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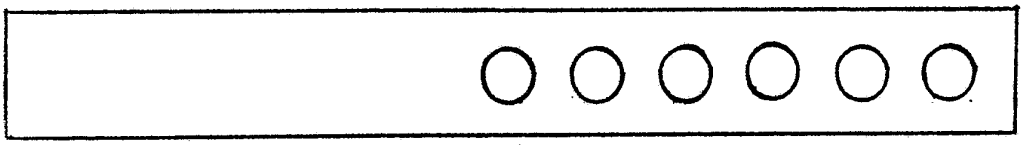

(57) Abstract

Devices, e.g. a test strip, and methods for using the devices for determining the presence of an analyte in a fluid. One embodiment involves a device containing a first reagent capable of reacting with the analyte to produce a detectable reaction product which comprises a support member impregnated with a first catalyst, e.g. an enzyme, for the production of the detectable product and a compensator means for inhibiting the first catalyst. The devices of this invention may also be impregnated with an indicator reagent and a second catalyst for the production of a compound which absorbs visible light thereby facilitating detection. In one embodiment the compensator means inhibits the second catalyst. The devices may also be impregnated with a known amount of a quantifying reagent which consumes the detectable reaction product. The device may contain more than one zone containing these constituents, with at least two zones containing different amounts of the quantifying reagent permitting the use of the device for quantitative assays. Embodiments of this invention can be used in various specific binding assays, e.g. for various immunoassays or as DNA probes. Numerous analytes of clinical significance can be assayed with these devices and methods, including blood enzymes, substrates for enzymes antigens, haptens and other substances.

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# DEVICE FOR RAPID QUANTITATIVE ANALYSIS OF A FLUID

This application is a continuation-in-part of U.S. Serial No. 542,601, filed October 17, 1983, the contents of which are hereby incorporated by reference into the present application.

This invention concerns devices for quantitative analysis of fluids which allows rapid, visual assays of analytes. The devices are useful in assays of biological fluids for enzymes and enzyme substrates and in particular in specific binding assays involving enzyme-linked reagents.

## Background of the Invention

Quantitative analysis of fluids is often necessary in industrial and medical laboratories. Diagnosis of disease, for instance, may depend on the results of quantitative chemical analysis of a body fluid for a particular component. Many methods are known today for analysis of body fluids such as blood and urine. They generally employ instruments which are very sensitive and accurate and are useful in detecting minute amounts of substances which may indicate disease. However, these instruments are expensive and require calibration, making them unsuitable for use outside a clinical laboratory.

Accordingly, efforts have been expended to develop simpler analytical devices for body fluids. Test strips have been devised which give observable color changes

upon contact with fluids. Glucose, for example, may be monitored in urine by means of a chromogenic paper strip (U.S. Patent 2,981,606). Proteins, ketone bodies and other substituents may likewise be assayed by paper strips or other inert surfaces impregnated with appropriate chromogens. Other devices with multi-layered components for improved sample handling and analysis have been developed (U.S. Patent Nos. 3,917,430 and 3,992,158).

These devices are usually made semi-quantitative by comparing them after reaction to a calibrated control. However, the accuracy of these measurements is limited since they involve observation and comparison of small color changes which are difficult to discern. Moreover, the reproducibility of such measurements is susceptible to variations in environmental conditions and in sample handling and application (Walter, B. et al., Analytical Chemistry 55:498 (1983). Meticulous care in handling the testing materials as well as proper technique, good lighting and good vision are important for achieving accurate results with such devices. Aziz, S. et al., Diabetes Care 6(6):529-532 (1983).

One approach, that of H.T. Hochstrasser, U.S. Patent Nos. 3,964,871 (1976) and 4,059,407 (1977), involves test strips for assays of glucose and other substances in a fluid. The test strips contain a plurality of reagent zones each of which contains a chemical reagent which reacts with the analyte resulting in the oxidation of an indicator to render the indicator colored. Each zone also contains a substance which consumes one of the reactants necessary for color formation. Since each zone contains an incrementally different amount of the substance, the test strip can quantify the concentration

range of the analyte present in the fluid.

Hochstrasser, however, does not disclose a means for preventing interference from substances such as hemoglobin. Accordingly, such devices cannot be used in direct accurate assays of whole blood. Furthermore, Hochstrasser does not disclose any internal means for standardizing the test strips against variations in the temperature of the fluid to be tested or in the time period in which the strip is contacted with the fluid. Overexposure of the device to the fluid, therefore, can produce a significant level of false positive results. More prolonged contact with the fluid can exhaust the quantifying substance and extinguish quantification completely.

Similarly, significant errors in results obtained with other test strips lacking an internal control or compensating means have been reported in cases of modest under- or overexposure of the strip to the fluid. Marshal, S.M. et al., Diabetes Care 6(6):543-547 (1983); Editorial, Diabetes Care 6(6):614-15 (1983).

Recently, the advent of monoclonal antibodies has led to an increased interest in simple quantitative devices. Immunoassay with monoclonal antibodies is very sensitive but is currently observed, in non-clinical settings, by small color changes. These small color changes are difficult to compare with accuracy to a standard.

Accordingly, it is an object of this invention to provide new devices and methods for improved analysis of fluids, especially body fluids.

## Summary of the Invention

This invention concerns simple, rapid methods and devices for carrying out such methods to determine the presence of analyte in a fluid.

In one embodiment the device comprises s support member impregnated with a first catlyst, e.g. an enzyme, for the production of a dectable product and a compensater means for inhibiting the first catalyst at a predetermined rate.

Another device is useful for determining the presence of an enzyme for the reaction of a substrate of the enzyme with a first reagent to produce a dectable reaction product in a fluid containing the first reagent. This device comprises a support member impregnated with the substrate of the enzyme and a compensator means for inhibiting the enzyme at a predetermined rate.

Another embodiment provides a device for determining the presence of an analyte containing a first reagent, a second reagent capable of reacting with the first reagent the second reagent being a substrate for a first catalyst for the production of the detectable reaction product and the first catalyst being bound to the analyte. The device comprises a support member impregnated with a biochemical reagent capable of binding to the analyte and a compensator means for inhibiting the first catalyst at a predetermined rate. This device is useful in specific binding assays, e.g. in immunoassays or as a DNA probe.

The various devices of this invention are designed for the production of a first reaction product, e.g. the

detectable reaction product. This product may but need not absorb visible light. The devices of this invention may also comprise a second catalyst, e.g. an enzyme, for the reaction of the first reaction product with an indicator reagent to produce a compound which does absorb visible light. The indiator reagent as well as other reagents may also be impregnated on the device or added to the fluid being tested.

The compensator means of this invention inhibits either the first catalyst or enzyme of the device, or the catalyst for the reaction of indicator reagent for producing the compound which absorbs visible light. The compensator means is suitable catalytic comprising a second catalyst, with the fluid containing a substrate of the second catalyst and a second reagent, the reaction of the second reagent with the substrate for the second catalyst producing the inhibitor. Alternatively the substrate for the second catalyst may be impregnated on the device with the second catalyst in the fluid, or both the second catalyst and its substrate may be impregnated on the device.

Alternatively, the compensator means may comprise an inhibitor of the first catalyst or enzyme or an inhibitor of the catalyst for the reaction of the indicator reagent for producing the compound which absorbs visible light, the inhibitor being encapsulated in a suitable material capable of releasing the inhibitor at a predetermined rate after the device contacts the fluid.

The compensator means of this invention, whether catalytic or encapsulated, serves to inhibit the relevant catalyst at a predetermined rate.

Devices of this invention may also comprise known amount of a quantifying reagent which reacts with the detectable reaction product or with the compound which absorbs visible light. The device may comprise the aforementioned constituents in at least two discrete zones on the suppport member. Where the known amount of quantifying reagent in at least two of the zones is different, the device is useful in quantitatives assays since the two zones will indicated the presence of the analyte in different concentrations.

