

## **Stabilization of dehydrogenases with stable coenzymes**

### **Description**

The present invention relates to a method for stabilizing an enzyme by storing the enzyme in the presence of a stable coenzyme. The present invention further relates to an enzyme stabilized with a stable coenzyme, and to the use thereof in test elements for detecting analytes.

Biochemical measurement systems are important constituents of clinically relevant methods of analysis. The priority here is to measure analytes, e.g. metabolites or substrates, which are determined directly or indirectly with the aid of an enzyme. The analytes are in this case converted with the aid of an enzyme-coenzyme complex and then quantified. This entails the analyte to be determined being brought into contact with a suitable enzyme and a coenzyme, with the enzyme usually being employed in catalytic amounts. The coenzyme is changed, e.g. oxidized or reduced, by the enzymatic reaction. This process can be detected directly, or electrochemically or photometrically through a mediator. A calibration provides a direct relationship between the measurement and the concentration of the analyte to be determined.

Coenzymes are organic molecules which are bound covalently or non-covalently to an enzyme and which are changed by the conversion of the analyte. Prominent examples of coenzymes are nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), respectively, from which NADH and NADPH, respectively, are produced by reduction.

Measurement systems known in the prior art are notable for being stable for a limited period and for the specific requirements on the environment, such as cooling or dry storage, for achieving this stability. For particular applications, e.g. tests carried out by the final user himself, such as, for example, in the self-monitoring of blood glucose, it is therefore possible for incorrect results to occur through incorrect, unnoticed faulty storage. The exhaustion of desiccants through the primary packaging being opened for too long in particular may lead to faulty measurements which, with some systems, can scarcely be identified by the user.

One known measure employed to increase the stability of biochemical measurement systems is the use of stable enzymes, e.g. the use of enzymes from thermophilic organisms. A further possibility is to stabilize enzymes by chemical modification, e.g. crosslinking, or by mutagenesis. In addition, enzyme stabilizers such as, for example, trehalose, polyvinylpyrrolidone and serum albumin can also be added, or the enzymes can be enclosed e.g. by photopolymerization in polymer networks.

Attempts have also been made to improve the stability of biochemical measurement systems by using stable mediators. Thus, the specificity of tests is increased, and interference during the reaction is eliminated, through the use of mediators with a redox potential which is as low as possible. However, the redox potentials of the enzyme/coenzyme complexes form a lower limit for the redox potential of mediators. Below these potentials, the reaction with the mediators is slowed down or even stopped.

An alternative possibility is also to use biochemical

measurement systems without mediators, in which for example there is direct detection of coenzymes, e.g. of the coenzyme NADH. One disadvantage of such measurement systems is, however, that coenzymes such as NAD and NADP are unstable.

NAD and NADP are base-labile molecules whose degradation pathways are described in the literature (N.J. Oppenheimer in *The Pyridine Nucleotide Coenzymes*, Academic Press New York, London 1982, editors J. Everese, B. Anderson, K. You, Chapter 3, pages 56-65). The degradation of NAD and NADP, respectively, essentially results in ADP-ribose through cleavage of the glycosyl linkages between the ribose and the pyridine unit. The reduced forms NADH and NADPH are on the other hand acid-labile: e.g. epimerization is one known degradation pathway. In both cases, the instability of NAD/NADP and NADH/NADPH derives from the lability of the glycosyl linkage between the ribose unit and the pyridine unit. However, even under conditions which are not drastic, such as, for example, in aqueous solution, the coenzymes NAD and NADP, respectively, are hydrolysed solely through the ambient moisture. This instability may lead to inaccuracies in the measurement of analytes.

A number of NAD/NADP derivatives is described for example in B.M. Anderson in *The Pyridine Nucleotide Coenzymes*, Academic Press New York, London 1982, editors J. Everese, B. Anderson, K. You, Chapter 4. Most of these derivatives are, however, not well accepted by enzymes. The only derivative which has to date therefore been used for diagnostic tests is 3-acetylpyridine adenine dinucleotide (acetyl NAD) which was described for the first time in 1956 (N.O. Kaplan, *J. Biol. Chem.* (1956), 221, 823). Also this coenzyme shows a poor acceptance by enzymes and a

change in the redox potential.

WO 01/94370 describes the use of further NAD derivatives with a modified pyridine group. Modifications of the nicotinamide group have, however, in general a direct influence on the catalytic reaction. In most cases, this influence is negative.

In a further idea for stabilization, the ribose unit has been altered in order thereby to influence the stability of the glycosyl linkage. This procedure does not directly interfere with the catalytic reaction of the nicotinamide group. However, there may be an indirect influence as soon as the enzyme exhibits a strong and specific binding to the ribose unit. Kaufmann et al. disclose in this connection in WO 98/33936 and US 5,801,006, and in WO 01/49247, respectively, a number of thioribose-NAD derivatives. A connection between the modification of the nicotinamide-ribose unit and the activity of the derivatives in enzymatic reactions has, however, not been shown to date.

CarbaNAD, a derivative without a glycosyl linkage, was described for the first time in 1988 (J.T. Slama, Biochemistry 1989, 27, 183 and Biochemistry 1989, 28, 7688). The ribose therein is replaced by a carbocyclic sugar unit. Although carbaNAD was described as a substrate of dehydrogenases, its activity has not to date been demonstrated clinically in biochemical detection methods.

A similar approach was described later by G.M. Blackburn, Chem. Comm., 1996, 2765, in order to prepare carbaNAD with a methylenebisphosphonate compound instead of the natural pyrophosphate. The methylenebisphosphonate shows increased stability

towards phosphatases and was used as inhibitor of ADP-ribosyl cyclase. An increase in hydrolysis stability was not the aim (J.T. Slama, G.M. Blackburn).

