100 ml.)	ml.	1
Magnesium sulfate, 0.1 molar solution	ml.	0.4
Triphosphopyridine nucleotide,		
10 mg./2 ml. water	ml.	0.2
Adenosine triphosphate, 9 mg./2 ml. water	ml.	0.2
Lyophilized enzymes	mg.	10
Water	ml.	1.2

The tris-succinate buffer solution is first formed by dissolving 1.7 grams of the buffer prepared in the first step of this example in 100 milliliters of water. The rest of the listed solutions are mixed with the buffer and the water to form a homogeneous mixture. The optical density of this solution is then measured at 340 millimicrons. A predetermined quantity of a glucose solution of known concentration is then added to the foregoing solution. This will cause a reaction wherein the TPN will be converted to TPNH. Since the TPN will be present in an abundant supply, the only factor which will limit the reaction is the quantity of glucose. After the reaction has been completed, the optical density of the solution is again measured at 340 millimicrons. The above assays are preferably made by employing several different standard solutions of glucose, for example, 200, 400 and 600 mg. percent of glucose.

The difference between the optical densities before and after the reaction should be equal to 0.230 times the mg. percent of the standard glucose solution divided by 100. In the event the difference is not equal to or very close this predicted value, the enzymes do not have an adequate amount of activity. However, of the changes of optical density are equal to or very close to the predicted value, the enzymes are sufficiently active and the mixture may be prepared for packaging into the capsules. This may be accomplished by employing the following chemicals in the indicated ranges:

	G.
Tris-succinate buffer (prepared above)	100–250
Magnesium sulfate, n-hydrate [1]	80–120
TPN	12–18
ATP	10–18
Lyophilized enzyme mixture	80–200

[1] An equivalent amount of magnesium may be added as the glutamate or aspartate salt.

All of these chemicals are dry solids that may be mixed directly together and then grounded by any suitable means such as a ball mill. At the completion of the grinding, the chemicals will have been properly ground to form a fine powder and have been thoroughly blended into a homogeneous mixture. This mixture forms the assay powder and contains the enzymes hexokinase and glucose-6-phosphate and dehydrogenase together with the stabilizers acacia, tris sulfate buffer and ammonium sulfate to insure the enzymes maintaining their activity for extended periods of time. In addition, this assay powder also contains the coenzymes ATP and TPN together with the buffer tris succinate to insure the desired assay reactions occurring when an assay is being made. Accordingly, this powder may now be divided into units which are of just the right size for performing a single assay of a single specimen. In order to determine the size of the units, an assay may be made to determine the amount of activity of the enzymes in the mixture. This assay may be accomplished in substantially the same manner as described above in connection with the assaying of the lyophilized powder containing the enzymes.

More particularly, several small specimens of the mixture may be dissolved in suitable quantities of water to form liquid reagents. The same standard values of glucose (i.e., 200, 400 and 600 mg. percent of glucose) may be mixed with the reagents and the resultant changes in optical density measured. From the results of these tests, the amount of activity of the enzymes contained in the powder can be determined.

When using the assay powder in performing an assay for glucose, the resultant reaction is allowed to continue until all of the glucose has been converted. The amount of time that is required for this conversion to be completed is determined by the amount of activity of the enzymes. Therefore, in order to determine the amount of activity to be included in each capsule, it is first necessary to determine the amount of time to be allowed for the reaction to be completed. To do this, a convenient test period or interval is arbitrarily selected, for example, 5 minutes. Some fractional part of this interval, for example, 3 minutes, is then selected during which the reaction is virtually completed. This will guarantee the reaction being completed at the end of the test period. The quantity of assay powder required for each capsule is then determined. With this quantity of activity in each capsule, it may be safely assumed that the reaction has been complete prior to the end of the test interval.

If it is desired that all of the capsules be of a standard size, the capsule size may be made larger than the largest units that may be encountered. A bulking agent such as mannitol may then be added to the assay powder so that the resultant units will just fill the capsule.

In order to make an assay of a serum for glucose using a capsule of this example, a specimen of the serum is first obtained in a suitable quantity for making an assay. The optical density of this specimen is first measured at 340 millimicrons. Following this, the contents of one of the capsules may be dissolved in a suitable quantity of water to thereby form a liquid reagent that is of the right size and activity for making one assay. This liquid reagent may then be combined with the serum so that the following reactions will occur:

$$\text{glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-P} + \text{ADP}$$

$$\text{glucose-6-P} + \text{TPN} \xrightarrow{\text{glucose-6-phosphate dehydrogenase}} \text{6-phosgluconate} + \text{TPNH}$$

As these reactions occur, the glucose that was originally present in the serum will be converted into glucose-6-P. This, in turn, will react with the TPN to produce the TPNH. Since the capsule supplies an excess of its chemicals, the only factor that limits the reaction is the quantity of the glucose originally present. Therefore, the amount of TPNH finally produced will be a function of the original quantity of glucose.

The foregoing reaction are allowed to continue for the duration of the test period or until they have been completed and the glucose is exhaused. After the reactions have been completed, the optical density of the specimen is measured again at 340 millimicrons. Since the TPNH will absorb the light at this wavelength, the difference in the optical density before and after the reactions will be a result of the change in the amount of the TPNH produced. Since the change in the quantity of TPNH will be a direct function of the quantity of glucose originally present in the specimen, the change in optical density may be employed to calculate the glucose originally present.

EXAMPLE E

The last exemple of the first embodiment in this group is a solid reagent that is particularly adapted to be employed for measuring the amount of activity of adenosine triphosphate or ATP present in a biological sample. This reagent fully prepared will consist of a dry mixture of the following solid substances:

Enzyme: hexokinase and glucose-6-phosphate dehydrogenase
Buffer: tris (hydroxymethyl) aminomethane succinate
Stabilizer: acacia, tris (hydroxymethyl) aminomethane and ammonium sulfate
Substrate: glucose
Accelerator: insulin and magnesium sulfate
Coenzyme: TPN
Bulking agent: mannitol

In order to prepare a large number of capsules of this reagent, the following procedure may be employed to produce a batch of dry reagent that may then be divided into small quantities and packaged into suitable capsules. Wherever quantities are specified in the example, they are suitable for preparing a batch that will yield on the order of 10,000 capsules. It should be understood, however, that whenever desired, these quantities may be varied to satisfy any particular requirements.

The first step in this procedure is to prepare a suitable buffer solution. The buffer employed is substantially identical to the tris buffer used in Example D and consists of a mixture of tris (hydroxymethyl) aminomethane and succinic acid. These chemicals may be mixed in substantially the same manner as described in Example D to produce a dried buffer powder that when dissolved in water wil produce a solution having a pH between 7.4 and 7.6.

A dry lyophilized powder containing the enzymes hexokinase and glucose-6-phosphate dehydrogenase may then be prepared by combining the following chemicals in the indicated ranges:

Gum acacia	gms	50–100
Tris-sulfate buffer 0.2 M pH 7.4 to 7.6 ammonium sulfate 1 M pH 7.5	ml	75–100
(adjusted with ammonia)	ml	75–100
Insulin	mg	50–750
Hexokinase	mg	100
Glucose-6-phosphate dehydrogenase	mg	100

The tris-sulfate solution is prepared first by dissolving tris (hydroxymethyl) aminomethane and adding a sufficient quantity of sulfuric acid to make a 0.2 molar solution with a pH 7.4 to 7.6.

The acacia, ammonium sulfate and insulin are completely mixed with the tris-sulfate to form a homogenous solution. After the solution is completely mixed, any air entrapped therein may be removed by placing the solution under a vacuum. The enzymes hexokinase and glucose-6-phosphate dehydrogenase are mixed into this solution. This solution should be thoroughly mixed to insure the enzymes being completely dispersed therein. While uniformly mixed, the solution is competely frozen. While in the frozen state, it is placed under a vacuum until all of the moisture is removed. This will leave a homogeneous dry powder containing the enzymes.

This lyophilized powder may then be assayed to determine the amount of activity of the enzyme present by employing the following chemicals in the indicated amounts to form a reagent:

Glucose solution 60 μM/ml. (1.08 g. in 100 ml.)	ml	0.5
Tris-succinate buffer solution	ml	1.0
Magnesium sulfate n hydrate 1 g./10 ml.	ml	0.4
TPN 10 mg./1 ml. water	ml	0.2
Lyophilized enzyme mixture	mg	10
Water	ml	1.2

The tris-succinate buffer solution is prepared by dissolving 3.4 grams of the buffer prepared in the beginning of this example in 100 milliliters of water. Following this, the indicated quantities of the rest of the solutions are combined and mixed with the indicated quantity of water. After the reagent has been completed, its optical density at 340 millimicrons is measured. Following this, a standard adenosine triphosphate solution containing 2 μM/ml. may be added to the mixture. When this occurs, the reaction subsequently described will occur. However, the solution is allowed to stand for an adequate period of time to insure that the reaction has been completed. After the completion of the reaction, the optical density at 340 millimicrons is again measured. By comparing the optical densities, the amount of change produced as a result of the reaction can be determined.