Various methods for using the devices of this invention in assays of various analytes and enzymes and in various specific binding assays including heterogeneous, homogeneous, and sandwich asssays as well as for nucleic acid hybridization assays are also disclosed herein.

## BRIEF DESCRIPTION OF DRAWINGS

Figures 1A and 1B illustrate a device with a plurality of reagent zones, each of which contain an indicator reagent and an amount of quantifying means differing from zone to zone.

Figure 1A illustrates the device before contact with the fluid.

Figure 1B illustrates the device after contact with the fluid. Three of the six zones show a change in the indicator reagent indicative of the presence of analyte in the fluid above a minimum concentration corresponding to the amount of quantifier in each of the three changed zones.

Detailed Description of the Invention

This invention concerns simple, rapid methods and devices to carry out such methods to determine the presence of an analyte in a fluid containing a first reagent capable of reacting with the analyte to produce a detectable reaction product. In one embodiment the device comprises a support member impregnated with a first catalyst, e.g. an enzyme for the production of the detectable product and a compensator means for inhibiting the first catalyst at a predetermined rate. The enzyme is suitably an oxidoreductase, transferase, hydrolase, lyase, isomerase or ligase enzyme.

The support member of the device of this and other embodiments of the invention may comprise any suitable material into which the various components and reagents may be impregnated. Insoluble polymers, for example, of cellulosics, acrylics, polyester or ceramics are suitable. The components may be impregnated into the matrix formed by the support material or may be immobilized upon the support member with suitable binding agents by conventional methods.

Suitably, the first catalyst is an enzyme which catalyzes the production of a detectable reaction product which may then be detected either directly, e.g. if the product is a compound which absorbs visible light, or indirectly with an indicator reagent as disclosed herein. Alternatively, where the analyte is an enzyme, the support member is suitably impregnated with an appropriate substrate of the enzyme. The enzyme may suitably be an oxidoreductase, transferase, hydrolase, lyase, isomerase or ligase enzyme. In one of the preferred embodi-

ments, the oxidoreductase enzyme is an enzyme capable of catalyzing the production of hydrogen peroxide, e.g. glucose oxidase or cholesterol oxidase, upon contact under suitable conditions with an appropriate substrate, e.g. glucose or cholesterol, respectively, in the presence of an appropriate first reagent, e.g. oxygen. Numerous other enzyme-substrate pairs suitable for use in this and other embodiments are known in the art as are methods for impregnating or immobilizing the enzyme onto various materials, see e.g. U.S. Patent Nos. 3,817,837 (1974) and 4,059,407 (1977). When an enzyme is used or detected in this and other embodiments, suitable buffers and ionic salts are added to the device or fluid to allow the enzymatic reaction to proceed.

The compensator means serves as an internal standard to control the extent to which the catalytic production of the detectable product proceeds by inhibiting the first catalyst at a predetermined rate which may be temperature dependent.

The compensator means of the device of this and other embodiments is suitably a second catalyst, e.g. an enzyme, where the fluid contains a substrate for the second catalyst and a second reagent, the reaction of the second reagent with the substrate for the second catalyst producing an inhibitor of the first catalyst.

Alternatively, the compensator means suitably comprises a substrate for a second catalyst, where the fluid contains the second catalyst and a second reagent, the reaction of the second reagent with the substrate for the second catalyst likewise producing an inhibitor of the first catalyst.

In other embodiments the compensator means suitably comprises a second catalyst and a substrate for the second catalyst and the fluid contains the second reagent, the reaction of the second reagent with the substrate for the second catalyst, as above, producing an inhibitor of the first catalyst.

In each of the above mentioned embodiments of the compensation means, the catalytic reaction proceeds independently of and concurrently with the reaction of the first reagent and the analyte. Although the reaction catalyzed by the first catalyst and the reaction catalyzed by the second catalyst proceed concurrently, the compensator means of this invention does not generate enough inhibitor to substantially completely inhibit the first catalyst until after a sufficient time has passed for a detectable amount of the detectable reaction product to have been produced. The amount of appropriate material for use as the compensator means can be chosen to predetermine the amount of time which will be allowed for the catalytic production of the detectable reaction product. Thus, for example, if a given amount of inhibitor is required to substantially completely inhibit the first catalyst, an amount of the second catalyst and its substrate necessary to generate this amount of inhibitor can be employed. The time in which this amount of inhibitor is produced can be predetermined by a simple kinetic study. Therefore, by choosing an appropriate amount of materials, the time allowed for the first catalyst to function can be internally standardized. This internal compensation for time removes variables caused by time of reaction which simplifies testing procedures and allows reliable comparison of different assays.

The compensator means can also be an internal standard for temperature if the rate of generation of compensator product varies in a predictable fashion with the temperature. By choosing a compensator means which varies with temperature with respect to production of inhibitor in the same manner as the catalytic activity of the first catalyst, the variation caused by the temperature parameter can also be eliminated. Thus, the compensator means can also eliminate the need for external temperature control or standardization.

In embodiments involving a first catalyst, e.g. an enzyme which is inhibited by cyanide, the second catalyst is suitably mandelonitrile lyase and the substrate for the second catalyst is mandelonitrile, where the second reagent is water and the inhibitor of the first catalyst produced by the reaction of the substrate and the second reagent is cyanide ion.

Alternatively, the second catalyst is suitably beta-glucosidase, the substrate for the second catalyst is amygdalin, the second reagent is water and the inhibitor of the first catalyst produced by the reaction of the second reagent is cyanide ion.

Numerous and varied other enzymes for catalyzing the production of a detectable product which are not inhibited by cyanide are also known in the art and useful as the first catalyst of this invention. In such a case, a suitable enzyme for catalyzing the production of an appropriate inhibitor, e.g. a sulfide, a specific amino acid or other substance, can readily be selected from those known in the art for use as the second catalyst for the compensator means.

In another embodiment, the compensator means is an inhibitor of the first catalyst encapsulated in a suitable material capable of releasing the inhibitor at a predetermined rate after the device contacts the fluid.

Suitable materials include porous materials and materials which decompose gradually or within a predetermined period of time, which in either case permit the release of the contained inhibitor at a predetermined rate. Suitable materials and methods for encapsulation are known. See e.g., U.S. Patent Nos. 3,092,463 (1963); 3,252,762 (1966); 3,240,117 (1966) and 3,341,466 (1967). Suitable inhibitors for many enzymes are also known, see e.g. The Enzymes, 3rd Ed., Academic Press, New York (1971); Enzyme Handbook, Vols. I and II, (Barman, T.E. Ed.) Springer-Verlag New York Inc.

In some embodiments the catalytic production of the detectable reaction product is a multi-step process, each step of which may be catalyzed by a different catalyst and require a different additional reagent. In such a case, the catalyst to serve as the first catalyst of this invention, i.e. the catalyst which will be inhibited by the compensator means, may be selected on the basis of convenience in inhibiting the catalyst.

The detectable reaction product in one embodiment is a compound which absorbs visible light. In another embodiment the device is also impregnated with a second catalyst for the reaction of the detectable reaction product with an indicator reagent to produce a compound which absorbs visible light. The device may also be impregnated with a suitable amount of the indicator reagent. Alternatively, the indicator reagent may be added to the fluid. Suitable indicator reagents include

redox, pH or chelometric indicators.

The indicator reagent is preferably a chromogen which undergoes a color change in the catalyzed reaction. Alternatively, the indicator reagent may undergo a density change or other visually observable physical change. If the device is to be used in a laboratory or clinic, the catalytic product of the indicator reagent and the detectable reaction product may be observed by instrumental methods.