WO 2007/012494 and US 11/460,366 disclose stable NAD/NADH and NADP/NADPH derivatives, respectively, enzyme complexes of these derivatives and the use thereof in biochemical detection methods and reagent kits.

The object on which the present invention was based was to provide methods for stabilizing enzymes, especially for the long-term stabilization of enzymes.

This object is achieved by a method for stabilizing an enzyme, where the enzyme is stored in the presence of a stable coenzyme. It has surprisingly been found that long-term stabilization of several weeks or months at high relative moisture or even in liquid phase and at elevated temperatures is possible with the aid of a stable coenzyme. This perception is surprising because it is known that although enzymes have an increased short-term stability for some hours in the presence of native coenzyme (Bertoldi et al., *Biochem. J.* 389, (2005), 885-898; van den Heuvel et al., (*J. Biol. Chem.* 280 (2005), 32115-32121; and Pan et al., (*J. Chin. Biochem. Soc.* Vol. 3 (1974), pp. 1-8), they show a lower stability over a longer period (*Nutrition Reviews* 36 (1978), 251-254).

Compared with these perceptions in relation to the prior art, it was surprising that an enzyme has a distinctly increased long-term stability in the presence of a stable coenzyme than does an enzyme in the presence of a native coenzyme, especially since the stable coenzymes have a lower binding constant with the enzyme than does the native coenzyme.

The enzyme stabilized by the method of the invention is a coenzyme-dependent enzyme. Examples of suitable enzymes are dehydrogenases selected from a glucose dehydrogenase (E.C.1.1.1.47), lactate dehydrogenase (E.C.1.1.1.27, 1.1.1.28), malate dehydrogenase (E.C.1.1.1.37), glycerol dehydrogenase (E.C.1.1.1.6), alcohol dehydrogenase (E.C.1.1.1.1), alpha-hydroxybutyrate dehydrogenase, sorbitol dehydrogenase or amino-acid dehydrogenase, e.g. L-amino-acid dehydrogenase (E.C.1.4.1.5). Further suitable enzymes are oxidases such as, for instance, glucose oxidase (E.C.1.1.3.4) or cholesterol oxidase (E.C.1.1.3.6) and amino transferases, respectively, such as, for example, aspartate or alanine amino transferase, 5'-nucleotidase or creatine kinase. The enzyme is preferably glucose dehydrogenase.

It has proved to be particularly preferable in the context of the method of the invention to employ a mutated glucose dehydrogenase. The term "mutant" as used in the context of the present application refers to a genetically modified variant of a native enzyme which, while the number of amino acids is the same, has an amino acid sequence which is modified compared with the wild-type enzyme, i.e. differs in at least one amino acid from the wild-type enzyme. The introduction of the mutation(s) can take place site-specifically or non-site-specifically, preferably site-specifically by using recombinant methods known in the art, whereas, appropriate for the particular requirements and conditions, at least one amino acid exchange within the amino acid sequence of the native enzyme results. The mutant particularly preferably has an increased thermal or hydrolytic stability compared with the wild-type enzyme.

The mutated glucose dehydrogenase can in principle comprise the amino acid(s) which is (are) modified by comparison with the corresponding wild-type glucose dehydrogenase at any position in its amino acid sequence. The mutated glucose dehydrogenase preferably includes a mutation at at least one of positions 96, 170 and 252 of the amino acid sequence of the wild-type glucose dehydrogenase, with particular preference for mutants with mutations at position 96 and position 170, and mutations at position 170 and position 252. It has proved advantageous for the mutated glucose dehydrogenase to comprise no further mutations besides these mutations.

The mutation at positions 96, 170 and 252 can in principle include any amino acid exchange which leads to a stabilization, e.g. an increase in the thermal or hydrolytic stability, of the wild-type enzyme. The mutation at position 96 preferably includes an amino acid exchange of glutamic acid for glycine, whereas in relation to position 170 an amino acid exchange of glutamic acid for arginine or lysine, in particular an amino acid exchange of glutamic acid for lysine, is preferred. In relation to the mutation at position 252, this preferably includes an amino acid exchange of lysine for leucine.

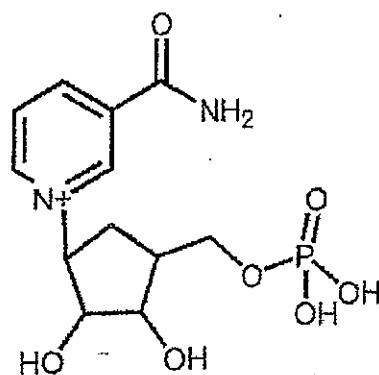
The mutated glyucose dehydrogenase can be obtained by mutation of a wild-type glucose dehydrogenase derived from any biological source, where the term "biological source" includes in the context of this invention both prokaryotes such as, for example, bacteria, and eukaryotes such as, for example, mammals and other animals. The wild-type glucose dehydrogenase is preferably derived from a bacterium, with particular preference for a glucose dehydrogenase from *Bacillus megaterium*, *Bacillus subtilis* or *Bacillus*

thuringiensis, especially from *Bacillus subtilis*.

In a particularly preferred embodiment of the present invention, the mutated glucose dehydrogenase is a glucose dehydrogenase obtained by mutation of wild-type glucose dehydrogenase from *Bacillus subtilis*, which has the amino acid sequence depicted in SEQ ID No.: 1 (GlucDH\_E96G\_E170K) or that depicted in SEQ ID No.: 2 (GlucDH\_E170K\_K252L).