Under the foregoing test conditions, 1 μM of the ATP in ml. should produce an optical density change at 340 millimicrons of 2.07. Accordingly, the 0.2 μM of the standard solution employed in this assay should produce a change in optical density of 0.414. If such a change is produced by the foregoing assay, the enzymes have sufficient activity to continue with the preparation of the described reagent.

To prepare the lyophilized powder for encapsulation, the following chemicals may be combined in the indicated ranges:

	G.
Tris-succinate buffer (prepared above)	100–250
Magnesium sulfate, heptahydrate	80–120
TPN	12–18
Glucose, anhydrous	25–100
Lyophilized enzyme mixture	80–200

All of these chemicals are in the form of a dry powder and may be directly mixed together. The mixture is then reduced to a fine powder by any suitable means such as placing in a ball mill for an extended period of time usually 24 hours. At this point, the powder is ready for dividing into suitable units and packaged into capsules. In order to determine the size of each unit, the activity of the powder must first be determined. This is accomplished by dissolving a small sample of the specimen in a suitable quantity of water such as 2.9 ml. The assay test described above may then be repeated. By measuring the change of optical density produced by the assay reaction, the amount of activity of the enzymes in the powder may be determined. When this powder is dissolved in water, it will make a reagent that will cause a reaction involving the adenosine triphosphate (ATP) present. This reaction will continue until all of the ATP has disappeared. The amount of time required for this to occur will be determined by the amount of activity of the enzymes present. Accordingly, in order to compute the amount of the assay powder to be placed in each capsule, an interval convenient for making the test must first be chosen. For example, this may be 5 minutes. The size of the units into which the assay powder is divided will then be such that the reaction will be virtually completed in some fraction of the test period, for example 3 minutes.

It is desired to have all of the capsules of some predetermined size, a standard capsule size is selected that will always accommodate the largest unit of powder. After the size of the units is determined, a suitable quantity of a bulking and stabilizing agent such as mannitol or its equivalent may be added to bring the volume to just the right size to fill the standard-sized capsule.

It may thus be seen that a large number of identical capsules will be produced that are suitable for assaying the amount of adenosine triphosphate (ATP) present in a serum. The stabilizers tris-sulfate, acacia and ammonium sulfate will be effective to stabilize the enzymes and preserve their activity for extended period of time. The tris-succinate buffer and the glucose substrate and TPN coenzyme will insure the assay reaction occurring.

To use one of the capsules to assay a serum to determine the amount of activity of adenosine triphosphate present in a serum, a suitable sample of the serum is first obtained. The optical density of the sample diluted with water to the same volume as the assay is first determined at a wavelength of 340 millimicrons. The contents of one of the capsules is then dissolved in a standard quantity of water such as 3 milliliters. This will create a liquid reagent of the correct size and activity for making an assay of one serum sample. This liquid reagent may then be combined with the sample of serum whereby the following reactions will occur:

$$\text{glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-P} + \text{ADP}$$

$$\text{glucose-6-P} + \text{TPN} \xrightarrow{\text{glucose-6-phosphate dehydrogenase}} \text{6-phosgluconate} + \text{TPNH}$$

As these reactions progress, the ATP will disappear and the TPN will be converted to TPNH. Since the components supplied by the reagent are in abundant supply, the only thing that will stop the reaction will be the exhausting of the ATP originally present. It may thus be seen that the amount of TPN converted into TPNH will be a function of the quantity of the ATP.

The reactions are allowed to continue for the duration of the test interval. However, long before the expiration of this interval, the reactions will have been completed. After the reactions have been completed or at the end of the test interval, the optical density of the sample is measured again at 340 millimicrons. Since the TPNH will absorb light at this wavelength, the difference between the otpical densities before and after the reactions will be a result of the changes in the amount of the TPNH produced. Since the change in the quantity of TPNH is a function of the quantity of ATP originally present in the specimen, this quantity of ATP can be computetd from the change in the optical density.

In the other embodiment of the invention, an assay material is provided that is very similar to the assay material in the previously-described embodiment. In this embodiment, the assay material is also a dry solid suitable for prepackaging into containers such as capsules or foil wrappers containing just the right quantity for making a single assay or predetermined number of assays. The assay material contains all of the components, except water, for making an assay. The contents of one package is dissolved in water to form a liquid reagent effective to create an enzymatic reaction of the same type created by the preceding embodiment.

The assay materials in this embodiment are particularly adapted for measuring the amount of activity of an enzyme originally present in the specimen. Accordingly, in this embodiment, the assay material does not necessarily include an enzyme. However, it does include at least one substrate for producing the desired assay reaction and a coenzyme that will enter into the reaction and be converted from one form to another form. The coenzyme may be of the same class as in the preceding embodiment. Accordingly, the reactions produced by these assay materials may also be observed by measuring the optical density of the specimen—assay mixture—at the same wavelength.

In order to prepare the assay material of this embodiment, a buffer mixture may be prepared first. The exact composition of the buffer will vary with the particular assay reaction to be produced. However, the buffers are very similar to those in the first embodiment and will be in a class that includes the salts of polyvalent inorganic anions and the organic amines together with the acids and salts thereof. By way of example, the salts of polyvalent inorganic anions may include at least sodium and potassium phosphates, sodium and potassium pyrophosphates. By way of example, the organic amines and their acids and salts may include at least tris (hydroxymethyl) amino methane and imidazole and their salts such as the hydrochloride, succinate, sulfate, succinic, aspartic and glutamic acids and their salts such as the sodium, potassium and lithium, glycylglycine and glycine. Normally, when these buffers have been fully prepared, they will be a dry powder mixture.

It is one of the purposes of the buffer to maintain the conditions such as the pH in the reagent and specimen—assay mixture—suitable for the desired assay reaction to occur.

The substrates employed in this embodiment will, of course, depend upon the particular assay reaction. However, they will normally be in a class of compounds whose chemical reactions will be specifically catalyzed by the classes of enzymes previously referred to and whose activity is being measured. These substrates may also be reduced to a dry powdered form suitable for mixing with the buffer materials. The coenzymes will also depend upon the particular assay reaction. However, the coenzyme is preferably from the same class as in the preceding embodiment. This will insure all of the assay reactions being effective to produce changes in the optical density of the specimens at a standard wavelength. More particularly, the coenzymes may be from the class that includes the pyridine nucleotides when in the oxidized form these coenzymes will also have an absorption in the region of about 340 millimicrons. The coenzyme may be in a solid form suitable for being powdered. Accordingly, the powdered buffers, substrates and coenzymes may all be mixed together to form the assay material.

It may thus be seen that irrespective of whether the assay to be made is to measure the amount of activity of an enzyme or not, the assay material will always be prepared as a dry solid material. The material may be finely powdered for prepackaging and for dissolving in

a liquid such as water to form a liquid reagent. By employing a bulking agent of the same type described in the preceding embodiment, the various assay materials of this embodiment may also have their volume increased to some predetermined standard level. This will insure all of the resultant units being of identical sizes.

It has also been found that bulking agents of this type are effective, increase the stability of the assay mixture of this embodiment. Among other things, because of its moisture absorbing abilities, it protects the assay material against deterioration resulting from exposure to moisture. Also, the bulking agents are effective to assist in stabilizing substrates such as oxaloacetic acid. This acid is an unstable compound particularly when it is in a buffer solution having a pH of about 7.5. By employing the present bulking agents and the methods of preparation described herein, the acid can be made very stable and in a form that is compatible with the other components in the desired reaction. Moreover, the oxaloacetic acid will be in a form that may be easily handled and used. As will become apparent, the oxaloacetic acid when in this prepared form will be particularly suitable for use in preparing an assay material for use in making assays for malate dehydrogenase.

It has also been found that use of the bulking agent in the preparation results in more rapid solution of the assay mixture into water and thereby decreases the time of mixing and increases the convenience of rapid dissolving by requiring less shaking or stirring.