Thus, for example, components of blood or urine such as glucose or triglycerides can be caused to form hydrogen peroxide upon contact under suitable conditions with glucose oxidase or lipase, glycerol kinase or GP oxidase (or alternatively lipase and glycerol dehydrogenase), respectively. The peroxide may be measured by the method and device of the present invention as set forth herein. In one of the presently preferred embodiments the indicator reagent is the redox indicator phenylenediamine. Also suitable is m-aminosalicylic acid and numerous other indicator reagents known in the art, including for example o-dianisidine, p-toluidine, o-toluidine, p-diphenylamine sulfonic acid and N,N-dimethylindoaniline. See e.g. U.S. Patent Nos. 4,059,407 (1977) and 4,391,906 (1983).

A device of this invention may also be impregnated with a known amount of a quantifying reagent which reacts with the detectable reaction product, thereby consuming it. Where the detectable reaction product is readily reducible the quantifying reagent may suitably be a reducing agent, e.g. ascorbic acid or a salt thereof. Where the detectable reaction product is pH-sensitive the quantifying reagent may suitably be a buffer. In

any case, a suitable quantifying reagent must be capable of inhibiting the accumulation of the detectable reaction product to a degree proportional to the amount of quantifying reagent impregnated on the device.

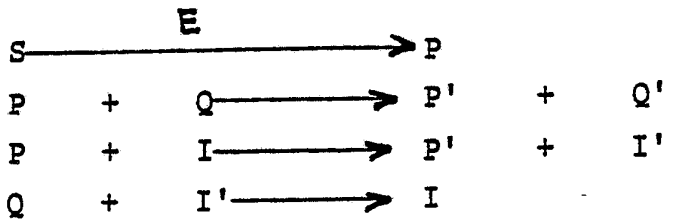
Numerous other quantifying reagents suitable for use in this invention are known in the art, including for example gentisic acid, hydroquinone, pyrogallol, hydroxylamine, sodium nitrite and sodium bisulfite. See e.g. U.S. Patent No. 4,059,407 (1977).

In one embodiment of this invention, the device is impregnated with a quantifying reagent and a second catalyst for the reaction of the detectable reaction product with an appropriate indicator reagent to produce a compound which absorbs visible light. In this embodiment the quantifying reagent is capable of competing with the indicator reagent and reacting with the detectable reaction product before that product can react with the indicator reagent.

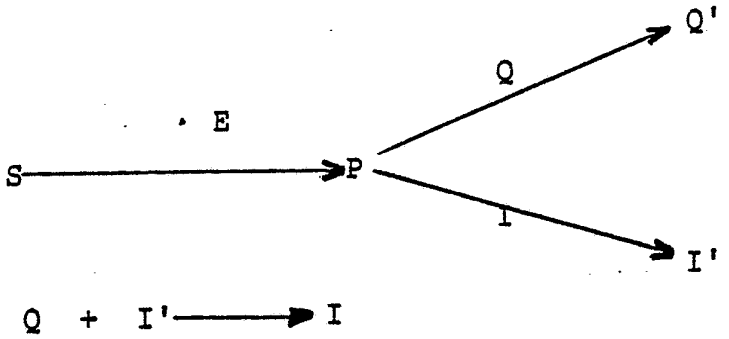
Although the present invention is not to be limited by any given mechanism, several explanations may be set forth for the mode of action of the quantifying reagent. The quantifying reagent may, for example, react faster with the product than does the indicator reagent, either because of a greater reaction rate constant or because of a higher concentration. It is possible also that the quantifier may have a more favorable reaction potential, e.g. oxidation potential, as in the case of redox reactions - than does the indicator reagent. Preferably, the quantifier should react essentially irreversibly with the product to prevent an observable, slow reaction of detectable reaction product with indicator. It should also be noted that the quantifying reagent may

also be capable of reacting with and consuming a compound which absorbs visible light produced by the catalytic reaction of the indicator reagent and the detectable reaction product.

Possible mechanisms for the action of the quantifying reagent are illustrated by the following equations:




- or -




wherein S is the analyte or other substrate of a first catalyst; E is the first catalyst (preferably an enzyme) which is impregnated in or immobilized on the device or is the material to be determined; P is the detectable reaction product; Q is the unreacted quantifying reagent; P' is an uncolored product derived from P, and Q' the consumed quantifier. I is the indicator reagent which reacts to produce the I', the compound which absorbs visible light. I' may also react with Q to produce I.

In a device of this invention the first catalyst, the

compensator means and the quantifying reagent are suitably each present in at least two discrete zones on the support member and the amount of quantifying reagent in at least two of the zones is different. Such a device is useful for indicating the presence of different concentrations of the analyte. In another embodiment, at least two of the zones may contain different components and indicate the presence of at least two different analytes.

The device may be constructed in any of the various physical forms known in the assay arts, e.g. in the form of a test strip with each zone located on a distinct region of the test strip. Alternatively the device may be a test plate with each reagent zone located in a distinct plate well. Whichever form the device takes, at least one zone may be protected from direct contact with the fluid by a semipermeable barrier means, e.g. by being coated laminated or otherwise firmly affixed with a semipermeable membrane material, to exclude substances capable of interfering with the operation of the device. Such barrier means comprise a layer capable of excluding substances such as hemoglobin or red blood cells, and permit the use of devices of this invention in direct assays of whole blood. Suitable material for such a layer is a polymer such as polyvinylacetate.

A preferred embodiment of the present invention is given in Figure 1 wherein quantifier, indicator and catalysts are located in discrete zones on the support member. Other embodiments may comprise different geometrical arrangements and shapes of areas where indicator and quantifier are affixed. Some or all of the areas may also be contiguous. Alternatively, the areas may be affixed to separate surfaces which are superimposed.

The device may be employed to determine quantitatively the concentration of an analyte which is a substrate of a catalyst, an enzyme, for example. In this procedure the solution containing an unknown amount of analyte is contacted with the first catalyst impregnated on the support member of the device along with appropriate buffers and ionic salts, or these reagents may be added independently to the sample while it is being analyzed. The analyte produces a detectable reaction product and may react with an indicator reagent if provided, to produce a compound which absorbs visible light in a reaction catalyzed by a second catalyst. Either type of product may be consumed completely or partially in a reaction with the quantifier means. All of the reactions must occur within the time determined by the compensator means, i.e., before the inhibition of the first catalyst is substantially complete. The reaction zone(s) are then monitored for detectable changes, i.e. the presence of the detectable product or the compound which absorbs light, and the identity and number of the zone(s) that change are correlated with amount of analyte present in the fluid.

This procedure is especially useful in measurement of glucose, triglycerides or other components of body fluids which produce, or can be coupled to other chemical reaction systems that produce hydrogen peroxide upon contact with suitable chemical or enzymatic catalysts. The hydrogen peroxide thus produced is generally observed by its reaction with an appropriate indicator in the presence of a peroxidase enzyme. In the method of the present invention this amount of hydrogen peroxide may be determined quantitatively by contacting it with the device wherein the quantifying reagent is a keto-

enol tautomer, ascorbic acid, for example, the indicator reagent is a chromagen such as phenylenediamine and the compensator means, mandelonitrile, in combination with mandelonitrile lyase, for example, produces cyanide ion.

In this and other embodiments involving a second catalyst and an indicator reagent, the compensator means may suitably function by inhibiting the second catalyst. Such a device for determining the presence of an analyte in a fluid containing a first reagent capable of reacting with the analyte to produce a detectable reaction product will thus comprise a support member impregnated with a first catalyst for the production of the detectable product, a second catalyst for the reaction of the detectable reaction product with an indicator reagent to produce a compound which absorbs visible light and a compensator means for inhibiting the second catalyst at a predetermined rate. Such a device may also be impregnated with the indicator reagent, or such reagent may be added to the fluid separately. It should be noted that the compensator means encompasses each of the previously described compensator means differing only in that the inhibitor so produced is in this embodiment an inhibitor of a second catalyst for the reaction with an indicator reagent.