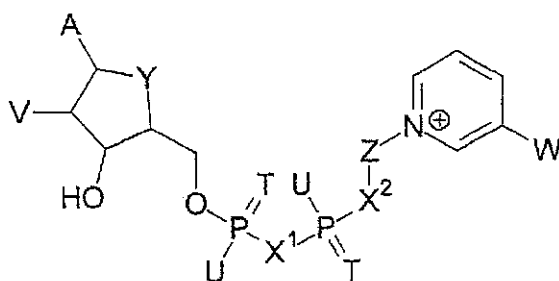
The stable coenzyme is a coenzyme which has been chemically modified by comparison with the native coenzyme and which has a higher stability than the native coenzyme (e.g. hydrolytic stability). The stable coenzyme is preferably stable to hydrolysis under test conditions. Compared with the native coenzyme, the stable coenzyme may have a reduced binding constant for the enzyme, for example a binding constant reduced by a factor of 2 or more.

Preferred examples of stable coenzymes are stable derivatives of nicotinamide adenine dinucleotide (NAD/NADH) or nicotinamide adenine dinucleotide phosphate (NADP/NADPH), or truncated NAD derivatives, e.g. without the AMP moiety or with non-nucleoside residues, e.g. hydrophobic residues. Likewise preferred as stable coenzyme in the context of the present invention is the compound of the formula (I)



(I).

Preferred stable derivatives of NAD/NADH and NADP/NADPH are described in the aforementioned references, the disclosure of which is hereby expressly incorporated by reference. Particularly preferred stabilized coenzymes are described in WO 2007/012494 and US 11/460366, respectively, the disclosure of which is hereby expressly incorporated by reference. The stable coenzyme is particularly preferably selected from compounds having the general formula (II)



(II)

with

A = adenine or an analogue thereof,

T = in each case independently O, S,

U = in each case independently OH, SH,  $\text{BH}_3^-$ ,  $\text{BCNH}_2^-$ ,

V = in each case independently OH or a phosphate group, or two groups forming a cyclic phosphate

group;

W = COOR, CON(R)<sub>2</sub>, COR, CSN(R)<sub>2</sub> with R = in each case independently H or C<sub>1</sub>-C<sub>2</sub>-alkyl,

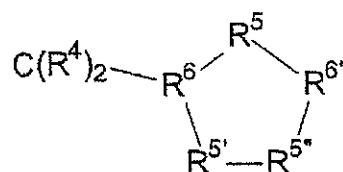
X<sup>1</sup>, X<sup>2</sup> = in each case independently O, CH<sub>2</sub>, CHCH<sub>3</sub>, C(CH<sub>3</sub>)<sub>2</sub>, NH, NCH<sub>3</sub>,

Y = NH, S, O, CH<sub>2</sub>,

Z = a linear or cyclic organic radical, with the proviso that Z and the pyridine residue are not linked by a glycosidic linkage, or a salt or, where appropriate, a reduced form thereof.

Z in the compounds of the formula (II) is preferably a linear radical having 4-6 C atoms, preferably 4 C atoms, in which 1 or 2 C atoms are optionally replaced by one or more heteroatoms selected from O, S and N, or a radical including a cyclic group which has 5 or 6 C atoms and which optionally comprises a heteroatom selected from O, S and N and optionally one or more substituents, and a radical CR<sup>4</sup><sub>2</sub>, where CR<sup>4</sup><sub>2</sub> is bonded to the cyclic group and to X<sup>2</sup>, with R<sup>4</sup> = in each case independently H, F, Cl, CH<sub>3</sub>.

Z is particularly preferably a saturated or unsaturated carbocyclic or heterocyclic 5-membered ring, in particular a compound of the general formula (III)



(III)

where a single or double bond may be present between R<sup>5</sup> and R<sup>5'</sup>, with

R<sup>4</sup> = in each case independently H, F, Cl, CH<sub>3</sub>,

$R^5 = CR^4_2$ ,

where  $R^{5'} = O, S, NH, NC_1-C_2\text{-alkyl}, CR^4_2, CHOH, CHOCH_3$ ,  
and

$R^{5''} = CR^4_2, CHOH, CHOCH_3$  if there is a single bond  
between  $R^{5'}$  and  $R^{5''}$ , and

where  $R^{5'} = R^{5''} = CR^4$  if there is a double bond between  
 $R^{5'}$  and  $R^{5''}$ , and

$R^6, R^{6'} =$  in each case independently  $CH$  or  $CCH_3$ .

In a preferred embodiment, the compounds of the invention comprise adenine or adenine analogues such as, for example,  $C_8$ - and  $N_6$ -substituted adenine, deaza variants such as 7-deaza, aza variants such as 8-aza or combinations such as 7-deaza or 8-aza or carbocyclic analogues such as formycin, whereas the 7-deaza variants may be substituted in position 7 by halogen,  $C_1-C_6$ -alkynyl, -alkenyl or -alkyl.

In a further preferred embodiment, the compounds comprise adenosine analogues which, instead of ribose, comprise for example 2-methoxydeoxyribose, 2'-fluorodeoxyribose, hexitol, altritol and polycyclic analogues, respectively, such as bicyclo-, LNA- and tricyclo-sugars.

It is possible in particular in the compounds of the formula (II) also for (di)phosphate oxygens to be replaced isotronically, such as, for example,  $O^-$  by  $S^-$  and  $BH_3^-$ , respectively,  $O$  by  $NH$ ,  $NCH_3$  and  $CH_2$ , respectively, and  $=O$  by  $=S$ .

$W$  in the compounds of the formula (II) of the invention is preferably  $CONH_2$  or  $COCH_3$ .

$R^5$  in the groups of the formula (III) is preferably  $CH_2$ . It is further preferred for  $R^{5'}$  to be selected from  $CH_2$ ,  $CHOH$  and  $NH$ . In a particularly preferred embodiment,  $R^{5'}$

and  $R^{5''}$  are each  $\text{CHOH}$ . In yet a further preferred embodiment,  $R^{5'}$  is  $\text{NH}$  and  $R^{5''}$  is  $\text{CH}_2$ . Specific examples of preferred stabilized coenzymes are depicted in Figure 1A and B.

