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DETERMINATION OF DEHYDROGENASE

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ABSTRACT OF THE DISCLOSURE

Concentration of nicotinamide—adenine nucleotide dependent enzymes determined in a body fluid by incubating said fluid in presence of reduced substrate, an oxidised coenzyme, a divalent cation and a reducible color developing metal chelate.

The present application is a continuation-in-part of our patent application S.N. 360,420, filed Apr. 16, 1964, and now abandoned.

The present invention relates to a method of determining nicotinamide-adenine nucleotide dependent enzymes in body fluids.

Nicotinamide-adenine nucleotide dependent enzymes are found in almost all body tissues and fluids. It is known that the alteration in the normal concentration of a constituent of blood serum is very commonly indicative of disease. Recently, tests for enzymes in body tissues and fluids have found application in clinical biochemistry. Where applied to a specific body tissue, an abnormally high enzyme level in the tissue frequently is a sign of disease in that tissue. Although an increase in the enzyme level of body fluids does not pinpoint disease in a specific body tissue, it does serve as an aid to diagnosis which is made in conjunction with other tests and clinical observations.

Diagnostic enzyme tests are usually performed with blood serum since this body fluid is easily obtained, but enzyme assays of other body fluids are also useful.

In the normal individual, the circulating plasma levels of intracellular enzymes are probably governed by several mechanisms. Normal levels are believed to arise from a balance between release from body cells and excretion or inactivation. During disease, however, a considerable increase in circulating levels may occur as a result of the alteration of cell membrane permeability with the consequent release of intracellular enzymes into the extracellular fluid. Severe tissue damage accompanied by necrosis may produce a similar effect on the level of circulating enzymes.

At the present time, there is considerable speculation with regard to the origin and fate of serum enzymes. Although the factors which regulate serum enzyme levels are only poorly understood, this lack of knowledge has not impeded the purely empirical development of diagnostic enzyme tests.

More than one hundred nicotinamide-adenine nucleotide dependent enzymes have been identified. Representative of such enzymes are L-malate dehydrogenase, L-lactate dehydrogenase, α-hydroxybutyrate dehydrogenase, D-sorbitol dehydrogenase, isocitric dehydrogenase and alcohol dehydrogenase. An abnormally elevated serum level of lactate dehydrogenase occurs in cases of myocardial infarction. Increased lactate dehydrogenase activity has also been reported in acute liver diseases, muscular dystrophy, neoplastic diseases, pernicious anemia and renal diseases and increased isocitrate dehydrogenase activity is a good indication of liver damage.

Enzyme catalyzed oxidation-reduction reactions which require the participation of nicotinamide-adenine nucleotide coenzymes may be formulated as follows:

$$\text{reduced substrate} + \text{oxidized coenzyme} \underset{}{\overset{\text{enzyme}}{\rightleftharpoons}} \text{oxidized substrate} + \text{reduced coenzyme}$$

Since these reactions are reversible, valid measurement of enzyme activity may be performed regardless of the direction in which a specific reaction is carried out. The initial rate of the reaction is directly proportional to the concentration of the enzyme.

It is an object of the present invention to provide a simple colorimetric test for the determination of nicotinamide-adenine nucleotide dependent enzymes in body fluids.

Other objects and advantages of this invention will become apparent from the following detailed description.

According to the present method, an acidic solution of a readily reducible metal chelate compound is added to a reaction mixture in which a reduced nicotinamide-adenine nucleotide has been allowed to accumulate under standardized conditions as a product of enzyme catalyzed reaction between reduced substrate and oxidized coenzyme. The enzyme reaction is stopped by reduction of pH and the reduced nicotinamide-adenine nucleotide coenzyme reacts with the oxidized metal chelate to form a reduced metal chelate compound. The oxidized metal chelate chosen is one which has an adsorption spectrum which differs appreciably from the adsorption spectrum of the reduced metal chelate, thus providing a simple colorimetric method for the determination of enzyme activity.

The oxidized nicotinamide-adenine nucleotide coenzyme which is reduced may be nicotinamide-adenine dinucleotide, nicotinamide-adenine dinucleotide phosphate or suitable derivatives of these compounds. The choice of the reduced substrate portion of the reaction mixture is dependent upon the nicotinamide-adenine nucleotide dependent enzyme which is being tested for. By reduced substrate is meant the substance which is acted upon by the enzyme under test. Such substrates include lactic acid and lactic acid salts, malic acid and malic acid salts, isocitric acid and isocitric acid salts, α-hydroxybutyric acid and α-hydroxybutyric acid salts, sorbital and ethanol.