Thus, an exemplary reaction system which constitutes the quantifying reaction and the time and temperature compensator reaction can be described by the following equations:

Quantifying Reactions:

$$\text{Ascorbate} + H_2O_2 \xrightarrow{HRP} \text{Dehydroascorbate}$$

$$I + H_2O_2 \xrightarrow{HRP} I^*$$

$$I^* + \text{Ascorbate} \longrightarrow I + \text{Dehydroascorbate}$$

Compensator Means Reactions:

$$\text{Amygdalin} \xrightarrow{\beta} CN^-$$

$$CN^- + HRP \longrightarrow HRP \text{ (Inactive)}$$

where:

$\beta$ = $\beta$-glucosidase
$I^*$ = Compound which absorbs visible light
$I$ = Indicator reagent (uncolored)
HRP = Horseradish Peroxidase
$CN^-$ = Cyanide ion

The compensator means using amygdalin and glucosidase can be replaced by one using mandelonitrile and mandelonitrile lyase to produce $CN^-$.

Using this technique the amount of peroxide present may be read out from the device and correlated to the amount of peroxide-generating analyte in the sample.

Quantitative assay of large numbers of other diagnostically significant components which generate peroxide may also be determined quickly, simply and reliable by this method and device.

In addition to the system described above, other quantifying reagents and materials for the compensator means may be effectively utilized in the device of the present

invention. This can be accomplished whenever the indicator reagent participates in a reversible reaction to produce the compound which absorbs visible light as with redox indicators, pH indicators and chelometric indicators. For example, in pH indicator-based systems, the quantifying reagent may be a buffering substance and the compensator means may inhibit the first, pH changing catalyst or enzyme.

The device is also useful in determining the quantities of various metabolites (urea, creatinine, etc.) and other substances (glucose, ethanol, etc.) found in biological fluids. See e.g., U.S. Patent No. 4,059,407 (1977).

A device of this invention is also useful for determining the presence of an enzyme for the reaction of a substrate with a first reagent to produce a detectable reaction product in a fluid containing the first reagent. Suitably the device comprises a support member impregnated with the substrate of the enzyme and a compensator means for inhibiting the enzyme at a predetermined rate. Such a device may also be impregnated with an indicator reagent capable of reacting with the detectable reaction product to produce a compound which absorbs visible light and a catalyst for the reaction of the indicator reagent with the detectable reaction product. Alternatively the indicator reagent may be separately added to the fluid. Enzyme assay devices of this invention may also be impregnated with an appropriate quantifying reagent and may be constructed with a plurality of zones for the detection of different levels of enzyme activity in an analogous fashion to the analyte assay devices previously discussed. Such enzyme assay devices may also be coated with a semipermeable

membrane material and are useful for numerous assays of clinical significance including assays for alkaline phosphatase, acid phosphatase, lipase, amylase, aspartate aminotransferase or glutamate oxalacetate transferase, alanine aminotransferase or glutamate pyruvate transferase, lactate dehydrogenase and creatine kinase or creatine phosphokinase.

A device of this invention is also suitable for use in specific binding assays, e.g. immunoassays or nucleotide probes, involving appropriately labeled reagents, e.g. reagents labeled with an enzyme. Methods for linking enzymes to various reagents, including monoclonal or serum antibodies, antigens, haptens and nucleotide sequences and methods for using such enzyme-linked reagents in various assays for numerous and varied analytes are known in the art, see e.g. Renz, M. et al., Acids Res. 12:3435-3444 (1984) and U.S. Patent Nos. 3,817,837 (1974) and 4,318,980 (1982), and are generally adaptable to the method and device of this invention. Many such enzyme-linked moieties are commercially available, e.g. from Boehringer Mannheim Biochemicals, Indianapolis, Indiana and other companies. As those of ordinary skill in the art will recognize, such assays are analogous to a direct assay of this invention, e.g. for glucose, in that in the course of the assay an enzyme is also linked to the reaction zone, albeit indirectly, and its activity measured by the production of a detectable product. Various conventional immunoassay configurations may be utilized, including competitive binding, homogeneous and sandwich techniques, for example. In such assays, the component of the antigen-antibody pair which contains an enzyme label is contacted with the device comprising a compensator means and one or more of substrate, indicator reagent and quanti-

fying reagent. The device may contain a plurality of discrete zones on the support member, the number and identity of zones in which a reaction is detected during the assay being a measure of the amount of enzyme and therefore of the amount of analyte being assayed. No comparison to a standard or control is necessary and variables due to the time of reaction, temperature and subjectivity of color measurements are eliminated. A reliable, simple immunoassay is thus provided.

One embodiment is a binding assay device for determining the presence of an analyte in a fluid containing a first reagent and a second reagent capable of reacting with the first reagent to produce a detectable reaction product, the second reagent being a substrate for a first catalyst and the first catalyst being bound to the analyte. The device comprises a support member impregnated with a biochemical reagent capable of binding the analyte and a compensator means capable of inhibiting the first catalyst at a predetermined rate.

In this embodiment the catalyst is suitably bound to the analyte by means of a second biochemical reagent, e.g. an antibody to the analyte, for use in a sandwich assay. Alternatively, the catalyst may suitably be bound directly to a known amount of the analyte, i.e. a molecule of the substance to be determined, for use in a homogeneous or heterogeneous competitive binding assay.

In one embodiment the analyte is an antigen and the biochemical reagent is an antibody to the antigen. Antigen, as the term is used herein, is also meant to encompass haptens. Suitably, the antigen is, or is derived from, a virus, bacterium, fungus, yeast, cancer cell or other disease causing agent, therapeutic drug,

drug of abuse, hormone or other protein.

In another embodiment the analyte is a ligand and the biochemical reagent is a receptor capable of binding the ligand. In still another embodiment the analyte is a nucleic acid sequence and the biochemical reagent is a complementary sequence.

The compensator means for such a device suitably comprises a substrate for a second catalyst where the fluid contains the second catalyst and a second reagent, the reaction of the second reagent with the substrate for the second catlayst producing an inhibitor of the first catalyst. Alternatively, the compensator means may comprise the second catalyst and the substrate for the second catalyst where the fluid contains the second reagent. The compensator means may also comprise the second catalyst where the fluid contains the substrate for the second catalyst and the second reagent. Suitable second catalysts and substrates therefor are as previously discussed and include mandelonitrile lyase and mandelonitrile, respectively, and amygdalin and beta glucosidase, respectively, where the first catalyst is inhibited by cyanide ion. Alternatively, where the first catalyst is inhibited by substances other than cyanide, e.g. by a sulfide, specific amino acid or other substance, a suitable second catalyst, preferably an enzyme, and a substrate and second reagent therefor capable of catalytically producing such substance may be selected from those known in the art.

As previously discussed, the compensator means may further comprise an inhibitor of the first catalyst encapsulated in a suitable material capable of releasing the inhibitor at a predetermined rate after the device

contacts the fluid.

The detectable reaction product may be a compound which absorbs visible light. Alternatively, as in previously described embodiments, the device may also be impregnated with a second catalyst for the reaction of the detectable reaction product with an indicator reagent to produce a compound whcih absorbs visible light. The device may also be impregnated with a suitable amount of the indicator reagent or the indicator reagent may be added to the fluid.

Again, as in previous embodiments the device may also be impregnated with a known amount of a quantifying reagent which reacts with the detectable reaction product.

In one embodiment, the biochemical reagent, the compensator means and the quantifying reagent are each present in at least two discrete zones on the support member and the amount of quantifying reagent in at leat two zones is different. Such a device is therefore useful in quantitative assays.

These devices again, may be constructed in various forms, e.g. as a test strip or test plate. The impregnated support member may also be coated with or laminated with a semipermeable membrane material. Such material can be selected to prevent the constituents of the support member from contacting interfering substances in the fluid, such as red blood cells or hemoglobin.