In the most preferred embodiment, the stable coenzyme is carbaNAD.

The method of the invention is particularly suitable for long-term stabilization of enzymes. This means that the enzyme stabilized with a stable coenzyme shows when stored, e.g. as dry substance, for example over a period of at least 2 weeks, preferably of at least 4 weeks and particularly preferably of at least 8 weeks, a decline in enzyme activity preferably of less than 50%, particularly preferably less than 30% and most preferably of less than 20% in relation to the initial enzyme activity.

The method of the invention further includes a storage of the enzyme stabilized with a stable coenzyme at elevated temperatures, for example at a temperature of at least  $20^\circ\text{C}$ , preferably of at least  $25^\circ\text{C}$ , and particularly preferably of at least  $30^\circ\text{C}$ . The enzyme activity in this case declines preferably by less than 50%, particularly preferably less than 30% and most preferably less than 20% in relation to its initial level.

It is possible by the stabilization according to the invention to store the enzyme stabilized with a stable coenzyme even without a drying reagent for a long time, as indicated above, and/or at high temperatures, as indicated above. It is further possible for the stabilized enzyme also to be stored at a high relative humidity, e.g. a relative humidity of at least 50%, in which case the enzyme activity declines preferably by

less than 50%, particularly preferably less than 30% and most preferably less than 20% in relation to the initial level.

The storage of the enzyme stabilized with a stable coenzyme can take place on the one hand as dry substance and on the other hand in liquid phase. The storage of the stabilized enzyme preferably takes place on or in a test element suitable for determining an analyte. The enzyme stabilized with a stable coenzyme is in this case preferably a constituent of a detection reagent which may where appropriate also comprise further constituents such as, for example, salts, buffers, etc. The detection reagent is in this case preferably free of a mediator.

The enzyme stabilized with a stable coenzyme can be employed for detecting analytes, for example parameters in body fluids such as, for instance, blood, serum, plasma or urine, and in sewage samples or food products, respectively.

Analytes which can be determined are any biological or chemical substances which can be detected by a redox reaction, e.g. substances which are substrates of a coenzyme-dependent enzyme or are themselves coenzyme-dependent enzymes. Preferred examples of analytes are glucose, lactic acid, malic acid, glycerol, alcohol, cholesterol, triglycerides, ascorbic acid, cysteine, glutathione, peptides, urea, ammonium, salicylate, pyruvate, 5'-nucleotidase, creatine kinase (CK), lactate dehydrogenase (LDH), carbon dioxide etc. The analyte is preferably glucose.

Another embodiment of the present invention relates to the use of a compound of the invention or of an enzyme stabilized with a stable coenzyme according to the

invention for detecting an analyte in a sample by an enzymatic reaction. The detection of glucose with the aid of glucose dehydrogenase (GlucDH) is particularly preferred in this connection.

The alteration in the stable coenzyme by reaction with the analyte can in principle be detected in any way. It is possible in principle to employ here all methods known from prior art for detecting enzymatic reactions. However, the alteration in the coenzyme is preferably detected by optical methods. Optical detection methods include for example the measurement of absorption, fluorescence, circular dichroism (CD), optical rotatory dispersion (ORD), refractometry etc.

An optical detection method which is preferably used in the context of the present application is photometry. Photometric measurement of an alteration in the coenzyme as a result of reaction with the analyte requires, however, the additional presence of at least one mediator which increases the reactivity of the reduced coenzyme and makes it possible for electrons to be transferred to a suitable optical indicator or an optical indicator system.

Mediators suitable for the purposes of the present invention are inter alia nitrosoanilines such as, for example, [(4-nitrosophenyl)imino]dimethanol hydrochloride, quinones such as, for example, phenanthrenequinones, phenanthrolinequinones or benzo[h]quinolinequinones, phenazines such as, for example, 1-(3-carboxypropoxy)-5-ethylphenazinium trifluoromethanesulfonate, or/and diaphorase (EC 1.6.99.2). Preferred examples of phenanthrolinequinones include 1,10-phenanthroline-5,6-quinones, 1,7-phenanthroline-5,6-quinones, 4,7-phenanthroline-5,6-quinones, and the N-alkylated and N,N'-dialkylated

salts thereof, with preference as counter ion in the case of N-alkylated and N,N'-dialkylated salts, respectively, for halides, trifluoromethanesulfonate or other anions which increase the solubility.

It is possible to use as optical indicator or as optical indicator system any substance which is reducible and on reduction experiences a detectable change in its optical properties such as, for example, colour, fluorescence, reflectance, transmission, polarization or/and refractive index. Determination of the presence or/and the amount of the analyte in the sample can take place with the unaided eye or/and by means of a detection device using a photometric method which appears to be suitable to a person skilled in the art.

Heteropolyacids and in particular 2,18-phosphomolybdic acid are preferably used as optical indicators and are reduced to the corresponding heteropoly blue.

The alteration in the coenzyme is particularly preferably detected by measuring the fluorescence. Fluorescence measurement is highly sensitive and makes it possible to detect even low concentrations of the analyte in miniaturized systems.

An alternative possibility is also to detect the alteration in the coenzyme electrochemically using a suitable test element such as, for example, an electrochemical test strip. The precondition for this is once again the use of suitable mediators which can be converted by the reduced coenzyme, by transfer of electrons, into a reduced form. The analyte is determined by measuring the current which is needed to reoxidize the reduced mediator and which correlates with the concentration of the analyte in the sample.

Examples of mediators which can be used for electrochemical measurements include in particular the aforementioned mediators employed for photometric measurements.