It will thus be seen that this embodiment may also provide a plurality of substantially identical packages such as metal foil packets, capsules, etc. Each of these packages will contain just a sufficient quantity of the assay material for making a single assay of a specimen or distinct and accurate multiples of a single assay. In order to make an assay, the contents of one package is dissolved in a standard amount of water so as to form a liquid reagent. This liquid reagent is then mixed with the specimen to produce an assay reaction. The extent of or the rate at which this reaction occurs will be a function of the amount of activity of the enzyme present in the specimen. Since every reaction, irrespective of the particular type of assay, will involve the same coenzyme as in the preceding embodiment, the rate at which this reaction occurs can be measured by measuring the optical density of the specimen different times to determine the rate of change of the optical density. From this, it will be possible to compute the amount of activity of the enzyme originally present.

While this embodiment may be employed to assay a large number of different enzymes or other biological substances, the following examples are illustrative of a limited number of different forms of this embodiment.

EXAMPLE F

The first example of the second embodiment is a solid assay material that is particularly adapted to be employed in preparing a reagent for assaying the amount of malate dehydrogenase or MDH present in a serum. When this assay material is fully prepared, it will consist of the dry mixture of the following substances:

Buffer: sodium phosphate and potassium phosphate
Substrate: oxaloacetic acid
Coenzyme: DPNH
Stabilizer: mannitol

In order to prepare a large number of capsules containing this assay material, the following procedure may be employed to produce a batch of dry assay material that may be divided into small quantities and packaged into standard size capsules. Wherever quantities are specified, they are suitable for preparing a batch that will yield about 10,000 capsules. It should be understood, however, that the amounts indicated may be varied to satisfy any particular requirements.

The assay material may be prepared by first combining the following materials in the indicated amounts to form a dry mixture:

	G.
Sodium phosphate	200–400
Potassium phosphate	40–80
Mannitol	1,000–2,000

These three ingredients are first ground into a fine powder by any suitable means such as in a ball mill. After these materials have been finely ground and are thoroughly mixed, a small quantity of the powder may be dissolved in a suitable quantity of water. The pH of the resultant solution should be in the range of about 7.4 to 7.6. If it is not within this range, additional quantities of one of the first two ingredients may be added to the mixture to bring the pH into the desired range. Although mannitol is used in the present instance, it may be replaced with sorbitol or lactose or one of the polymers thereof having from 1 to 5 hydroxyl groups per monomeric unit. The powder may then be dried under a vacuum of 0.2 mm. Hg at 25° for about 48 to 72 hours. It is also desirable for the individual powders to be dried in this manner before they are mixed together.

Following this, the coenzyme DPNH may be prepared as a fine powder and added to the foregoing mixture. Alternatively, the coenzyme may be powdered and added at the same time as the other powders. The quantity of the oxaloacetic acid to be added to the mixture should be carefully determined by the following assay. In order to make the assays, a reagent is prepared by combining the indicated amounts of the following solutions:

	Ml.
0.10 molar potassium phosphate pH 7.4	2.7
DPNH 4 mg./ml.	0.1
Oxaloacetic acid 1 mg./ml.	0.1
Malate dehydrogenase	0.1

The phosphate may be prepared by dissolving the phosphate to form a 0.10 molar solution. The DPNH solution is formed by dissolving approximately 4 milligrams of the DPNH in 1 milliliter of water. The reagent is then prepared with a sufficient quantity of the malate dehydrogenase in 0.1 ml. to cause a change of the optical density at 340 millimicrons on the order of 0.02 per minute under the conditions of the assay. The quantity of the oxaloacetic acid added to the foregoing powder is then adjusted to produce the same rate of change in the optical density.

The amount of oxaloacetic acid that is required to be added to the assay mixture to produce the optimum rate of change in the optical density can thus be determined. This quantity of the oxaloacetic acid and the DPNH are then added to the assay mixture. The amount of DPNH that is added will be determined by the optical density which is desired in the specimen and reagent mixture. Normally, this will be in the general range of about 0.8 O.D. at 340 mμ.

After the assay material has been dried under vacuum, a dry powder is provided that contains the substrate oxaloacetic acid and reduced diphosphopyridine nucleotide together with mannitol (as an example). As long as this powder is maintained dry, it is very stable and will have a very long shelf life. Accordingly, it may now be divided into a plurality of small parts that are just large enough to be mixed with water to form a liquid reagent 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. If it is desirable that the same size capsule be employed at all times, the standard size may be large enough to accept the largest quantity of the powder that will ever be needed. The quantity of the bulking and stabilizing agent, mannitol, added to the assay material may be selected to bring the total volume of each part up to a size that will just fill the capsule.

In order to use one of the capsules to make an assay of the amount of activity of the malate dehydrogenase present in a serum, a specimen of the 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 may thus be mixed with the specimen. As soon as the reagent and the specimen are mixed together, the following reaction will occur:

$$\text{oxaloacetic acid} + \text{DPNH} \xrightarrow{\text{MDH}} \text{malic acid} + \text{DPN}$$

Since the oxaloacetic acid and the DPNH are supplied from the assay material, they will be in excess of the minimum amount required to complete the foregoing assay reaction. However, this reaction is dependent upon being catalyzed by the enzyme malate dehydrogenase present in the serum. Accordingly, the only factor which will limit the rate at which the DPNH which is converted into DPN will be the amount of activity of the malate dehydrogenase originally present in the specimen. The optical density of the specimen-assay mixture is then measured at 340 millimicrons at periodic intervals. This will permit the rate of change of the optical density to be compiled which, in turn, will permit the amount of activity of the malate dehydrogenase to be computed.

EXAMPLE G

The second example of the second embodiment is a solid assay material that is particularly adapted to be employed in preparing a reagent for assaying the amount of lactate dehydrogenase or LDH presents in a serum. When this assay material is fully prepared, it will consist of the dry mixture of the following substances:

Buffer: disodium hydrogen phosphate and potassium dihydrogen phosphate
Substrate: sodium pyruvate
Coenzyme: DPNH
Stabilizer: Mannitol

In order to prepare a large number of capsules containing this assay material, the following procedure may be employed to produce a batch of dry assay material that may be divided into small quantities and packaged into standard size capsules. Wherever quantities are specified, they are suitable for preparing a batch that will yield about 10,000 capsules. It should be understood, however, that the amounts indicated may be varied to satisfy any particular requirements.

The assay material may be prepared by first combining the following materials to form a dry buffer mixture in the indicated amounts:

	G.
Disodium hydrogen phosphate	200–400
Potassium dihydrogen phosphate	40–80
Mannitol	1,000–2,000

These three ingredients are first ground into a fine powder by any suitable means such as in a ball mill. After these materials have been finely ground and are thoroughly mixed, a small quantity of the powder may be dissolved in a suitable quantity of water. The pH of the resultant solution should be in the range of about 7.4 to 7.6. If it is not within this range, additional quantities of one of the first two ingredinents may be added to the mixture to bring the pH into the desired range. The buffer powder may then be dried under vacuum and in the presence of a moisture absorbing agent such as phosphorus pentaoxide (P_2O_5) at a temperature of about 50° C. until all of the moisture is removed.

In addition to the buffer powder, a coenzyme and substrate powder may be prepared by combining the following materials in the indicated ranges:

	G.
Sodium pyruvate	2.7–4
DPNH	6–8

In order to obtain the optimum assay reaction from the assay material, the exact amount of the sodium pyruvate is critical. Accordingly, the quantity of the sodium pyruvate to be added to the mixture should be carefully determined by means of a series of assays. In order to make the assays, a plurality of assay liquids may be prepared by combining the indicated amounts of the following solutions:

Phosphate buffer 1.0 g. phosphate powder above/10 ml.	ml.	2.8
DPNH 4 mg./ml.	mg.	0.1
Serum	ml.	0.020

The phosphate buffer may be prepared by dissolving 1 gram of the phosphate containing the powder described above in 10 milliliters of water. The DPNH solution is formed by dissolving approximately 4 milligrams of the DPNH in 1 milliliter of water. A serum containing a suitable quantity of lactate dehydrogenase may be used as a source of enzyme. At the same time, about 32 milligrams of the sodium pyruvate to be employed is dissolved into about 10 milliliters of water.