In this embodiment, the first catalyst bound to the analyte catalyzes, upon subsequent contact with an appropriate substrate and reagent the production of the detectable reaction product. Preferably, the first

catalyst is an enzyme, e.g. an oxidoreductase, transferase, hydrolase, lyase, isomerase or ligase enzyme. In one embodiment the substrate for the first catalyst is also present on the support member, but is encapsulated in a suitable material or otherwise kept from contacting the first catalyst until the device is contacted with the fluid.

The support member may further comprise a removably mounted, permeable layer containing a suitable amount of the analyte to which the first catalyst is bound. Such a layer is suitably capable of permitting the first catalyst bound to the analyte to diffuse to at least one zone of the support under suitable conditions. Whatman No. 1 paper is suitable for use as the permeable layer. By initially segregating the analyte to which the first catalyst is bound in a separate layer, premature binding of the labeled analyte and the biochemical component or the support member can be avoided. This reduces the time required for the analyte and analyte to which the first catalyst is bound to achieve an equilibrium level of competitive binding with the corresponding biochemical component, and thus permits a more rapid assay.

The devices of this invention are useful in various assay methods and are adaptable to a broad range of conventional assay techniques. One method is useful for determining the presence of an analyte in a fluid containing a first reagent capable of reacting with the analyte to produce a detectable reaction product. The method comprises contacting the fluid with a first catalyst for the reaction of the analyte with the first reagent to produce the detectable reaction product and with a compensator means for inhibiting the first catalyst under suitable conditions permitting the first

catalyst to catalyze the reaction and permitting the compensator means to inhibit the first catalyst at predetermined rates.

By detecting the presence of the detectable reaction product the presence of the analyte in the fluid is determined. By reacting the detectable reaction product with an indicator reagent to produce a compound which absorbs visible light, detection can be effected by visually observing the appearance of the compound.

One method of this invention is useful for determining the presence of an enzyme in a fluid containing a first reagent capable of reacting with a substrate of the enzyme to produce a detectable reaction product. The method involves contacting the fluid with the substrate of the enzyme for the production of the detectable reaction product and with a compensator means for inhibiting the enzyme. The contacting is effected under suitable conditions for the enzyme to catalyze the reaction and the compensator means to inhibit the enzyme at predetermined rates.

By detecting the presence of the detectable reaction product the presence of the enzyme in the fluid is thereby determined.

Another method is useful for determining the presence of an analyte at a concentration greater than a predetermined concentration in a fluid containing a first reagent capable of reacting with the analyte to produce a detectable reaction product. This method involves contacting the fluid with a first catalyst for the production of the detectable reaction product, with a known amount of a quantifying reagent capable of react-

ing with and thereby consuming an amount of the detectable reaction product corresponding to the predetermined concentration of the analyte and with a compensator means for inhibiting the first catalyst. The contacting is effected under suitable conditions permitting the first catalyst to catalyze the production of the detectable reaction product, the quantifying reagent to consume the detectable reaction product and the compensator means to inhibit the first catalyst at predetermined rates. By detecting the presence of the detectable reaction product, the presence of the analyte in the fluid at a concentration greater than the predetermined concentration can be determined.

Another embodiment also concerns a competitive binding assay method for determining the presence or concentration of a given analyte in a fluid. This method involves a device of this invention containing a removably-mounted permeable layer containing labeled analyte, as previously mentioned. The method involves contacting the device with the fluid, the contacting being under suitable conditions and for a sufficient period of time permitting analyte in the fluid and the labeled analyte in the removably mounted, permeable layer to diffuse to at least one reaction zone and permitting the analyte and the labeled analyte to bind competitively with the biochemical reagent in the reaction zones of the device; removing excess fluid and labeled analyte from the device after a suitable period of time e.g. by washing or blotting; contacting the reaction zone(s) of the device with a suitable amount of an appropriate substrate of the reaction reagent label under suitable conditions permitting the substrate to react with the reaction reagent of the labeled analyte to produce a reaction product, identifying the reaction zone(s) in

which the presence of the reaction product is detected, and correlating the zones identified with the concentration of the analyte in the fluid.

Another method is a heterogeneous binding assay for determining the presence of an analyte in a fluid containing a first reagent capable of reacting with a substrate of a first catalyst to produce a detectable reaction product. This method involves adding to the fluid a first known amount of the first catalyst to which a second known amount of the analyte is bound The resulting fluid is then contacted with a biochemical reagent capable of binding to the analyte under suitable conditions so as to form a complex thereof. The complex is separated from uncomplexed first catalyst to which the analyte is bound, e.g. by washing or removing excess fluid, and contacted with the substrate of the first catalyst and with a compensator means for inhibiting the first catalyst under suitable conditions permitting the first catalyst to catalyze the production of the detectable reaction product and the compensator means to inhibit the first catalyst at predetermined rates. By detecting the presence of the detectable reaction product so produced and the presence of the analyte in the fluid can be determined.

Another method of this invention is a homogeneous assay for determining the presence of an analyte in a fluid containing a first reagent capable of reacting with a substrate of a first catalyst to produce a detectable reaction product. This method involves adding to the fluid a first known amount of the first catalyst to which a second known amount of the analyte is bound. The resulting fluid is then contacted with a biochemical reagent capable of binding to the analyte under suitable

conditions to as to form a complex. The catalytic activity of a suitable first catalyst bound to uncomplexed analyte is detectably greater than the catalytic activity of the first catalyst bound to complexed analyte, as in prior homogeneous assay methods. The fluid is then contacted with the substrate of the first catalyst and with a compensator means for inhibiting the first catalyst under suitable conditions permitting the first catalyst to catalize the production of the detectable reaction product and the compensator means to inhibit the first catalyst at predetermined rates. by detecting the presence of the detectable reaction product so produced, and comparing the amount of detectable reaction product to detected with the amount produced in the absence of analyte, the presence of the analyte in the fluid can be determined.

Another method is useful for determining the presence of an analyte in a fluid containing a first reagent capable of reacting with a substrate of a first catalyst to produce a detectable reaction product. This method is a sandwich technique and involves adding to the fluiddd a first biochemical reagent and the first catalyst to which a second biochemical reagent is bound, both biochemical reagents being capable of binding to the analyte under suitable binding conditions to produce a complex comprising the analyte bound to both biochemical reagents. The complex is separated from uncomplexed first catalyst to which the second biochemical reagent is bound. The separated complex is then contacted with the substrate of the first catalyst in the presence of a compensator means for inhibiting the first catalyst under suitable conditions permitting the first catalyst to catalyze the production of the detectable reaction product and the compensator means to inhibit the first

catalyst at predetermined rates. By detecting the presence of the detectable reaction product so produced, the presence of the analyte in the fluid can be determined.

These methods may all be conducted using the devices of this invention. Thus, in a method for determining the presence of an analyte in a fluid containing a first reagent capable of reacting with the analyte to produce a detectable reaction product a devioe comprising a support member impregnated with a first catalyst for the production of a detectable reaction product and a compensator means for inhibiting the first catalyst at a predetermined rate, as previously described, can be used. In this method the device is contacted with the fluid, removed the device from the fluid and the presence of the detectable reaction production detected on the device. The presence of the analyte in the fluid is thereby determined.

Another method is of general utility for controlling the catalytic reaction of a reagent and a substrate for a catalyst. The method comprises contacting the reagent with the substrate for the catalyst, the catalyst and a compensator means under suitable conditions permitting the catalyst to catalyze the reaction and the compensator means to inhibit the catalyst at predetermined rates, in accordance with this invention.

The following examples are presented to illustrate the present invention but are not intended to and should not be construed to limit the scope of the invention as claimed hereinafter.