It is possible to use a liquid test to detect an analyte, in which case the reagent is for example in the form of a solution or suspension in an aqueous or nonaqueous liquid or as powder or lyophilisate. However, it is also possible to use a dry test, in which case the reagent is applied to a support, a test strip. The support may include for example a test strip including an absorbent or/and swellable material which is wetted by the sample liquid to be investigated.

A particularly preferred test format includes the use of the enzyme glucose dehydrogenase with a stable NAD derivative for detecting glucose, in which case a derivative of the reduced coenzyme NADH is formed. NADH is detected by optical methods, e.g. by photometric or fluorometric determination after UV excitation. A particularly preferred test system is described in US 2005/0214891, to which express reference is made here.

The present invention further relates also to an enzyme stabilized with a stable coenzyme, where the stabilized enzyme shows on storage for preferably at least 2 weeks, particularly preferably at least 4 weeks and most preferably at least 8 weeks at a temperature of, preferably, at least 20°C, particularly preferably at least 25°C and most preferably at least 30°C, where appropriate with high humidity and without a drying reagent, a decline in the enzymatic activity of less than 50%, preferably less than 30% and most preferably less than 20% compared with the initial level.

The invention further relates also to a detection reagent for determining an analyte which comprises an enzyme stabilized with a stable coenzyme as indicated above. The invention additionally relates to a test element which comprises an enzyme stabilized according to the invention and a detection reagent, respectively, according to the invention. The detection reagent and the test element, respectively, may be suitable for carrying out dry or liquid tests. The test element is preferably a test strip for fluorometric or photometric detection of an analyte. Such a test strip comprises the enzyme stabilized with a stable coenzyme and immobilized on an absorbent or/and swellable material such as, for instance, cellulose, plastics, etc.

The invention is to be explained in more detail by the following figures and examples:

#### **Description of the Figures**

**Figure 1A:** depiction of the stable coenzyme carba-NAD (cNAD).

**Figure 1B:** depiction of the stable coenzyme pyrrolidiny-NAD.

**Figure 2:** depiction of the results of the glucose dehydrogenase enzyme kinetics in the presence of NAD and in the presence of cNAD, respectively, before and after storage.

**2A:** kinetics of GlucDH in the presence of NAD after 1 day.

**2B:** kinetics of GlucDH in the presence of cNAD after 1 day.

**2C:** kinetics of GlucDH in the presence of NAD after storage at 32°C and 85% relative humidity for 5 weeks.

**2D:** kinetics of GlucDH in the presence of cNAD after storage at 32°C and 85% relative humidity for 5 weeks.

**Figure 3:** comparison of the blank values for glucose dehydrogenase in the presence of NAD and of GlucDH in the presence of cNAD, respectively, over a period of up to 5 weeks at 32°C and 85% humidity.

**Figure 4:** depiction of various function plots of glucose dehydrogenase after storage of glucose dehydrogenase in the presence of NAD at 32°C and 85% humidity. The storage time varied between 1 day and 5 weeks.

**Figure 5:** depiction of various function plots of glucose dehydrogenase after storage of glucose dehydrogenase in the presence of cNAD at 32°C and 85% humidity. The storage time varied between 1 day and 5 weeks (Figure 5A) and between 1 day and 24 weeks (Figure 5B), respectively.

**Figure 6:** depiction of the residual content of NAD and cNAD after storage of glucose dehydrogenase in the presence of NAD and cNAD, respectively, at 32°C and 85% humidity for 24 weeks.

**Figure 7:** depiction of the GlucDH activity after storage of glucose dehydrogenase in the presence of NAD and cNAD, respectively, at 32°C and 85% humidity for 5 weeks (Figure 7A) and 24 weeks (Figure 7B), respectively.

**Figure 8:** depiction of the GlucDH activity after storage of glucose dehydrogenase (GlucDH-wt), the double mutant GlucDH\_E96G\_E170K (GlucDH-Mut1) and the double mutant GlucDH\_E170K\_K252L (GlucDH-Mut2) in the presence of NAD and cNAD, respectively, at 32°C and 83% relative humidity over a period of 25 weeks.

**Figure 9:** analysis of glucose dehydrogenase by gel electrophoresis after storage in the presence of NAD and cNAD, respectively. Test conditions: MW, 10-220 kDa markers; 1: GlucDH/NAD, 5 weeks at 6°C; 2: GlucDH/NAD, 5 weeks at 32°C/85% relative humidity; 3: GlucDH/cNAD, 5 weeks at 6°C; 4: GlucDH/cNAD, 5 weeks at 32°C/85% relative humidity.

**Figure 10:** analysis of glucose dehydrogenase by gel electrophoresis after storage at 50°C in the presence of NAD and cNAD, respectively. Test conditions: MW, 10-220 kDa markers; 1: GlucDH 8.5 mg/ml, NAD, 0 hours; 2: GlucDH 8.5 mg/ml, NAD, 22 hours; 3: GlucDH 8.5 mg/ml, NAD, 96 hours; 4: GlucDH 8.5 mg/ml, NAD, 118 hours; 5: GlucDH 8.5 mg/ml, NAD, 140 hours; 6: GlucDH 8.5 mg/ml, NAD, 188 hours; 7: GlucDH 8.5 mg/ml, NAD, 476 hours; 8: GlucDH 8.5 mg/ml, cNAD, 0 hours; 9: GlucDH 8.5 mg/ml, cNAD, 188 hours; 10: GlucDH 8.5 mg/ml, cNAD, 476 hours.

**Figure 11:** depiction of the stability of glucose dehydrogenase in the presence of NAD and cNAD, respectively, in liquid phase at 50°C over a period of 4 days (Figure 11A) and 14 days (Figure 11B), respectively. Test conditions: GlucDH 10 mg/ml; NAD and cNAD, respectively, 12 mg/ml; buffer: 0.1 M Tris, 1.2 M NaCl, pH 8.5; temperature 50°C.