After all of these solutions have been prepared, the indicated quantities of the serum, DPNH and buffer solutions may be combined with each other to form an assay liquid. A suitable quantity of the pyruvate solution, for example, 0.05 milliliter is then added to the assay liquid. The optical density of the solution at 340 millimicrons is measured as periodic intervals and recorded. Following this, the foregoing procedure is repeated with increasing amounts of the pyruvate solution (for example, 0.10 milliliter, 0.15 milliliter, 0.20 milliliter, etc.). The optical density of the assay liquids at 340 millimicrons is periodically measured and recorded. This procedure is continued until the exact amount of the pyruvate solution required to produce the maximum rate of change of the optical density at 340 millimicrons is determined.

The amount of sodium pyruvate that is required to be added to the assay mixture to produce the optimum rate of change in the optical density can then be determined. This quality of the pyruvate and the DPNH are then added to the assay mixture. The amount of DPNH that is added will be determined by the optical density which is desired in the specimen-reagent mixture. Normally, this will be in the general range of about 0.8 O.D. at 340 mμ. After the sodium pyruvate and DNPH have been added to the previously prepared mixture, the resultant mixture may be ground and pulverized by any suitable means such as ball mill to form the powdered assay material. Following this, the assay material may be dried under vacuum.

At this point, a dry powder is provided that contains the substrate sodium pyruvate and reduced diphosphopyridine nucleotide together with mannitol (as an example). As long as this powder is maintained dry, it is very stable and will have a very long shelf life. Accordingly, it may now be divided into a plurality of small parts that are just large enough to be mixed with water to form a liquid reagent 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. If it is desirable that the same size capsule be employed at all times, the standard size may be large enough to accept the largest quantity of the powder that will ever be needed. The quantity of the bulking and stabilizing agent, mannitol, added to the assay material may be selected to bring the total volume of each part up to a size that will just fill the capsule.

In order to use one of the capsules to make an assay of the amount of activity of the lactate dehydrogenase present in a serum, a specimen of the 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 may thus be mixed with the specimen. As soon as the reagent and the specimen are mixed together, the following reaction will occur:

$$\text{pyruvate} + \text{DPNH} \xrightarrow{\text{LDH}} \text{lactate} + \text{DPN}$$

This reaction is dependent upon being catalyzed by the enzyme lactate dehydrogenase present in the serum. Accordingly, the only factor which will limit the amount of the DPNH which is converted into DPN under the test conditions will be the amount of activity of the lactate dehydrogenase originally present in the specimen.

EXAMPLE H

This exemplifies a solid assay material that is particularly adapted to be employed in preparing a reagent for assaying the amount of alpha-hydroxybutyrate dehydrogenase or HBDH present in a serum. When this assay material is fully prepared, it will consist of the dry mixture of the following substances:

buffer: disodium hydrogen phosphate and potassium dihydrogen phosphate
Substrate: sodium alpha-ketobutyrate
Coenzyme: DPNH
Stabilizer: mannitol

In order to prepare a large number of capsules containing this assay material, the following procedure may be employed to produce a batch of dry assay material that may be divided into small quantities and packaged into standard size capsules. Wherever quantities are specified, they are suitable for preparing a batch that will yield about 10,000 capsules. It should be understood, however, that the amounts indicated may be varied to satisfy any particular requirements.

The assay material may be prepared by first combining the following materials to form a dry buffer mixture in the indicated amounts:

	Grams
Disodium hydrogen phosphate	200–400
Potassium dihydrogen phosphate	40–80
Mannitol	1,000-2,000

The first two ingredients are first ground into a fine powder by any suitable means such as in a ball mill. After these materials have been finely ground and are thoroughly mixed, a small quantity of the powder may be dissolved in a suitable quantity of water. The pH of the resultant solution should be in the range of about 7.4 to 7.6. If it is not within this range, additional quantities of one of the first two ingredients may be added to the mixture to bring the pH into the desired range. The buffer powder may then be dried under vacuum and in the presence of a moisture absorbing agent such as phosphorus pentoxide (P_2O_5) at a temperature of about 50° C. for two days.

In this example, the buffer consists of a mixture of disodium hydrogen phosphate and potassium dihydrogen phosphate; however, equivalent buffers such as those composed of alkali metal phosphate or tris(hydroxymethyl) aminomethane and succinic acid may be employed. The buffer used should be an anhydrous powder before dissolving in water at which time the resultant solution should have a pH in the range of 7.4 to 7.6.

After the first two ingredients have been mixed and pulverized, vacuum-dried mannitol is mixed with them. The resulting buffer-mannitol powder is kept dry throughout the succeeding steps of processing.

In addition, to the buffer-mannitol powder, a coenzyme and substrate powder may be prepared by combining the following materials in the indicated ranges:

	G.
Sodium alpha-ketobutyrate	25–50
DPNH	5–7.5

In order to obtain the optimum assay reaction from the assay material, the exact amount of the sodium alpha-ketobutyrate is critical. Accordingly, the quantity of the sodium alpha-ketobutyrate to be added to the mixture should be carefully determined by means of a series of assays. In order to make the assays, a plurality of assay liquids may be prepared by combining the indicated amounts of the following solutions:

	Ml.
Phosphate buffer 1.0 g. buffer-mannitol above/10 ml.	2.8
DPNH 4 mg./ml.	0.1
Serum	0.020

The phosphate buffer may be prepared by dissolving 1 gram of the phosphate containing the powder described above in 10 milliliters of water. The DPNH solution is formed by dissolving approximately 4 milligrams of the DPNH in 1 milliliter of water. A serum containing a suitable quantity of lactate dehydrogenase may be used as a source of enzyme. As the same time, about 320 milligrams of the sodium alphaketobutyrate to be employed is dissolved into about 10 milliliters of water.

After all of these solutions have been prepared, the indicated quantities of the serum, DPNH and buffer solutions may be combined with each other to form an assay liquid. A suitable quantity of the alpha-ketobutyrate solution, for example, 0.05 milliliter is then added to the assay liquid. The optical density of the solution at 340 millimicrons is measured at periodic intervals and recorded. Following this, the foregoing procedure is repeated with increasing amounts of the keto butyrate solution (for example, 0.10 milliliter 0.15 milliliters, 0.20 milliliter, etc.). The optical density of the assay liquids at 340 millimicrons is periodically measured and recorded. This procedure is continued until the exact amount of the ketobutyrate solution required to produce the maximum rate of change of the optical density at 340 millimicrons is determined.

The amount of sodium alpha-ketobutyrate that is required to be added to the assay mixture to produce the optimum rate of change in the optical density can then be determined. This quantity of the sodium alpha-ketobutyrate and the DPNH are then dried under vacuum at room temperature for 12 to 24 hours and added to the buffer-mannitol mixture. The amount of DPNH that is added will be determined by the optical density which is desired in the specimen. Normally, this will be in the general range of about 0.8. After the sodium pyruvate and DPNH have been added to the previously prepared mixture, the resultant mixture may be ground and pulverized by any suitable means (which prevents exposure to excess moisture) such as a ball mill form the thoroughly mixed powdered assay material. Following this, the assay material may be dried under vacuum.

At this point, a dry powder is provided that contains the substrate sodium alpha-ketobutyrate and coenzyme diphosphopyridine nucleotide (reduced) together with mannitol (as an example). As long as this powder is maintained dry, it is very stable and will have a very long shelf life. Accordingly, it may now be divided into a plurality of small parts that are just large enough to be mixed with water to form a liquid reagent 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. It it is desirable that the same size capsule be employed at all times, the standard size may be large enough to accept the largest quantity of the powder that will ever be needed. The quantity of the buliking and stabilizing agent, mannitol, added to the assay material may be selected to bring the total volume of each part up to a size that will just fill the capsule.

Mannitol has been found to be the preferably bulking-stabilizing agent; however, any equivalent material which dissolves readily, does not interfere with the chemical reaction of the assay, is not hygroscopic and contains negligible moisture, may be an equivalent contemplated by the inventor for carrying out his invention.

Equivalent materials are typically polyhydric substances and polymers of said polyhydric substances with from 1 to 5 hydroxyl groups per monomeric unit.

In order to use one of the capsules to make an assay of the amount of activity of the alpha-hydroxybutyrate dehydrogenase present in a serum, a specimen of the 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 may thus be mixed with the specimen. As soon as the reagent and the specimen are mixed together, the following reaction will occur:

$$\text{alpha-ketobutyrate} + \text{DPNH} \xrightarrow{\text{HDBH}} \text{alpha-hydroxybutyrate} + \text{DPN}$$

Since the sodium alpha-ketobutyrate and the DPNH are supplied from the assay material, this reaction is dependent upon being catalyzed by the enzyme alpha-hydroxybutyrate dehydrogenase present in the serum. Accordingly, the only factor which will limit the amount of the DPNH which is converted into DPN under the test conditions will be the amount of activity of the alpha-hydroxybutyrate dehydrogenase originally present in the specimen.