Materials used in the following examples are commercial-

ly available from suppliers including Sigma Chemical Co. (St. Louis, MO), Aldrich Chemical Co. (Milwaukee, WI) and Calbiochem-Behring (LaJolla, CA).

Examples

Example I

This example illustrates the preparation of a device to determine blood glucose.

The following solutions were prepared:

Solution A

8.3 mM sodium ascorbate in deionized water

Solution B

2000 units glucose oxidase
1000 units horseradish peroxide
.1M mole phenylenediamine
50 units mandelonitrile lyase
.1 gram polyvinyl alcohol
.01 M phosphate buffer to pH 7.4
(all in 50 cc deionized water)

Solution C

.5% mandelonitrile in acetone
.1% polyvinylacetate

Using the above solutions, a series of solutions were prepared for paper impregnation as follows:

| Solution No. | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| ml Solution A | 10 | 20 | 30 | 40 | 50 |
| ml Solution B | 10 | 10 | 10 | 10 | 10 |
| Deionized $H_2O$ | 40 | 30 | 20 | 10 | 0 |

Preparation of Paper Strips

A separate strip of Whatman #1 paper was immersed in each solution. The paper was allowed to soak in the solution for about two minutes and then dried in vacuo without heating. After drying lightly, each strip was sprayed on one side with Solution C. Each paper strip was designated by the number of the solution (1, 2, 3, 4 and 5) from which it was prepared.

Preparation of Quantitative Device

The five paper strips prepared above were sliced into 1/8" wide strips and fixed onto a heavy plastic sheet previously coated with a pressure sensitive adhesive. The combined assembly was then cut into 1/8" strips containing five discrete zones. Each zone was designated #1, #2, #3, #4 or #5, corresponding to the paper strip of the same number from which the zone was prepared. These devices were stored with dessicanta in a sealed container.

Example II

The device prepared in Example I was used in an assay of glucose in whole blood as follows:

A drop of blood was applied to each of the five zones of the device from Example 1. After about two minutes, the blood was wiped away with a moist paper towel. When the blood specimen contained 120 mg/dl glucose, zones 1 and 2 became reddish-orange in color while 3-5 remained off-white.

The corresponding glucose concentration readout for zone segment is:

| Zone | Glucose, mg/dl |
|---|---|
| 1 | 60 |
| 2 | 120 |
| 3 | 180 |
| 4 | 240 |
| 5 | 300 |

Example III

A device for determining blood glucose was prepared as follow. Solutions A, B, C and D were prepared as indicated below:

Solution A

20% polyvinyl alcohol, 98% hydrolyzed (ave. MW 75K) in deionized water (hereinafter $H_2O$)

Solution B

4000 units glucose oxidase
2000 units horseradish peroxidase
600 units β-glucosidase
1 cc m-amino salicylic acid
(all in 20 cc $H_2O$)

Solution C

0.5% amygdalin (w/v) in 90% acetone/10% $H_2O$

Solution D

0.1 M sodium ascorbate (aq.)

Solutions $B_1$, $B_2$ and $B_3$ were then prepared as follows:

Solution $B_1$: 0.014 cc of Solution D was added to 5 cc of Solution B

Solution $B_2$: 0.042 cc of Solution D was added to 5 cc of Solution B

Solution $B_3$: 0.070 cc of Solution D was added to 5 cc of Solution B

Preparation of Quantitative Device

The paper strips prepared as above were sliced into 1/8" wide strips which were then used to prepare a quantitative device as in Example I, but containing three zones designated #1, #2 and #3, corresponding to the paper strip of the same number from which each zone was prepared.

Example IV

The device prepared in Example III was used in an assay of glucose in whole blood. The assay method of Example II was followed. Zone #1 turned red when the glucose concentration was above 80 mg/dl, #2 when above 110 mg/dl and #3 when above 140 mg/dl.

Example V

Device and Method for Analysis of Lactate Dehydrogenase (LDH) in Blood

$$NAD + Lactate \xrightarrow{(LDH)} Pyruvate + NADH$$

$$NADH + \underset{(oxidized)}{Methylene\ Blue} \longrightarrow NAD + \underset{(reduced)}{Methylene\ Blue}$$

$$\underset{(reduced)}{Methylene\ Blue} + O_2 \longrightarrow \underset{(oxidized)}{Methylene\ Blue} + H_2O_2$$

The above reaction sequences generate $H_2O_2$ at a rate proportional to LDH concentration when excess lactate and sufficient NAD are present.

When lactate and catalytic amounts of NAD and methylene blue are substituted for glucose oxidase in a test strip according to Example I, a device for measuring LDH in blood is produced.

Example VI

Use of the Device in Immunoassay for Pregnancy Determination

Purified antibodies to human corionic gonodatropin (HCG) are reacted with an anhydride containing polymer such as Gantrez AN 11% (available from GAF Corporation) in anhydrous dimethyl sulfoxide (DMSO) to couple the protein to the polymer. Mandelonitrile lyase is also coupled to the polymer at the same time. The mandelonitrile lyase is the internal time and temperature compensator. The coupling reaction requires from one to five days at room temperature depending on the concentration of reactants.

After the coupling reaction is completed, 1,4 butonediol is added to the DMSO solution as a crosslinking agent for the Gantrez.

Strips of Whatman #1 filter paper are immersed in the DMSO solution, removed and drained of excess solution and held under $N_2$ for about three to five days at room temperature until the crosslinking is completed.

The strips are then immersed in an aqueous 1M phosphate buffer at pH about 7.4 for three days to hydrolyze any unreacted anhydride remaining in the Gantrez backbone. Finally, the strips are immersed in a solution of sodium ascorbate, are dried under vacuum, mounted on plastic support pieces and cut into test strips.

A specimen of human urine is collected for analysis. Human HCG labeled with horseradish peroxidase is added

to the urine, and the test strip is placed in the urine sample for about 20 minutes. The test strip is removed and washed in .01m phosphate buffer at pH=7.

A developing solution is prepared containing $H_2O_2$, phenylenediamine, and mandelonitrile. Excess fluid is blotted from the test strip and several drops of developing solution are put onto the test strip. After about five minutes, the color of the paper test area is examined. A reddish-brown color is a negative indication for pregnancy while an unchanged test area is positive.

WHAT IS CLAIMED IS:

1. A device for determining the presence of an analyte in a fluid containing a first reagent capable of reacting with the analyte to produce a detectable reaction product which comprises a support member impregnated with a first catalyst for the production of the detectable reaction product and a compensator means for inhibiting the first catalyst at a predetermined rate.

2. A device of claim 1, wherein the catalyst is an enzyme and the analyte is a substrate of the enzyme.

3. A device of claim 1, wherein the compensator means comprises a second catalyst and the fluid contains a substrate for the second catalyst and a second reagent, the reaction of the second reagent with the substrate for the second catalyst producing an inhibitor of the first catalyst.

4. A device of claim 1, wherein the compensator means comprises a substrate for a second catalyst and the fluid contains the second catalyst and a second reagent, the reaction of the second reagent with the substrate for the second catalyst producing an inhibitor of the first catalyst.

5. A device of claim 1, wherein the compensator means comprises a second catalyst and a substrate for the second catalyst and the fluid contains a second reagent, the reaction of the second reagent with the substrate for the second catalyst producing an inhibitor of the first catalyst.

6. A device of claim 5, wherein the first catalyst is an enzyme, the second catalyst is mandelonitrile lyase, the substrate for the second catalyst is mandelonitrile, the second reagent is water and the inhibitor of the first catalyst produced by the reaction of the substrate for the second catalyst and the second reagent is cyanide ion.

7. A device of claim 5, wherein the first catalyst is an enzyme, the second catalyst is beta-glucosidase, the substrate for the second catalyst is amygdalin, the second reagent is water and the inhibitor of the first catalyst produced by the reaction of the substrate for the second catalyst and the second reagent is cyanide ion.