**Figure 12:** analysis of alcohol dehydrogenase from yeast by gel electrophoresis after storage in the presence of NAD and cNAD, respectively. Test conditions: MW, 10-220 kDa markers; 1: ADH, 65 hours at 6°C; 2: ADH/cNAD, 65 hours at 6°C; 3: ADH/NAD, 65 hours at 6°C; 4: ADH, 65 hours at 35°C; 5: ADH/cNAD, 65 hours at 35°C; 6: ADH/NAD, 65 hours at 35°C.

**Figure 13:** analysis of alcohol dehydrogenase from yeast

by gel electrophoresis after storage at 35°C in the presence of NAD and cNAD, respectively. Test conditions: MW, 10-220 kDa markers; 1: ADH/NAD, 0 days; 2: ADH/NAD, 1 day; 3: ADH/NAD, 2 days; 4: ADH/NAD, 3 days; 5: ADH/NAD, 5 days; 6: ADH/cNAD, 0 days; 7: ADH/cNAD, 1 day; 8: ADH/cNAD, 2 days; 9: ADH/cNAD, 3 days; 10: ADH/cNAD, 6 days.

**Figure 14:** depiction of the stability of alcohol dehydrogenase from yeast in the presence of NAD and cNAD, respectively, in liquid phase at 35°C over a period of 65 hours. Test conditions: ADH 5 mg/ml; NAD and cNAD, respectively, 50 mg/ml; buffer: 75 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , glycine, pH 9.0; temperature 35°C.

**Figure 15:** depiction of various function plots of glucose dehydrogenase after storage in the presence of NAD and various mediators at room temperature for 11 weeks.

**Figure 16:** depiction of the results of the glucose dehydrogenase enzyme kinetics in the presence of NAD and 1-(3-carboxypropoxy)-5-ethylphenazinium trifluoromethanesulfonate at various glucose concentrations.

**Figure 17:** diagrammatic depiction of glucose detection with GlucDH as enzyme and diaphorase as mediator.

**Figure 18:** depiction of the function plots of glucose dye oxidoreductase (GlucDOR) in the presence of pyrroloquinolinequinone (PQQ) and [(4-nitrosophenyl)imino]dimethanol hydrochloride as mediator, and of glucose dehydrogenase in the presence of NAD and diaphorase/[(4-nitrosophenyl)imino]dimethanol hydrochloride as mediator, respectively.

**Figure 19:** depiction of the results of the glucose

dehydrogenase enzyme kinetics in the presence of NAD and diaphorase at various glucose concentrations.

**Figure 20:** depiction of the current measured as a function of the glucose concentration in the electrochemical determination of glucose using glucose dehydrogenase in the presence of NAD and cNAD, respectively. Test conditions: 25 mM NAD and cNAD, respectively; 2.5 seconds delay; 5 seconds measurement time.

**Figure 21:** depiction of the amino acid sequences of the glucose dehydrogenase double mutants GlucDH\_E96G\_E170K and GlucDH\_E170K\_K252L.

### Example 1

Carba-NAD (Fig. 1A) or NAD were added to the glucose-specific GlucDH. These formulations were in each case applied to Pokalon film (Lonza) and, after drying, stored under warm and moist conditions (32°C, 85% relative humidity). The reaction kinetics and the function plot were subsequently determined at regular intervals. In parallel, at each of the times of measurement a cNAD/NAD analysis and a determination of the residual activity of the enzyme were carried out.

The kinetics plots for NAD (Figure 2A) and cNAD (Figure 2B) determined on the first day are comparable and also show a similar rise in the glucose dependence. However, a distinct difference in the kinetics plots is evident after 5 weeks. Whereas the kinetics for NAD (Figure 2C) decrease greatly in their dynamics, the kinetics of the enzyme stabilized with cNAD remain virtually unchanged (Figure 2D).

There is also a distinct difference in the blank values

(dry blank value before application of a blood sample), as is evident from Figure 3. The rise in the dry blank value for NAD is attributable to the formation of fluorescent particles (Oppenheimer (1982), Supra). Surprisingly, this does not occur with cNAD.

The differing stability of glucose dehydrogenase in the presence of NAD and cNAD, respectively, is also evident from comparison of Figures 4 and 5. After 5 weeks, the function plot for the enzyme stabilized with cNAD is still in the bunch of previous measurements (Figure 5A), whereas the plot for the enzyme treated with NAD (Figure 4) shows a fall-off at higher concentrations, which is a typical sign that the amounts of enzyme/coenzyme are too low. Figure 5B shows various function plots of the glucose dehydrogenase stabilized with cNAD over a period of 24 weeks. It is clear in this connection that the function of the enzyme is only slightly changed at high glucose concentrations throughout the period and approximately corresponds after 24 weeks to the value obtained after 5 weeks.

The relation between structure of the coenzyme and its stability over a predetermined period is evident from Figure 6. According to this, the residual content of cNAD in a glucose detection reagent after storage (at 32°C and 85% relative humidity) for 24 weeks is still about 80% of the initial level, whereas the content of NAD in a glucose detection reagent stabilized with NAD declines after only 5 weeks to about 35% of the initial level and, by extrapolation, is reduced to zero after about 17 weeks.

The result of the determination of residual activity of the active GlucDH enzyme after 5 weeks at 32°C and 85% relative humidity (Figure 7A) is completely surprising.

The enzyme stabilized with NAD now shows only an extremely low enzyme activity (0.5%), whereas the enzyme stabilized with cNAD still has a residual activity of 70% (in each case by comparison with samples stored in a refrigerator with desiccant). After 24 weeks at 32°C and 85% relative humidity (Figure 7B), the residual activity of the enzyme on stabilization with cNAD is still about 25%.