EXAMPLE I

The fourth example of the second embodiment is a solid assay material that is particularly adapted for making a liquid reagent that may be employed for assaying the amount of activity of glucose-6-phosphate dehydrogenase. This material when fully prepared will consist of a dry mixture of the following:

Buffer: tris-succinate
Substrate: glucose-6-phosphate, sodium salt
Coenzyme: TPN
Accelerator: magnesium sulfate, glutamate or aspartate
Stabilizer: mannitol

In order to prepare a large number of capsules of this assay material, the following procedure may be employed to produce a batch of a dry reagent or assay powder that may be divided into small quantities and packaged into capsules. Wherever quantities are specified in this example, they are suitable for preparing a batch that will yield about 10,000 capsules. It should be noted that the values are for illustrative purposes only and may be varied to satisfy any particular requirement.

Normally, the first step in making an assay material of this form is to prepare a buffer. In the present example, the mixture of the buffer includes the following materials in about the indicated ranges:

	G.
Tris (hydroxymethyl) aminomethane	180–350
Succinic acid	75–150
Magnesium sulfate—nH_2O	80–160

The tris (hydroxymethyl) aminomethane and the succinic acid are first mixed together and ground into a fine powder in the same manner as described before. This will provide a tris-succinate buffer that is in the form of a fine powder, which when dissolved in water will form a solution having a pH of 7.4 to 7.6.

Following the preparation of the tris-succinate buffer, the magnesium sulfate may be added thereto (an equivalent amount of magnesium as its glutamate or aspartate salt may be used). This will be in solid form and accordingly may be mixed directly with the buffer and the resultant mixture ground into a fine powder.

It is desirable to make sure that there are no inhibitors present in the sulfate-buffer which might interfere with the final assays. To do this, the following solutions are prepared in the indicated manner:

The first solution is formed by dissolving 10 milligrams of glucose-6-phosphate sodium salt in 10 milliliters of water (10μ molar test).

The second solution is also formed by dissolving 11.5 milligrams of triphosphopyridine nucleotide in 1 milliliter of water (1.5μ molar test).

A third solution is prepared by dissolving 430 milligrams of the mixture of the magnesium sulfate and tris-succinate buffer in 1.8 milliliters of water.

A fourth solution is also prepared that includes tris (hydroxymethyl) aminomethane, succinic acid and magnesium sulfate which are obtained from sources differing from those used in preparing the magnesium-buffer mixture, but are dissolved to form a solution that is the equivalent of the third solution. A first test solution is formed by combining 0.1 milliliter of the first or glucose-6-phosphate solution, 0.1 milliliter of the second or triphosphopyridine solution, 1.8 milliliters of the third or buffer mixture and enough water to make the final volume equal to 2.9 millilters.

A second test solution is formed by combining the buffer solution obtained from the original or step 1, 0.1 milliliter of the glucose-6-phosphate solution, 0.1 milliliter of the second or triphosphopyridine nucleotide solution, 1.8 milliliters of the fourth or equivalent of the prepared buffer and sufficient water to make a total of 2.9 milliliters.

After the two solutions are prepared, 0.1 milliliter of a solution of glucose-6-phosphate dehydrogenase is placed in one of the test solutions. The changes in the optical density of the solution occurring at 340 millimicrons are measured. The same amount of glucose-6-phosphate dehydrogenase (0.1 milliliter) is then added to the second test solution. The changes in the optical density of this solution are then measured. If the changes for the two solutions are of the same, the magnesium-buffer mixture contains no inhibitors and the assay material may be prepared.

The assay material may be prepared by mixing the following materials together in the indicated amounts:

	G.
Magnesium-tris succinate buffer	400–600
Glucose-6-phosphate	27–33
Triphosphopyridine nucleotide	10–13

The indicated quantities of the chemicals are mixed together and placed in a ball mill until they are ground into a fine powder.

At this point, a dry powder is provided that contains the substrate and coenzyme that are to react with each other to form the assay reaction. This powder is very stable and will have a long shelf life. Accordingly, it may be divided into a plurality of small parts that are just large enough to be mixed with water to form a liquid reagent 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. If it is desirable that the same size capsule be employed at all times, the standard size may be large enough to accept the largest quantity of the powder that will ever be needed. A bulking and stabilizing agent such as mannitol is added so as to increase the total volume and improve the stability.

In order to use one of the capsules to make an assay of a serum to determine the amount of activity of glucose-6-phosphate dehydrogenase, a specimen of the 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 may thus be mixed with the specimen. As soon as the reagent and the specimen are mixed together, the following reaction will occur:

$$\text{glucose-6-phosphate} + \text{TPN} \xrightarrow{\text{glucose-6-phosphate dehydrogenase}} \text{6-phospho-gluconate} + \text{TPNH}$$

Since the glucose-6-phosphate and the TPN are supplied by the capsule, they will be in a very abundant supply whereby they can react with each other. However, this reaction is dependent upon being catalyzed by the enzyme glucose-6-phosphate dehydrogenase. Accordingly, the only

factor which will limit the rate of which the TPN which is converted into TPNH will be the amount of activity of the glucose-6-phosphate dehydrogenase originally present in the specimen. Accordingly, by placing the specimen in a suitable spectrophotometer and measuring the optical density at 340 millimicrons, the rate at which the TPN is being converted into TPNH may be determined. From this rate of change, it will be possible to calculate the amount of activity of the glucose-6-phosphate dehydrogenase that was originally present in the specimen.

It may thus be seen that a solid assay material and the method of preparing same have been provided. The assay material is particularly adapted for use in assaying specimens involving simple or complex enzyme systems or biochemical metabolites or intermediate. The assay material is in the form of a very stable solid that may be prepackaged in accurately measured quantities so as to permit a relatively inexperienced technician to produce an optimum assay reaction. Since the assay material is very stable and accurately premeasured, the assay reactions and the results thereof can be obtained very rapidly and conveniently while still providing very accurate and reproducible results. In addition, the assay material will permit a more efficient, inexpensive and economical assay to be obtained since the amount of time, equipment, storage space, etc., is greatly reduced.

While only a limited number of embodiments of the present invention have been disclosed herein, it will be readily apparent to persons skilled in the art that numerous changes and modifications may be made thereto. For example, the quantity of such materials employed may be modified so as to fit any particular application and equivalent materials may be substituted for those described herein. Accordingly, the foregoing disclosure is for illustrative purposes only and does not in any way limit the invention which is defined only by the claims which follow.

EXAMPLE J

Another example of the first embodiment is a solid reagent that is particularly adapted to be employed for measuring the amount of urea or urea nitrogen present in a serum. This reagent when fully prepared will consist of a dry mixture containing the following substances:

Enzyme—urease and glutamic dehydrogenase
Stabilizers—dithioerythritol,[1] and sodium potassium tartrate
Buffer-tris (hydroxymethyl) aminomethane
Substrates—alpha-ketoglutaric acid or its salts
Coenzyme—reduced diphosphopyridine nucleotide (DPNH)
Activator—adenosine diphosphate, sodium salt

In order to prepare a large number of units of this reagent, the following procedure may be employed to produce a batch of dry reagent, that may be then divided into a large number of aliquots and packaged into capsules. Wherever quantities for a substance are specified, the quantities are suitable for preparing a batch of reagent that will yield about 1,000 capsules each of which makes up to 3 ml. size assay mix. It should be understood, however, that these values are for illustrative purposes only and may be varied to satisfy the demands of the particular application to which the reagent is to be used.

The dry buffer-substrate mixture of tris (hydroxymethyl) aminomethane and alpha-ketoglutaric acid may be prepared by thoroughly mixing and grinding together the appropriate quantities of each. When dissolved in water, the tris (hydroxymethyl) aminomethane alpha-ketoglutarate salt is formed in solution to form a buffer of the appropriate pH. Tris (hydroxymethyl) aminomethane has been found a preferred buffer; however, phosphate glycine or bicarbonate buffers are useful.

Urease is extracted from finely ground jack beans with dilute aqueous sodium phosphate buffer (pH 6.3 to 6.7). The extract is dialyzed to remove ammonia, clarified by filtration, dithioerythitol added and the resulting solution lyophilized. This forms a dry stable powder containing urease.

Following this, a dry lyophilized powder containing glutamate dehydrogenase is prepared. This may be accomplished by employing the following chemicals in the indicated ranges:

Glutamate dehydrogenase, 1,000,000 International units;
Dithioerythitol, 300–600 mg.;
Sodium potassium tartrate, 25 ml. saturated solution.