8. A device of claim 1, wherein the compensator means is an inhibitor of the first catalyst encapsulated in a suitable material capable of releasing the inhibitor at a predetermined rate after the device contacts the fluid.

9. A device of claim 1, wherein the detectable reaction product is a compound which absorbs visible light.

10. A device of claim 1 which is also impregnated with a second catalyst for the reaction of the detectable reaction product with an indicator reagent to produce a compound which absorbs visible light.

11. A device of claim 10 which is also impregnated with a suitable amount of the indicator reagent.

12. A device of claim 1 which is also impregnated with a known amount of a quantifying reagent which reacts with

the detectable reaction product.

13. A device of claim 12, wherein the first catalyst, the compensator means and the quantifying reagent are each present in at least two discrete zones on the support member and the amount of quantifying reagent in at least two zones is different.

14. A device of claim 1, wherein the device is a test strip.

15. A device of claim 1, wherein the impregnated support member is coated with a semipermeable membrane material.

16. A device for determining the presence of an enzyme for the reduction of a substrate of the enzyme with a first reagent to produce a detectable reaction product in a fluid containing the first reagent which comprises a support member impregnated with the substrate of the enzyme, an indicator reagent capable of reacting with the detectable reaction product to produce a compound which absorbs visible light, a catalyst for the reaction of the indicator reagent with the detectable reaction product and a compensator means for inhibiting the catalyst at a predetermined rate.

17. A device for determining the presence of an analyte in a fluid containing a first reagent capable of reacting with the analyte to produce a first reaction product which comprises a support member impregnated with a first catalyst for the production of the first reaction product, a second catalyst for the reaction of the first reaction product with an indicator reagent to produce a detectable compound, the indictor reagent and a compensator means for inhibiting the second catalyst at a

predetermined rate.

18. A device of claim 17, wherein the first reaction product is a peroxide and the second catalyst is a perooxidase enzyme which is inhibited by cyanide ion.

19. A device for determining the presence of an analyte in a fluid containing a first reagent capable of reacting with the analyte to produce a first reaction product and containing an indicator reagent which comprises a support member impregnated with a first catalyst for the production of the first reaction product, a second catalyst for the reaction of the first reaction product with an indicator reagent to produce a detectable compound and a compensator means for inhibiting the second catalyst at a predetermined rate.

20. A device for determining the presence of an enzyme for the reaction of a substrate of the enzyme with a first reagent to produce a detectable reaction product in a fluid containing the first reagent whcih comprises a support member impregnated with the substrate of the enzyme and a compensator means for inhibiting the enzyme at a predetermined rate.

21. A device for determining the presence of an analyte in a fluid containing a first reagent, a second reagent capable of reacting with the first reagent to produce a detectable reaction product, the second reagent being a substrate for a first catalyst for the production of the detectable reaction product and the first catalyst being bound to the analyte, the device comprising a support member impregnated with a biochemical reagent capable of binding to the analyte and a compensator means for inhibiting the first catalyst at a predetermined rate.

22. A device of claim 21, wherein the catalyst is bound to the analyte by means of an antibody.

23. A device of claim 21, wherein the catalyst is bound directly to a known amount of the analyte.

24. A device of claim 21, wherein the analyte is an antigen and the biochemical reagent is antibody to the antigen.

25. A device of claim 21, wherein the analyte is a ligand and the biochemical reagent is a receptor capable of binding the ligand.

26. A device of claim 21, wherein the analyte is a nucleic acid sequence and the biochemical reagent is a complementary sequence.

27. A device of claim 21, wherein the compensator means comprises a substrate for a second catalyst and the fluid contains the second catalyst and a second reagent, the reaction of the second reagent with the substrate for the second catalyst producing an inhibitor of the first catalyst.

28. A device of claim 21, wherein the compensator means comprises a second catalyst and a substrate for the second catalyst and the fluid contains a second reagent, the reaction of the second reagent with the substrate for the second catalyst producing an inhibitor of the first catalyst.

29. A device of claim 28, wherein the first catalyst is an enzyme, the second catalyst is mandelonitrile lyase,

the substrate for the second catalyst is mandelonitrile, the second reagent is water and the inhibitor of the first catalyst produced by the reaction of the substrate for the second catalyst and the second reagent is cyanide ion.

30. A device of claim 28, wherein the first catalyst is an enzyme, the second catalyst is beta-glucosidase, the substrate for the second catalyst is amygdalin, the second reagent is water and the inhibitor of the first catalyst produced by the reaction of the substrate for the second catalyst and the second reagent is cyanide ion.

31. A device of claim 21, wherein the compensator means is an inhibitor of the first catalyst encapsulated in a suitable material capable of releasing the inhibitor at a predetermined rate after the device contacts the fluid.

32. A device of claim 21, wherein the detectable reaction product is a compound which absorbs visible light.

33. A device of claim 31 which is also impregnated with a second catalyst for the reaction of the detectable reaction product with an indicator reagent to produce a compound which absorbs visible light.

34. A device of claim 33 which is also impregnated with a suitable amouant of the indicator reagent.

35. A device of claim 21 which is also impregnated with a known amount of a quantifying reagent which reacts with the detectable reaction product.

36. A device of claim 35, wherein the biochemical reagent, the compensator means and the quantifying reagent are each present in at least two discrete zones on the support member and the amount of quantifying reagent in at least two zones is different.

37. A device of claim 21, wherein the device is a test strip.

38. A device of claim 21, wherein the impregnated support member is coated with a semipermeable membrane material.

39. A method for determining the presence of an analyte in a fluid containing a first reagent capable of reacting with the analyte to produce a detectable reaction product which comprises:

a) contacting the fluid with a first catalyst for the reaction of the analyte with the first reagent to produce the detectable reaction product and with a compensator means for inhibiting the first catalyst under suitable conditions permitting the first catalyst to catalyze the reaction and permitting the compensator means to inhibit the first catalyst at predetermined rates;

b) detecting the presence of the detectable reaction product; and

c) determining thereby the presence of the analyte in the fluid.

40. A method of claim 39, wherein the detecting comprises reacting the detectable reaction product with an

indicator reagent to produce a compound which absorbs visible light and visually observing the appearance of the compound.

41. A method for determining the presence of an enzyme in a fluid containing a first reagent capable of reacting with a substrate of the enzyme to produce a detectable reaction product which comprises:

a) contacting the fluid with the substrate of the enzyme for the production of the detectable reaction product and with a compensator means for inhibiting the enzyme under suitable conditions for the enzyme to catalyze the reaction and the compensator means to inhibit the enzyme at predetermined rates;

b) detecting the presence of the detectble reaction product; and

c) determining thereby the presence of the enzyme in the fluid.

42. A method for determining the presence of an analyte at a concentration greater than a predetermined concentration in a fluid containing a first reagent capable of reacting with the analyte to produce a detectable reaction product which comprises:

a) contacting the fluid with a first catalyst for the production of the detectable reaction product, with a known amount of a quantifying reagent capable of reacting with and thereby consuming an amount of the detectable reaction product corresponding to the predetermined concentration of the analyte and with a compensator means for inhibiting the first catalyst under suitable

conditions permitting the first catalyst to catalyze the production fo the detectable reaction product, the quantifying reagent to consume the detectable reaction product and the compensating means to inhibit the first catalyst at predetermined rates;

b) detecting the presence of the detectable reaction product, and

c) determining thereby the presence of the analyte in the fluid at a concentration greater than the predetermined concentration.

