

nitrophenyl)-5-phenyl(-2H tetrazolium (NBT), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium (MTT), 2-phenyl-3-(4-carboxyphenyl)-5-methyl tetrazolium (PCPM), tetrazolium blue (TB), thiocarbonyl nitroblue tetrazolium (TCNBT), tetranitroblue tetrazolium (TNBT), tetrazolium violet, (TV), 2-benzothiazothiazolyl-3-(4-carboxy-2-methoxyphenyl)-5-[4-(2-sulfoethylcarbamoyl)phenyl]-2H-tetrazolium (WST-4), and 2,2'-dibenzothiazolyl-5,5'-bis [4-di(2-sulfoethyl)carbamoylphenyl]-3,3'-(3,3'-dimethoxy-4,4'-biphenylene)ditetrazolium, disodium salt (WST-5). WST-5 is preferred, because it readily dissolves in an aqueous medium, which is most compatible with biological samples. Further, the resulting formazan compound exhibits strong spectral absorption at the purple-blue region, thus reducing the need for correcting the background signal from hemoglobin.

Finally, a hemoglobin suppressor is present in the reagent to curtail the undesirable dye-forming reaction between hemoglobin and the tetrazolium compound. The role of the hemoglobin suppressor is to oxidize the hemoglobin to methemoglobin, which does not react with the tetrazolium. Surprisingly, nitrite salts, such as sodium nitrite, potassium nitrite, and their derivatives, are very effective in suppressing the hemoglobin, while not destroying the NADH. The nitrites are effective, as well, at elevated temperature and with high hematocrit samples. Sodium nitrite is preferred, because it has high aqueous solubility, is not toxic, and is relatively inexpensive.

Although the reagent of this invention can be used in a wet chemical mode, such as in a cuvette, in a preferred embodiment, the invention is a dry strip for assaying beta-hydroxybutyrate in whole blood. It consists of a membrane test pad, preferably of nylon, that is placed between a support and a top layer. The support is preferably of polyester sheet. The top layer can be any bibulous material known in the art. A preferred material is a porous polyethylene treated with sodium methyl oleoyl taurate, available from the Porex Corp. of Fairburn, Ga. We refer to this material as "Porex". The test pad contains a reagent comprising beta-hydroxybutyrate dehydrogenase, NAD, diaphorase, and WST-5 (Table 1, below). The Porex top layer contains a nitrite reagent (Table 2).

In operation, a user applies a drop of whole blood to the upper surface of the Porex top layer. As the whole blood or lysed blood comes into contact with the Porex, the sodium nitrite is reconstituted and reacts with the available free hemoglobin, thus rendering the hemoglobin harmless to the assay. The resulting, substantially hemoglobin-free sample is transferred to the test pad below, via capillary or gravitational force. On the test pad, the sample initiates the cascade reaction depicted in FIG. 4 to yield a colored dye, whose concentration is proportional to the beta-hydroxybutyrate in the sample and can be determined directly with a photometer.

FIG. 5 depicts the effect of nitrite on the color-forming reaction in this system, using blood samples containing 0 and 15 mg/dL. In the absence of nitrite, hemoglobin reduces the tetrazolium to form a continually increasing dye concentration, with a corresponding increase in optical density. Nitrite, by removing the hemoglobin (by oxidation), limits the color formation to that which results solely from the ketone bodies (i.e., beta-hydroxybutyrate) in the sample.

The following example demonstrates a preferred embodiment of the present invention, in which the analyte is beta-hydroxybutyrate and the enzyme is beta-hydroxybutyrate dehydrogenase. The composition can readily be modified for application to other analyte-enzyme combinations listed earlier. (See, for example, *Tietz Textbook of Clinical Chemistry*, 2<sup>nd</sup> Ed., ed. by C. Burtis et al., W. B.

Saunders Co., Philadelphia, Pa., 1994, pp 976-978 and 1174-1175.) The Example is not intended to be in any way limiting.

#### EXAMPLE 1

A 0.8  $\mu$ m nylon membrane obtained from Cuno (Meriden, Conn., USA) was dipped into the reagent of Table 1, until saturated. The excess reagent was scraped off gently with a glass rod. The resulting membrane was hung to dry in a 56° C. oven for 10 minutes. Porex (0.6 mm thick) was soaked in the nitrite solution of Table 2 and then hung to dry in a 100° C. oven for ten hours. Finally, the membrane was laminated between a polyester stock (0.4 mm Melenex® polyester from ICI America, Wilmington, Del.) and the nitrite-impregnated Porex.

TABLE 1

Reagent for the Test Pad	
Components	Quantity
Water	100ml
Tris(hydroxymethyl)Aminomethane (MW 121, Sigma, St. Louis, MO, USA) (Adjust pH to 8.5 by adding 6M HCl)	1.2gm
Sodium Chloride (MW 56.44, Sigma, St. Louis, MO, USA)	560mg
Magnesium Chloride (MW 203, Sigma, St. Louis, MO, USA)	2.5gm
PSSA, polystyrenesulfonic acid, sodium salt (MW 70,000, Polysciences, Inc., Warrington, PA, USA)	3gm
Croton (Croda Inc. Parsippany, NJ, USA)	3gm
Oxamic acid, sodium salt (MW 111.03, Aldrich Chemicals, Milwaukee, WI, USA)	250mg
Tetronic 1307 (BASF Corporation, Mount Olive, New Jersey, USA)	2gm
Sucrose (MW 342.30, Aldrich Chemicals, Milwaukee, WI, USA)	5gm
NAD (MW 663.4, N-7004, Sigma, St. Louis, MO, USA)	450mg
D-3-hydroxybutyrate dehydrogenase (Origin: Pseudomonas sp., HBD-301, 125 U/mg, Toyobo, Japan)	50,000U
Diaphorase (Origin: <i>B. Stearothermophilus</i> , New, 1033 U/mg, Toyobo, Japan)	340890U
WST-5 (MW 1331.37, Dojindo, Japan)	1.8gm

TABLE 2

Nitrite Reagent	
Components	Quantity
10 mM Phosphate Buffer Saline, pH 7.4, (P-3813, Sigma, St. Louis, MO, USA)	70ml
Ethanol	30ml
Sodium Nitrite (MW 69, Aldrich Chemicals, Milwaukee, WI, USA)	5gm
Polyvinylpyrrolidone (MW 40,000, Sigma, St. Louis, MO, USA)	200mg
Oxamic acid, sodium salt (MW 111.03, Aldrich Chemicals, Milwaukee, WI, USA)	500mg

We claim:

1. A reagent for measuring a concentration of an analyte in a hemoglobin-containing biological fluid, comprising
  - a) a dehydrogenase enzyme that has specificity for the analyte,
  - b) nicotinamide adenine dinucleotide (NAD) or an NAD derivative,
  - c) a tetrazolium dye precursor,
  - d) a diaphorase enzyme or an analog thereof, and
  - e) a nitrite salt.
2. The reagent of claim 1 in which the analyte is beta-hydroxybutyrate and the enzyme is beta-hydroxybutyrate dehydrogenase.