The glutamate dehydrogenase may first be made free of ammonia if necessary by passing over a gel filtration column. To this enzyme solution, dithioerythritol and sodium potassium tartrate may be added and a stabilized solution formed.

After the stabilized solution has been thoroughly mixed, it may then be placed under a vacuum for a sufficient interval of time to remove any entrapped air. Following this, the solution may be frozen and placed under a vacuum until all of the moisture is removed therefrom.

This will produce a dry lyophilized powder which contains the glutamate dehydrogenase, together with stabilizers, which will be effective to maintain the activity of the enzyme at its desired level.

After the lyophilized powders containing the urease and glutamate dehydrogenease have been prepared, they may be assayed to determine the amount of activity of the enzyme urease present in the powder. This may be accomplished by employing a reagent containing the following chemicals in the indicated quantities:

Substrates: urea (.01 M)
Coenzyme: DPNH (4 mg./ml.)
Buffer: 0.1 M, pH 7.5 containing alpha-ketoglutarate
Activator: ADP 10 mg./ml.

After the foregoing solutions have been prepared in the indicated concentrations, they may be all mixed together to form a liquid reagent suitable for performing the present assay. A suitable quantity of each lyophilized powder containing the enzyme glutamate dehydrogenase and unrease may be dissolved in water. This may be accomplished by dissolving about 100 milligrams of each powder in about 10 milliliters of water, to form two separate enzyme solutions. About 0.1 milliliter of each solution may then be combined with the reagent. When this mixture is made, the DPNH will immediately begin to be converted into DPN. While this reaction is occurring, the solution may be placed in a suitable spectrophotometer and the optical density in the reagent measured at suitable increments of time, for example, every minute. After several readings have been taken, an average may be provided which will indicate the amount of activity of the urease in the lyophilized powder. A change in the optical density of 0.001 per minute indicates an activity of 1 unit. From this, it will be possible to calculate the amount of the lyophilized powders containing the urease and glutamate dehydrogenase that are required to produce an activity capable of bringing the reaction (for 0.05 micromoles of urea) to completion in 5 to 10 minutes.

Following this, buffer-substrate, activators, and cofactors may be added to the dry lyophilized powders containing ureas and glutamic dehydrogenase, ground and mixed in a dry atmosphere. The resulting mixture is then suitable for packaging in a capsule. For the production of reagent for 10,000 tests, the following chemicals in the indicated ranges as described below:

[1] Dithioerythritol has been found a preferred sulfhydryl; however, the other compounds, such as cysteine and glutathione are useful. Sodium potassium tartrate has been found a preferred salt; however, sodium sulfate, tartrate and glutamate, and potassium sulfate are useful.

Tris (hydroxymethyl) aminomethane	g.	300–500
Alpha-ketoglutaric acid	g.	50–80
Adenosine diphosphate, sodium salt	g.	5–15
Mannitol	g.	500–1000
DPNH	g.	3–6
Lyophilized glutamate dehydrogenase (International units [1])		7–15
Lyophilized ureas (International units [1])		15–40

[1] As previously stated the amount of each enzyme is most conveniently established by determination of those amounts required to bring the reaction of 0.05 micromoles of urea to completion in 5 to 10 minutes.

The alpha-ketoglutaric acid, tris (hydromethyl) aminomethane, ADP and mannitol may all be mixed together and ground into a fine powder by any suitable means such as placing in a ball mill about 6 to 10 hours. Following the mixing, a sample such as about 100 milligrams of the mixture may be dissolved in a suitable quantity of water such as 3 milliliters. The pH of this solution should be between 8.0 and 8.5.

At this point, a powder is provided that contains the two enzymes, together with the mannitol, tris (hydroxymethyl) aminomethane, alpha-ketoglutaric acid and adenosine diphosphate. The powder is now dried under vacuum for 2 days or longer so as to insure the powder very stable and having a very long shelf life wherein the enzyme activity will not deteriorate.

After drying, the DPNH is added under dry conditions; the amount added is generally sufficient to cause an absorbancy at 340 mμ of 1. In addition, the powder includes a buffer, enzymes, adenosine diphosphate sodium salt, and alphaketoglutaric acid, that will insure the desired reaction occurring when an assay is made.

Accordingly, this powder may be divided into a plurality of small parts that are just large enough to form a liquid reagent suitable for making one assay of a specimen. The entire bulk of the powder may then be divided into units or parts which contain the desired amount of activity. Each of these parts may then be packaged in a suitable container such as a capsule. It is desirable that the same size capsule be employed at all times irrespective of which batch the capsule is produced from. Accordingly, the capsule may be of a sufficiently large size to insure packaging of all variations. In such a case, the mannitol acts as a bulking agent such as which may be added to the mixture so as to bring its volume up to an amount that will just fill the capsule.

In order to use one of the capsules to make an assay of a serum for urea, a specimen of a serum or other biological fluid is first obtained in a suitable quantity. Following this, a capsule of this example is dissolved in a suitable quantity of water. The resultant solution will form a liquid reagent that is of just the right size and strength to make a sing'e assay of the serum. Accordingly, this liquid reagent may then be mixed with the specimen. As soon as the reagent and specimen are mixed together, the following reactions will occur:

$$\text{urea} + \text{water} \xrightarrow{\text{urease}} 2NH_4^+ + HCO_3^- NH_4^+ + \text{alpha-ketoglutarate}$$

$$+ \text{DPNH glutamate dehydrogenase} \longrightarrow \text{glutamate} + \text{DPN}$$

Since the alpha-ketoglutaric acid, DPNH, urease, and glutamic dehydrogenase are supplied in the capsule in sufficient amounts, the extent of the foregoing reactions will only be limited by the amout of urea originally present. This, in turn, will cause the DPNH to be converted to DPN in direct proportion to the amount of the urea originally present. By placing the specimen in a suitable spectrophotometer and measuring the optical density at 340 millimicrons, the amount of DPNH being converted can be determined. This, in turn, will permit the amount of urea in the original specimen specification to be calculated.

EXAMPLE K

Another example of the first embodiment of this invention is a solid reagent that is particularly adapted to be employed for measuring the amount of activity of creatine kinase, creatine phosphokinase or CPK present in a biological sample. This reagent fully prepared will consist of a dry mixture of the following solid substances:

Enzyme: hexokinase and glucose-6-phosphate dehydrogenase
Buffer: tris-succinate
Stabilizer: acacia, tris-sulfate, ammonium sulfate and apteine
Substrates: creatine phosphate and glucose
Activator: magnesium sulfate
Accelerator: insulin
Coenzymes: adenosine diphosphate and triphosphopyridine nucleotide (TPN)
Inhibitor: adenosine monophosphate

In order to prepare a large number of capsules of this reagent the following procedure may be employed to produce a batch of dry reagent that may then be divided into small quantities and packaged into suitable capsules. Wherever quantities are specified in this example they are suitable for preparing a batch that will yield on the order of 10,000 capsules. It should be understood, however, that whenever desired these quantities may be varied to satisfy any particular requirements.

The first step in this procedure is to prepare a suitable buffer solution. The buffer employed is substantially identical to the tris buffer used in Example D and consists of a mixture of tris (hydroxymethyl)aminomethane and succinic acid. These chemicals may be mixed in substantially the same manner as described in Example D to produce a dried buffer powder that when dissolved in water will produce a solution having a pH between 7.4 and 7.6.

Tris-succinate has been found to be a preferred buffer; however, any compound which can maintain the pH between 6.5 and 7.5, may be used, such as an alkali metal phosphate, or triethanol amine. A dry lyophilized powder containing the enzymes hexokinase and glucose-6-phosphate dehydrogenase may then be prepared by combining the following chemicals in the indicated ranges:

Gum acacia	gms.	50–100
Tris-buffer 0.2 M pH 7.4 to 7.6	ml.	75–100
Ammonium sulfate 1 M pH 7.5 (adjusted with ammonia)	ml.	75–100
Insulin	mg.	50–750
Hexokinase	mg.	100
Glucose-6-phosphate dehydrogenase	mg.	100

The tris-sulfate solution is prepared first by dissolving tris (hydroxymethyl) aminomethane and adding a sufficient quantity of sulfuric acid to make a 0.2 molar solution with a pH 7.4 to 7.6.