43. A method for determining the presence of an analyte in a fluid containing a first reagent capable of reacting with a substrate of a first catalyst to produce a detectable reaction product which comprises:

a) adding to the fluid a first known amount of the first catalyst to which a second known amount of the analyte is bound;

b) contacting the resulting fluid with a biochemical reagent capable of binding to the analyte under suitable conditions so as to form a complex thereof;

c) separating the complex from uncomplexed first catalyst to which the analyte is bound;

d) contacting the complex with the substrate of the first catalyst and with a compensator means for inhibiting the first catalyst under suitable conditions permitting the first catalyst to catalyze the production of the detectable reaction product and the compensator means to inhibit the first catalyst at predetermined

rates;

e) detecting the presence of the detectable reaction product so produced; and

f) determining thereby the presence of the analyte in the fluid.

44. A method for determining the presence of an analyte in a fluid containing a first reagent capable of reacting with a substrate of a first catalyst to produce a detectable reaction product which comprises:

a) adding to the fluid a first known amount of the first catalyst to which a second known amount of the analyte is bound;

b) contacting the resulting fluid with a biochemical reagent capable of binding to the analyte under suitable conditions so as to form a complex thereof, the catalystic activity of the first catalyst bound to uncomplexed analyte being detectably greater than the catalytic activity of the first catalyst bound to complexed analyte;

c) contacting the fluid with the substrate of the first catalyst and with a compensator means for inhibiting the first catalyst to catalyze the production of the detectable reaction product and the compensator means to inhibit the first catalyst at predetermined rates;

d) detecting the presence of the detectable reaction product so produced;

e) comparing the amount of detectable reaction product

so detected with the amount produced in the absence of analyte; and

f) determining thereby the presence of the analyte in the fluid.

45. A method for determining the presence of an analyte in a fluid containing a first reagent capable of reacting with a substrate of a first catalyst to produce a detectable reaction product which comprises:

a) adding to the fluid a first biochemical reagent and the first catalyst to which a second biochemical reagent is bound, both biochemical reagents being capable of binding to the analyte under suitable binding conditions to produce a complex comprising the analyte bound to both biochemical reagents;

b) separating the complex from uncomplexed first catalyst to which the second biochemical reagent is bound;

c) contacting the complex with the substrate of the first catalyst in the presence of a compensator means for inhibiting the first catalyst under suitable conditions permitting the first catalyst to catalyze the production of the detectable reaction product and the compensator means to inhibit the first catalyst at predetermined rates;

d) detecting the presence of the detectable reaction product so produced; and

e) determining thereby the presence of the analyte in the fluid.

46. A method for determining the presence of an analyte in a fluid containing a first reagent capable of reacting with the analyte to produce a detectable reaction product which comprises contacting a device of claim 1 with the fluid, removing the device from the fluid and detecting the presence of the detectable reaction product on the device.

47. A method for controlling the catalytic reaction of a reagent and a substrate for a catalyst which comprises contacting the reagent with the substrate for the catalyst, the catalyst and a compensator means under suitable conditions permitting the catalyst to catalyze the reaction and the compensator means to inhibit the catalyst at predetermined rates.

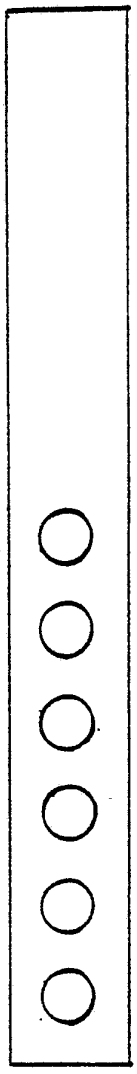


FIG. 1A

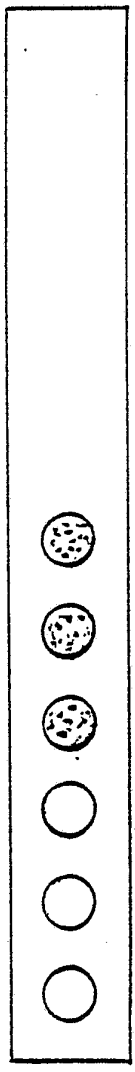


FIG. 1B

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US84/01665

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.[3] C12Q 1/00,1/68,1/54,1/28; G01N 33/54; C12N 9/00,9/99
US 435/4,6,7,14,18,28,183,184,192,805;436/501,530,531,536,537,538,810,

## II. FIELDS SEARCHED

Minimum Documentation Searched [4]

| Classification System | Classification Symbols |
|---|---|
| U.S. | 435/4,6,7,14,18,28,183,184,192,805<br>436/501,530,531,536,537,538,810,814,818,34,37<br>422/56 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Chemical Abstracts (Enzymes-inhibition and inhibitors) 1976-1983
Lexpat

## III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

| Category * | Citation of Document, [16] with indication, where appropriate, of the relevant passages [17] | Relevant to Claim No. [18] |
|---|---|---|
| X | US,A, 4,038,485, published 26 July 1977 Johnston et al. | 1,2,8-11,14, 17-19,39,40, 46,47 |
| Y | US,A, 4,038,485, published 26 July 1977 Johnston et al. | 3-7,12,13,15, 16,20-38,41-45 |
| Y | US,A, 4,059,407, published 22 November 1977 Hochstrasser | 12,13,16,20, 35,36,41,42 |
| Y | US,A, 3,964,871, published 22 June 1976 Hochstrasser | 12,13,35,36, 42 |
| Y | US,A, 3,092,465, published 04 June 1963 Adams et al. | 15,38 |
| Y | US,A, 4,256,693, published 17 March 1981 Kondo et al. | 15,38 |
| Y | US,A, 3,814,668, published 04 June 1974 Blake et al. | 15,38 |

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search [2] | Date of Mailing of this International Search Report [2] |
|---|---|
| 28 December 1984 | 04 JAN 1985 |
| **International Searching Authority [1]** | **Signature of Authorized Officer [20]** |
| ISA/US | |

Form PCT/ISA/210 (second sheet) (October 1981)

# INTERNATIONAL SEARCH REPORT

PCT/US84/01665

International Application No



**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

**II. FIELDS SEARCHED**

Minimum Documentation Searched [4]

| Classification System | Classification Symbols |
|---|---|
| US Cl. Continued | 814,818,34,37: 422/56 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

**III. DOCUMENTS CONSIDERED TO BE RELEVANT** [14]

| Category * | Citation of Document, [16] with indication, where appropriate, of the relevant passages [17] | Relevant to Claim No. [18] |
|---|---|---|
| | | |

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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|---|---|
| | |
| **International Searching Authority** [1] | **Signature of Authorized Officer** [20] |
| | |

**FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET**

| | | |
|---|---|---|
| Y | N, Enzymes, issued 1964, Dixon et al., New York, Academic Press, pp.. 337-340,766-767 | 6,18,29 |
| Y | N, Enzyme-Immunoassay, issued 1980, Maggio editor, Boca Raton, Florida, CRC Press, pages 106-108, 183, 184 | 21-25,31-34, 37, 43-45 |
| Y | US,A, 4,390,343, published 28 June 1983 Walter | 21-25,31-34, 37, 44 |

**V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]**

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers....., because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

**VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]**

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, [16] with indication, where appropriate, of the relevant passages [17] | Relevant to Claim No [18] |
|---|---|---|
| Y,T | US,A, 4,442,204, published 10 April 1984, Greenquist et al. | 21-26,31-34, 37, 44 |
| Y | US,A, 4,358,535, published 09 November 1982 Falkow et al. | 26 |
| Y | US,A, 3,817,837, published 18 June 1974 See Col. 8, lines 38-41 and Col. 36, lines 35-36, Rubenstein et al. | 26 |
| A | EP,A, 0075293 , published 30 March 1983 | 1-47 |
| A | US,A, 4,341,866, published 27 July 1982 Yoshida | 1-47 |
| A | US,A, 4,218,535, published 19 August 1980, Ray | 1-47 |
| A | US,A, 4,234,680, published 18 November 1980 Hevey et al. | 1-47 |