The enzyme catalyzed reaction is carried out in a solution which is buffered to a pH within a range which gives measurable enzyme activity. For example, the pH range in which the activity of lactate dehydrogenase is most marked is between about 7.5 and about 10.5, preferably about 10.0. The reaction is permitted to proceed until a measurable amount of the reduced nicotinamide-adenine nucleotide coenzyme is formed. This will be between about 5 to about 20 minutes at a temperature of about 37° C. The reaction is then stopped by acidifying the reaction mixture to reduce to pH to a range of from about 2.8 to about 5.0, preferably about 3.2. The solution used to stop the reaction may contain a color developing metal chelate, or the color developing metal chelate may be added after the reaction is stopped.

The chelate system used should be such that (1) the oxidized system will be reduced by the reduced nicotinamide-adenine dinucleotide, and (2) there be a significant difference in some property, such as light absorption, between the oxidized and reduced chelate.

Color developing metal chelates which have been found to be useful include ferri chelates of 1,10-phenanthroline and sulfonated 4,7-diphenyl-1,10-phenanthroline, cupric chelates of 2,9-dimethyl-1,10-phenanthroline and sulfonated 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline.

The formation of reduced nicotinamide-adenine dinucleotide ($NADH_2$) is illustrated by the following equations wherein nicotinamide-adenine nucleotide dependent enzymes catalyze the reversible oxidation of the substrate

in the presence of nicotinamide-adenine dinucleotide (NAD):

(1) Malate dehydrogenase

$$\text{L-malate} + \text{NAD} \rightleftharpoons \text{oxalacetate} + \text{NADH}_2$$

(2) Alcohol dehydrogenase

$$\text{ethanol} + \text{NAD} \rightleftharpoons \text{acetaldehyde} + \text{NADH}_2$$

(3) Lactate dehydrogenase

$$\text{L-lactate} + \text{NAD} \rightleftharpoons \text{pyruvate} + \text{NADH}_2$$

Isocitrate dehydrogenase is representative of an enzyme which requires the coenzyme nicotinamide-adenine dinucleotide phosphate (NADP) to carry out the oxidation reaction. In addition, the enzyme is activated by certain divalent cations. The most effective divalent cation tested is the manganous ion (Mn^{++}). The equation for this reaction is as follows:

$$\text{(+)-isocitrate} + \text{NADP} \overset{Mn^{++}}{\rightleftharpoons} \alpha\text{-ketoglutarate} + CO_2 + \text{NADPH}_2$$

NAD is also known as coenzyme I and NADP is also known as coenzyme II.

The following examples are intended to illustrate, but not to limit, the scope of the present invention:

EXAMPLE I

Lactate dehydrogenase

Reagent A is prepared having the following composition:

	Gm.
Epsilon-aminocaproic acid	0.346
Sodium epsilon-aminocaproate	0.087
Lithium lactate	0.461
Nicotinamide-adenine dinucleotide (NAD)	0.048

Reagent B is prepared having the following composition:

	Gm.
Epsilon-aminocaproic acid	0.346
Sodium epsilon-aminocaproate	0.087
Lithium lactate	0.461

Reagents A and B are each dissolved in 32 ml. of water to give a pH of 10.0.

To 5 ml. of each of reagents A and B is added 0.10 ml. of the serum sample to be tested. The reaction is permitted to proceed at 37° C. for exactly 12 minutes.

The reaction is stopped by the addition to each of the reaction mixtures of 5 ml. of reagent C which has the following composition:

	Gms. per liter of solution
Copper sulfate pentahydrate	0.25
2,9-dimethyl-1,10-phenanthroline	0.833
Neutronyx	0.50
Sulfuric acid	11.30

The addition of reagent C reduces the pH of the solutions to 3.2. The reaction mixtures are kept at 37° C. for 12 minutes.

The optical density of each of the reaction mixtures is read using a spectrophotometer set at wavelength 455 mμ.

The units of lactate dehydrogenase per milliliter are read from a calibration curve prepared in accordance with the procedure set out in Example II.

EXAMPLE II

Preparation of calibration curve for use in lactate dehydrogenase activity determination

For the determination of the lactate dehydrogenase activity of serum, a calibration curve must be prepared using known concentrations of enzyme. Enzyme activities are expressed in terms of a lactate dehydrogenase unit defined as the amount of enzyme which catalyzes the conversion of one micromole NAD to one micromole of $NADH_2$ per minute during the initial stage of the reaction carried out in the presence of very high concentrations of DL-lactate and NAD at pH 10.0 and 25° C. in a 0.1 M glycine-sodium glycinate buffer.