The acacia, ammonium sulfate and insulin are completely mixed with the tris-sulfate to form a homogenous solution. After the solution is completely mixed any air entrapped therein may be removed by placing the solution under a vacuum. The enzymes hexokinase and glucose-6-phosphate dehydrogenase are mixed into this solution. This solution should be thoroughly mixed to insure the enzymes being completely dispersed therein. While uniformly mixed, the solution is completely frozen. While in the frozen state it is placed under a vacuum until all of the moisture is removed. This will leave a homogenous dry powder containing the enzymes. This lyophilized powder may then be assayed to determine the amount of activity of the enzyme present by employing the following chemicals in the indicated amounts to form a reagent:

Glucose solution 60 μMoles/ml. (1.08 g. in 100 ml.)	ml.	0.5
Creatine phosphate 100 mg./ml.	ml.	0.2
Tris succinate buffer solution	ml.	1.0
Adenosine diphosphate 15 mg./ml.	ml.	0.2
Magnesium sulfate hydrated 1 g./10 ml.	ml.	0.4
Adenosine monophosphate 60 mg./1 ml.	ml.	0.2
TPN 10 mg./1 ml. water	ml.	0.2
Water	ml.	0.4
Lyophilized enzyme mixture	mg.	10
Cysteine hydrochloride 12.5 mg./ml.	ml.	0.2

The tris succinate buffer solution is prepared by dissolving 3.4 grams of the buffer prepared in the beginning of this example in 100 milliliters of water. Following this the indicated quantities of the rest of the solutions are combined and mixed with the indicated quantity of water. After the reagent has been completed its optical density at 340 millimicrons is measured. Following this a standardized creatine phosphokinase solution containing 0.02 International Enzyme Units [1] may be added to the mixture. When this occurs the reaction subsequently described will occur. The change with time of the optical density at 340 millimicrons is measured. By comparing the change in optical density the activity of CPK can be as result of the reaction be determined.

Under the foregoing test conditions one International Enzyme Unit of the CPK should produce an optical density change at 340 millimicrons of 2.07 per minute at 30° C. Accordingly, the 0.02 units of the standardized solution employed in this assay should produce a change in optical density of 0.414 per 10 minutes at 30° C. If such a change is produced by the foregoing assay, the reagent enzymes have sufficient activity to continue with the preparation of the described reagent.

To prepare the lyophilized powder for encapsulation, the following chemicals may be combined in the indicated ranges:

	G.
Tris-succinate buffer (prepared above)	100–250
Disodium creatine phosphate tetrahydrate	100–200
Magnesium sulfate, hydrated	80–120
Sodium adenosine-5-diphosphate dihydrate	15–30
TPN	12–18
Adenosine-5-minophosphate, monohydrate	60–120
Glucose, anhydrous	25–100
Cysteine hydrochloride	5–20
Iyophilized enzyme mixture	80–200

All of these chemicals are in the form of a dry powder and may be directly mixed together. The mixture is then reduced to a fine powder by any suitable means such as placing in a ball mill for an extended period of time. At this point the powder is ready for dividing into suitable units and packaged into capsules. In order to determine the size of each unit, the activity of the powder must first be determined. This is accomplished by dissolving a small sample of the specimen in a suitable quantity of water such as 2.9 ml. The assay test described above may then be repeated. By measuring the change of optical density produced by the assay reaction, the amount of activity of the enzymes in the powder may be determined. When this powder is dissolved in water it will make a reagent that will cause a reaction involving the adenosine triphosphate or ATP generated by the CPK reaction. This reaction will continue to increase in rate with increasing amounts of CPK until it is limited. The amount of CPK required for this to occur will be determined by the amount of activity of the reagent enzymes present. Accordingly, in order to compute the amount of the assay powder to be placed in each capsule, a convenient maximum amount of CPK for making the test must first be chosen. For example, this may be 0.05 units. The size of the units into which the assay powder is divided will then be such that the rate of reaction will increase proportionally with the amount of CPK up to 0.05 unit or the change in optical density at 340 mμ for 0.05 unit should be 0.518 for 5 minutes at 30° C.

If it is desired to have all of the capsules of some predetermined size, a standard capsule size is selected that will always accommodate the largest unit of powder. After the size of the units is determined a suitable quantity of a bulking and stabilizing agent such as mannitol or its equivalent may be added to bring the volume to just the right size to fill the standard-sized capsule.

It may thus be seen that a large number of identical capsules will be produced that are suitable for assaying the amount of creatine phosphokinase or CPK present in a serum. The stabilizers tris-sulfate, acacia and ammonium sulfate will be effective to stabilize the enzymes and preserve their activity for extended periods of time. The tris-succinate buffer and the glucose substrate and TPN coenzyme will insure the assay reaction occurring.

To use one of the capsules to assay a serum to determine the amount of activity of adenosine triphosphate present in a serum, a suitable sample of the serum is first obtained. The optical density of the sample is first measured at a wavelength of 340 millimicrons. One of the capsules is then dissolved in a standard quantity of water such as 3 milliliters. This will create a liquid reagent of the correct size and activity for making one assay of serum sample. This liquid reagent may then be combined with the sample of serum whereby the following reactions will occur:

$$\text{creatine phosphate} + \text{ADP} \xrightarrow{\text{Creatine phosphokinase}}$$

$$\text{creatine} + \text{ATP}; \text{glucose} + \text{ATP} \xrightarrow{\text{hexokinase}}$$

$$\text{glucose-6-P} + \text{ADP}; \text{glucose-6-P} + \text{TPN}$$

$$\xrightarrow{\text{glucose-6-phosphate dehydrogenase}} \text{6-phoshogluconate} + \text{TPNH}$$

As these reactions progress the ATP will form and then disappear and the TPN will be converted to TPNH. Since the components supplied by the reagent are in abundant supply, the only thing that will control the rate of the reaction will be the CPK present. It may thus be seen that the rate at which TPN is converted into TPNH will be a function of the quantity of the CPK present in the biological sample.

The reactions are allowed to continue for the duration of the normal test interval (5 to 10 minutes). However, before the expiration of this interval, the rate of reaction may be determined. At the end of the test interval, the optical density of the sample is measured again at 340 millimicrons. Since the TPNH will absorb light at this wavelength, the difference between the optical densities before and after the reactions will be a result of the changes in the amount of the TPNH produced. Since the change in the quantity of TPNH with time is a function of the quantity of CPK originally present in the specimen this activity of CPK can be computed from the change in the optical density.

Adenosine monophosphate or AMP is added to the reagent mixture in order to reduce the activity of myokinase or adenylate kinase, which is at times present in biological samples. Myokinase would cause the following reaction to occur:

$$2\,\text{ADP} \xrightarrow{\text{myokinase}} \text{ATP} + \text{AMP}$$

The ATP formed by this reaction would lead to false CPK determination; therefore, AMP is added which inhibits the formation of ATP by this reaction.

Cysteine is added to protect or stabilize under some conditions the CPK activity.

EXAMPLE L

Another example of the second embodiment of a solid assay material that is particularly adapted to be employed

[1] An International Ensyme Unit is that amount of activity which will convert one micromole of substrate per minute at 30° C.

in preparing a reagent for assaying the amount of lactate dehydrogenase or LDH present in a serum is as follows. When this assay material is fully prepared, it will consist of the dry mixture of the following substances:

Buffer: glycine and sodium carbonate
Substrate: lithium lactate
Coenzyme: (DPN) diphosphopyridine nucleotide
Stabilizer: mannitol

In order to prepare a large number of capsules containing this assay material, the following procedure may be employed to produce a batch of dry assay material that may be divided into small quantities and packaged into standard size capsules. Wherever quantities are specified, they are suitable for preparing a batch that will yield about 10,000 capsules. It should be understood, however, that the amounts indicated may be varied to satisfy any particular requirements.

The assay material may be prepared by first combining the following materials to form a dry buffer mixture in the indicated amounts:

	G.
Glycine	180–315
Sodium carbonate	80–120
Mannitol	1,000–2,000

These three ingredients are first ground into a fine powder by any suitable means such as in a ball mill. After these materials have been finely ground and are thoroughly mixed, a small quantity of the powder may be dissolved in a suitable quantity of water. The pH of the resultant solution should be in the range of about 8.4 to 8.6. If it is not within this range, additional quantities of one of the first two ingredients may be added to the mixture to bring the pH into the desired range. The buffer powder may then be dried under vacuum and in the presence of a moisture absorbing agent such as phosphorus pentaoxide (P_2O_5) at a temperature of about 40° C. until all of the moisture is removed.