If a mutant is used instead of the wild-type enzyme (from *Bacillus subtilis*), it is possible to increase the residual GlucDH activity even further. After storage at 32°C and 85% relative humidity in the presence of cNAD for 24 weeks, the residual activity of a GlucDH\_E96G\_E170K mutant with the amino acid replacements glutamic acid → glycine at position 96 and glutamic acid → lysine at position 170 (GlucDH-Mut1) of the wild-type enzyme is about 70%, whereas the residual activity of a GlucDH\_E170K\_K252L mutant with the amino acid replacements glutamic acid → lysine at position 170 and lysine → leucine at position 252 (GlucDH-Mut2) is about 50% (Figure 8).

The analysis of glucose dehydrogenase by gel electrophoresis in an SDS gel (Figures 9 and 10) also shows clearly the difference between storage in the presence of NAD and cNAD, respectively. Whereas the enzyme is still identifiable as a band with the expected mobility after storage for 5 weeks at 32°C and 85% relative humidity in the presence of cNAD, the enzyme stored in the presence of NAD has completely disappeared (Figure 9). It is evident at the same time from Figure 10 that the band of the enzyme stabilized by NAD and stored at 50°C becomes weaker as the storage time increases and has virtually disappeared after 476 hours, whereas the corresponding band of the enzyme stored in the presence of cNAD shows an only slight

change compared with a band detected at the start of the experiment.

This result can also be confirmed on storage in liquid phase (Figures 11A and 11B). After 95 hours at 50°C, the residual activity of glucose dehydrogenase in the presence of the native coenzyme NAD is  $\approx$  5%, whereas the residual activity of GlucDH in the presence of the artificial coenzyme cNAD is 75% (Figure 11A). After storage at 50°C for 336 hours, the residual activity of the enzyme stabilized with NAD is now only about 1%; the residual activity of the enzyme stored in the presence of cNAD is observed to be still about 70%. The corresponding SDS gels likewise show a change in the GlucDH band in the presence of the native coenzyme NAD: new bands appear at higher molecular masses and there is a shift in the 30 kDa band.

Overall, it is an extremely surprising result that stabilization of the cofactor simultaneously brings about a stabilization of the enzyme - and not just through the cooperative effect of the better cohesion of the enzyme. Decomposition of the cofactor NAD has a negative effect on the stability of the enzyme GlucDH and in fact speeds up inactivation thereof. Replacement of native NAD by artificial analogues permits GlucDH to be stored under stress conditions (e.g. elevated temperature) even in the presence of a cofactor.

It is possible with such a system to produce blood glucose test strips with considerably improved stability properties, for which a presentation without desiccant is possible.

## Example 2

cNAD or NAD were added to an alcohol detection

solution. These mixtures were stored at 35°C. The stability of the enzyme was then checked at regular intervals, and the residual activity of the enzyme was determined.

Once again, an analysis by gel electrophoresis in an SDS gel (Figures 12 and 13) shows the difference between storage in the presence of NAD and cNAD. Thus, the bands of the alcohol dehydrogenase stabilized with cNAD differ only slightly after storage at 6°C and 35°C, respectively, for 65 hours, indicating a stabilization of the enzyme by artificial coenzyme. By contrast, the band of the enzyme stored in the presence of NAD at 35°C has completely disappeared (Figure 12).

It is further clear from Figure 13 that the band of the enzyme stabilized with NAD and stored at 35°C becomes weaker as the storage time increases and has almost completely disappeared after 5 days. A band of the enzyme stabilized with cNAD detected after storage at 35°C for 6 days shows distinctly less decomposition of the enzyme and thus an increased stability.

This result can be confirmed also on storage in liquid phase (Figure 14). After 65 hours at 35°C, the residual activity of alcohol dehydrogenase in the presence of the native coenzyme NAD is about 6%, whereas the residual activity of the enzyme in the presence of the artificial coenzyme cNAD is still about 60%.

### Example 3

To determine glucose, various test systems which included in each case glucose dehydrogenase, NAD, a mediator and, where appropriate, an optical indicator were measured photometrically and electrochemically.

For photometric measurements, initially four test elements which had in each case been stored at room temperature for 11 weeks and comprised 2,18-phosphomolybdic acid, besides glucose dehydrogenase, NAD and a mediator, were investigated with various glucose concentrations.

As is evident from Figure 15, a fall in the reflectance was observed with increasing glucose concentration for all four mediators employed, i.e. [(4-nitrosophenyl)imino]dimethanol hydrochloride (Med A), 1-methyl-5,6-dioxo-5,6-dihydro-1,10-phenanthroline trifluoromethanesulfonate (Med B), 7-methyl-5,6-dioxo-5,6-dihydro-1,7-phenanthroline trifluoromethanesulfonate (Med F) and 1-(3-carboxypropoxy)-5-ethylphenazinium trifluoromethanesulfonate (Med G), and thus the abovementioned mediators are in principle suitable for determining glucose by photometry.

At high glucose concentrations in the region of 800 mg/dl, the reflectance of the measured sample on use of [(4-nitrosophenyl)imino]dimethanol hydrochloride and 1-(3-carboxypropoxy)-5-ethylphenazinium trifluoromethanesulfonate, respectively, is still about 20%, suggesting that these two mediators are particularly suitable for photometric measurements using the glucose dehydrogenase/NAD system, and thus also the glucose dehydrogenase/cNAD system. The kinetics of the conversion of glucose using the glucose dehydrogenase, NAD, 1-(3-carboxypropoxy)-5-ethylphenazinium trifluoromethanesulfonate and 2,18-phosphomolybdic acid system at glucose concentrations in the range from 0 to 800 mg/dl are depicted in Figure 16.