A series of solutions of known concentrations of lactate dehydrogenase activity are prepared from a lyophilized preparation of dialyzed human plasma having an elevated lactate dehydrogenase activity. Each of the solutions is assayed according to the following procedure.

At zero time, 0.10 ml. of enzyme solution is mixed with 5.0 ml. of buffered substrate solution having a temperature of 37° C. The buffered substrate solution contains 0.15 M DL-lactate, 0.0021 M NAD, and 0.1 M epsilon-aminocaproate buffer. The substrate solution has a pH of 10.0. The mixture is incubated for 12 minutes at 37° C. 5.0 ml. of a solution containing 0.115 M sulfuric acid, 0.004 M 2,9-dimethyl-1,10-phenanthroline, 0.001 M cupric sulfate, and 0.05% neutronyx is added. The mixture is permitted to stand at 37° C. for 12 minutes and the absorbance of the solution is measured at 455 mμ against a blank prepared in the identical manner, except that the NAD is omitted from the buffered substrate.

A calibration curve is prepared from the data in the following table:

Enzyme concentration, units per ml.:	Measured absorbance
0	0.012
0.04	0.130
0.08	0.255
0.16	0.488
0.32	0.823
0.63	1.319

EXAMPLE III

Malate dehydrogenase

At zero time 0.2 ml. of enzyme solution is mixed with 5.0 ml. of buffered substrate solution having a temperature of 37° C. The buffered substrate solution contains 0.10 M DL-malate, 0.002 M NAD and 0.10 M epsilon-aminocaproate buffer. The substrate solution has a pH of 10.0. The mixture is incubated for 12 minutes at 37° C. The $NADH_2$ formed is determined by mixing 1.0 ml. of the incubation mixture with 4.0 ml. of solution containing 0.25 M acetic acid, 0.175 M sodium acetate, 0.115 N sulfuric acid, 0.0005 M cupric sulfate and 0.002 M 2,9-dimethyl-1,10-phenanthroline. After fifteen minutes, the absorbance of the solution is measured at 455 mμ against a blank prepared in an identical manner, except that the NAD is omitted from the buffered substrate solution.

The assay of varying amounts of crystalline malic dehydrogenase give the following results:

Enzyme per test, μg.:	Absorbance at 455 mμ
0	0.025
0.09	0.108
0.19	0.179
0.28	0.260
0.46	0.420

EXAMPLE IV

Alcohol dehydrogenase

At zero time 0.2 ml. of enzyme solution is mixed with 5.0 ml. of buffered substrate solution having a temperature of 37° C. The buffered substrate solution contains 0.10 M ethanol, 0.002 M NAD, and 0.10 M epislon-aminocaproate buffer. The substrate solution has a pH of 10.0. The mixture is incubated for 10 minutes at 37° C. The $NADH_2$ formed is determined by mixing 1.0 ml. of the incubation mixture with 4.0 ml. of solution containing 0.25 M acetic acid, 0.175 M sodium acetate, 0.115 N sulfuric acid, 0.0005 M cupric sulfate and 0.002 M 2,9-dimethyl-1,10-phenanthroline. The mixture is permitted to stand for 15 minutes and the absorbance of the solution is measured at 455 mμ against a blank prepared in the

identical manner, except that the NAD is omitted from the buffered substrate.

Known concentrations of crystalline yeast alcohol dehydrogenase are prepared in pooled normal human serum and assayed to give the following results:

Enzyme per test, μg.:	Absorbance at 455 mμ
0	0.007
0.52	0.062
1.04	0.143
1.56	0.186
2.08	0.238
2.60	0.321

EXAMPLE V

Isocitrate dehydrogenase

At zero time 0.2 ml. of enzyme solution is mixed with 2.8 ml. of buffered substrate solution containing 0.0035 M trisodium isocitrate, 0.15 M sodium chloride, 0.0017 M manganese chloride, 0.0018 M NADP and 0.054 M tris (hydroxymethyl)-aminomethane. The pH of the solution is 7.5. The mixture is incubated at 37° C. for 12 minutes. The $NADPH_2$ formed is determined by the addition of 3.0 ml. of a solution containing 0.23 N surfuric acid, 0.001 M cupric sulfate and 0.004 M 2,9-dimethyl-1,10-phenanthroline. The mixture is allowed to stand for 12 minutes at 37° C. and the absorbance is measured at 455 mμ against a blank prepared in the identical manner except that the NADP is omitted from the buffered substrate solution.