In addition to the buffer powder, a coenzyme and substrate powder may be prepared by combining the following materials in the indicated ranges:

	G.
Lithium lactate	50–100
DPN	30–40

In order to obtain the optimum assay reaction from the assay material, the exact amount of the lithium lactate is critical. Accordingly, the quantity of the lithium lactate to be added to the mixture should be carefully determined by means of a series of assays. In order to make the assays, a plurality of assay liquids may be prepared by combining the indicated amounts of the following solutions:

	Ml.
Glycine-sodium carbonate buffer 1 g. above powder/10 ml. water	2.8
DPN 33 mg./ml. water	0.1
Serum	0.020

The buffer may be prepared by dissolving 1.0 g. of the glycine-sodium carbonate containing powder described above in 10 milliliters of water. The DPN solution is formed by dissolving approximately 33 milligrams of the DPN in 1 milliliter of water. A serum containing a suitable quantity of lactate dehydrogenase may be used as a source of enzyme. At the same time, about 750 milligrams of the lithium lactate to be employed is dissolved into about 10 milliliters of water.

After all of these solutions have been prepared, the indicated quantities of the serum, DPN and buffer solutions may be combined with each other to form an assay liquid. A suitable quantity of the lactate solution, for example, 0.05 milliliters is then added to the assay liquid. The optical density of the solution at 340 millimicrons is measured at periodic intervals and recorded. Following this, the foregoing solution is repeated with increasing amount of the lactate solution (for example, 0.10 milliliters, 0.15 milliliters, 0.20 milliliters, etc.). The optical density of the assay liquids at 340 millimicrons is periodically measured and recorded. This procedure is continued until the exact amount of the lactate solution required to produce the maximum rate of change of the optical density at 340 millimicrons is determined.

The amount of lithium lactate that is required to be added to the assay mixture to produce the optimum rate of change in the optical density can then be determined. This quantity of the lactate and the DPN are then added to the assay mixture. The amount of DPN that is added will be determined by performing the above assay as for lactate but where the optimum amount of lactate is used and the volume of DPN solution is varied. Normally, this will be in the general range of 0.1 ml. After the lithium lactate and DPN have been added to the previously prepared mixture, the resultant mixture may be ground and pulverized by any suitable means such as ball mill to form the powdered assay material. Following this, the assay material may be dried under vacuum.

At this point, a dry powder is provided that contains the substrate lithium lactate and coenzyme diphosphopyridine nucleotide together with mannitol (as an example). As long as this powder is maintained dry, it is very stable and will have a very long shelf life. Accordingly, it may now be divided into a plurality of small parts that are just large enough to be mixed with water to form a liquid reagent 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. If it is desirable that the same size capsule be employed at all times, the standard size may be large enough to accept the largest quantity of the bulking and stabilizing agent, mannitol, added to the assay material may be selected to bring the total volume of each part up to a size that will just fill the capsule.

In order to use one of the capsules to make an assay of the amount of activity of the lactate dehydrogenase present in a serum, a specimen of the 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 may thus be mixed with the specimen. As soon as the reagent and the specimen are mixed together, the following reaction will occur:

$$\text{lactate} + \text{DPN} \xrightarrow{\text{LDH}} \text{pyruvate} + \text{DPNH}$$

This reaction is dependent upon being catalyzed by the enzyme lactate dehydrogenase present in the serum. Accordingly, the only factor which will limit the rate at which the DPN is converted into DPNH will be the amount of activity of the lactate dehydrogenase originally present in the specimen.

What is claimed is:

1. An assay material for assaying a specimen for adenosine triphosphate comprising a solid, water-soluble, substantially anhydrous, storage stable mixture of:

(a) glucose:
(b) the coenzyme triphosphopyridine nucleotide;
(c) a source of magnesium ions;
(d) a buffer;
(e) the enzymes hexokinase and glucose-6-phosphate dehydrogenase;
(f) a stabilizer selected from the group consisting of mucilagenous gums, hydroxyalkylamines, ethylenediamine tetraacetic acid and its salts, and a source of sulfate anion, and mixtures thereof; and
(g) a stabilizing-bulking agent selected from the group consisting of mannitol, sorbitol, lactose, and polyvinyl alcohol;

further wherein (d) and (e) are a lyophilized mixture.

2. The method of assaying a specimen for glucose,

using an assay material comprising a solid, water soluble, substantially anhydrous, storage stable, mixture of:
(a) the coenzymes adenosine triphosphate and triphosphopyridine nucleotide;
(b) a source of magnesium ions;
(c) a buffer;
(d) the enzymes hexokinase and glucose-6-dehydrogenase;
(e) a stabilizer selected from the group consisting of mucilagenous gums, hydroxyalkylamines, ethylenediamine tetraacetic acid and its salts, and a source of sulfate anion and mixtures thereof; and
(f) a stabilizing-bulking agent selected from the group consisting of mannitol, sorbitol, lactose, and polyvinyl alcohol;
further wherein (d) and (e) are a lyophilized mixture; which includes the steps of:
(i) dissolving in water said material, thereby producing a liquid reagent having a measurable optical density;
(ii) mixing said liquid reagent with said specimen to form a specimen-reagent assay mixture; and
(iii) determining the amount of change in the optical density of the reacted specimen-reagent assay mixture.

3. The method of assaying a specimen for adenosine-triphosphate using an assay material comprising a solid, water soluble, substantially anhydrous mixture of:
(a) glucose;
(b) the coenzyme triphosphopyridine nucleotide;
(c) a source of magnesium ions;
(d) a buffer;
(e) enzymes comprising hexokinase and glucose-6-phosphate dehydrogenase;
(f) a stabilizer selected from the group consisting of mucilagenous gums, hydroxyalkylamines, ethylenediamine tetraacetic acid and its salts, and source of sulfate anion, and mixtures thereof;
(g) a stabilizing-bulking agent selected from the group consisting of mannitol, sorbitol, lactose and polyvinyl alcohol;
further wherein (e) and (f) are a lyophilized mixture; which includes the steps of:
(i) dissolving in water said material, thereby to produce a liquid reagent having a measurable optical density;
(ii) mixing said liquid reagent with said specimen to form a specimen-reagent assay mixture, and
(iii) determining the amount of change in optical density of the reacted specimen-reagent assay mixture.

4. The method of assaying a specimen for the enzyme glucose-6-phosphate dehydrogenase, using an assay material comprising a solid, water soluble, susbtantially anhydrous mixture of:
(a) glucose-6-phosphate;
(b) the coenzyme triphosphopyridine nucleotide;
(c) a source of magnesium ions; and
(d) a buffer;
(e) a stabilizing-bulking agent selected from the group consisting of mannitol, sorbitol, lactose and polyvinyl alcohol;
which includes the steps of:
(i) dissolving in water said material, thereby producing a liquid reagent having a measurable optical density;
(ii) mixing said liquid reagent with said specimen to form a specimen-reagent assay mixture; and
(iii) measuring the rate of change in optical density of the reacting specimen-reagent assay mixture.

5. The material of claim 1 in which (f) is a mixture of gum acacia, tris(hydroxymethyl)aminomethane sulfate and ammonium sulfate.

6. The material of claim 1 in which (g) is mannitol.

7. The material of claim 1 in which (g) is mannitol and (f) is a mixture of gum acacia, tris(hydroxymethyl aminomethane sulfate and ammonium sulfate.

8. The material of claim 2 in which (e) is a mixture of gum acacia, tris(hydroxymethyl)aminomethane and its sulfate salt, and ammonium sulfate.

9. The material of claim 2 in which (f) is mannitol.

10. The material of claim 2 in which (f) is mannitol and (e) is a mixture of gum acacia, tris(hydroxymethyl) aminomethane sulfate, and ammonium sulfate.

11. The method of claim 3 in which (f) is a mixture of gum acacia, tris(hydroxymethyl)aminomethane and its sulfate salt, and ammonium sulfate.

12. The method of claim 3 in which (g) is mannitol.

13. The method of claim 3 in which (g) is mannitol and (f) is gum acacia, tris(hydroxymethyl)aminomethane and its sulfate, and ammonium sulfate.

14. The method of claim 4 in which (e) is mannitol.

References Cited

UNITED STATES PATENTS

2,990,338	6/1961	Gibson	195—103.5
3,072,532	1/1963	Innerfield	195—63 X
3,133,001	5/1964	Puig	195—68

OTHER REFERENCES

Colowick et al., Methods in Enzymology, vol. II, pp. 497 and 498 (1955); vol. III, pp. 109 and 110 (1957).

ALVIN E. TANENHOLTZ, *Primary Examiner.*