Aliquots of a human liver homogenate diluted with serum are assayed for isocitrate dehydrogenase activity with the following results:

Relative concentration homogenate:	Absorbance at 455 mμ
0	0.003
1	0.242
2	0.450
4	0.770
8	0.875

While the foregoing examples illustrate the use of the cupric chelate of 2,9-dimethyl-1,10-phenanthroline as the reducible color developing metal chelate, the reduction of other reducible color developing metal chelates by reduced nicotinamide-adenine dinucleotide ($NADH_2$) are illustrated in the following examples:

Example VI

A solution containing 0.01 M ferric chelate of 2,9-dimethyl-1,10-phenanthroline in 0.5 M acetate buffer, pH 4.0 is prepared. 0.2 ml. of this solution is diluted with 5.0 ml. of 0.5 M acetate buffer, pH 4.0, and 0.1 ml. of 0.01 M $NADH_2$ is added. The characteristic absorption spectrum of ferrous 4,7-diphenyl-1,10-phenanthroline is observed in the resulting solution. The final absorbance of the mixture, at the absorption maximum of 525 mμ, is recorded as 2.378 when compared with the blank solution which was of similar composition except that the NADH was not added.

Example VII

A 0.2 ml. aliquot of an aqueos solution 0.01 M in the ferric chelate of 1,10-phenanthroline is added to 4.5 ml. of 0.5 M acetate buffer, pH 4.0. This solution is mixed with 0.5 ml. of a solution containing 1.74×10^{-4} M $NADH_2$, and the absorbance of the resulting mixture is recorded at 510 mμ, the absorption maximum of ferrous 1,10-phenanthroline. A maximum absorbance of 0.351 was recorded after 30 min.

Example VIII

A 0.2 ml. aliquot of an aqueous solution 0.01 M in the ferric chelate of sulfonated 4,7-diphenyl-1,10-phenanthroline was added to 4.5 ml. of 0.5 M acetate buffer, pH 4.0. This solution was mixed with 0.5 ml. of a solution containing 1.74×10^{-4} M $NADH_2$, and the absorbance of the resulting mixture was recorded at 525 mμ, the absorption maximum of the ferrous chelate of sulfonated 4,7-diphenyl-1,10-phenanthroline. A maximum absorbance of 0.611 was recorded after 24 min.

Example IX

A 0.2 ml. aliquot of an aqueous solution 0.01M in the cupric chelate of 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline is added to 4.5 ml. of 0.5 M acetate buffer, pH 4.0. This solution is mixed with 0.5 ml. of a solution containing 1.74×10^{-4} M $NADH_2$, and the absorbance of the resulting mixture is recorded at 480 mμ, the absorption maximum of the cuprous chelate of 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline. A maximum absorbance of 0.400 is recorded after 15 min.

What is claimed is:

1. A method of determining the concentration in body fluid of nicotinamide-adenine nucleotide dependent enzymes which comprises incubating in a small volume of body fluid a buffered reduced substrate selected from the group consisting of lactic acid and lactic acid salts, malic acid and malic acid salts, α-hydroxybutyric acid and α-hydroxybutyric acid salts, sorbitol, isocitric acid and isocitric acid salts and ethanol, and an oxidized coenzyme selected from the group consisting of nicotinamide-adenine dinucleotide and nicotinamide-adenine dinucleotide phosphate and a divalent cation, reducing the pH of the reaction mixture to a pH between about 2.8 and about 5.0 and adding a reducible color developing metal chelate selected from the group consisting of ferri chelates of 1,10-phenanthroline, 4,7-diphenyl-1,10-phenanthroline and sulfonated 4,7-diphenyl-1,10-phenanthroline and cupric chelates of 2,9-dimethyl-1,10-phenanthroline and sulfonated 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline.

2. A method according to claim 1 of determining the concentration in body fluid of lactate dehydrogenase wherein the substrate is selected from the group consisting of lactic acid and lactic acid salts.

3. A method according to claim 1 of determining the concentration in body fluid of malate dehydrogenase wherein the substrate is selected from the group consisting of malic acid and malic acid salts.

4. A method according to claim 1 of determining the concentration in body fluid of alcohol dehydrogenase wherein the substrate is ethanol.

5. A method according to claim 1 of determining the concentration in body fluid of isocitrate dehydrogenase wherein the substrate is selected from the group consisting of isocitric acid and isocitric acid salts.

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