As is evident from the diagrammatic depiction in Figure 17, the photometric determination of glucose can also take place with (additional) use of diaphorase as

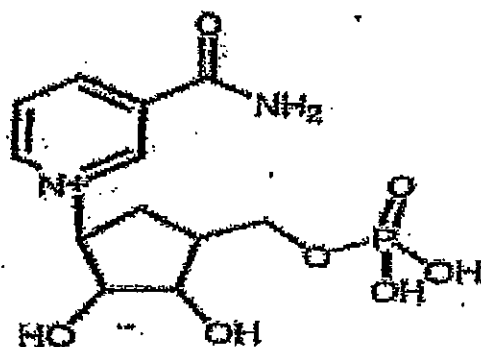
intermediary mediator. Figure 18 shows a concentration-dependent decrease in reflectance for the glucose dehydrogenase, NAD, diaphorase, [(4-nitrosophenyl)imino]dimethanol hydrochloride and 2,18-phosphomolybdic acid system (system 1). The system which served as comparison was glucose dye oxidoreductase, pyrroloquinolinequinone, [(4-nitrosophenyl)imino]dimethanol hydrochloride and 2,18-phosphomolybdic acid (system 2), which likewise causes a concentration-dependent decrease in the reflectance, but has disadvantages because of the low specificity of glucose dye oxidoreductase. The kinetics of the conversion of glucose using system 1 at glucose concentrations in the range from 0 to 800 mg/dl are depicted in Figure 19.

As alternative to photometry it is also possible to employ an electrochemical measurement for the purpose of determining analytes. Thus, the current required to reoxidize the reduced mediator was found to be linearly dependent on the glucose concentration (Figure 20) both with a test element which, besides glucose dehydrogenase, comprised NAD as coenzyme and 1-(3-carboxypropoxy)-5-ethylphenazinium trifluoromethanesulfonate as mediator, and with a corresponding system which included the stabilized coenzyme cNAD instead of NAD.

It has thus been shown that determination of analytes using the dehydrogenase/stable coenzyme system is also possible by means of electrochemical detection and evaluation with another wavelength which is independent of the coenzyme. The overall formulation ought also to be further stabilized through the use of the stable enzyme/coenzyme pair.

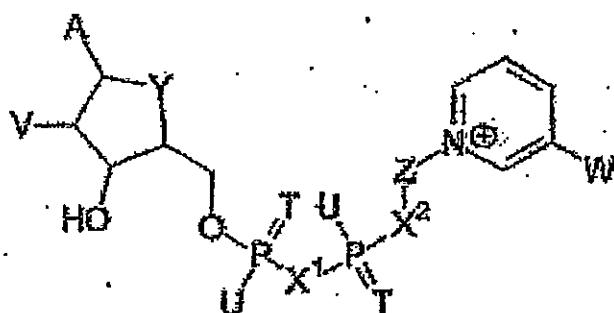
The claims defining the invention are as follows:

1. A method for stabilizing an enzyme, wherein the enzyme is stored in the presence of a stable coenzyme.
2. The method according to Claim 1, wherein the enzyme is a dehydrogenase.
3. The method according to Claim 2, wherein the enzyme is a dehydrogenase selected from the group consisting of a glucose dehydrogenase (E.C.1.1.1.47), lactate dehydrogenase (E.C.1.1.1.27, 1.1.1.28); malate dehydrogenase (E.C.1.1.1.37), glycerol dehydrogenase (E.C.1.1.1.6); alcohol dehydrogenase (E.C.1.1.1.1), alpha-hydroxybutyrate dehydrogenase, sorbitol dehydrogenase and an amino-acid dehydrogenase.
4. The method according to claim 3 wherein the amino-acid dehydrogenase is L-amino-acid dehydrogenase (E.C.1.4.1.5)
5. The method according to any one of Claims 1-4, wherein the enzyme is a glucose dehydrogenase.
6. The method according to any one of Claims 1-5, wherein the stable coenzyme is selected from stable nicotinamide adenine dinucleotide (NAD/NADH) compounds, nicotinamide adenine dinucleotide phosphate (NADP/NADPH) compounds and the compound of the formula (I).



(I)

7. The method according to Claim 6, wherein the stable coenzyme is selected from a compound having the general formula (II)



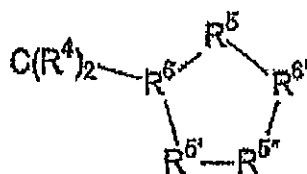
(II)

wherein

- A = adenine or an analogue thereof,  
 T = in each case is independently selected from O and S;  
 U = in each case is independently selected from OH, SH, BH<sub>3</sub><sup>-</sup> and BCNH<sub>2</sub><sup>-</sup>;  
 V = in each case is selected from OH, a phosphate group, and two groups forming a cyclic phosphate group;  
 W = COOR, CON(R)<sub>2</sub>, COR or CSN(R)<sub>2</sub> wherein R = in each case is selected from H and C<sub>1</sub>-C<sub>6</sub>alkyl.

$X^1, X^2 =$  is independently selected from O,  $\text{CH}_2$ ,  $\text{CHCH}_3$ ,  $\text{C}(\text{CH}_3)_2$ , NH and  $\text{NCH}_3$ ;  
 $Y =$  NH, S, O or  $\text{CH}_2$ ;  
 $Z =$  a linear or cyclic organic radical with the proviso that Z and the pyridine residue are not linked by a glycosidic linkage;  
 or a salt or a reduced form thereof.

8. The method according to Claim 7, wherein Z is selected from  
 (i) a linear radical having 4-6 C atoms, in which 1 or 2 atoms are optionally replaced by one or more heteroatoms selected from O, S and N, or  
 (ii) a radical including a cyclic group having 5 or 6 C atoms which optionally comprises a heteroatom selected from O, S and N and optionally one or more substituents, and a radical  $\text{CR}^4_2$ , where  $\text{CR}^4_2$  is bonded to the cyclic group and to  $X^2$ , wherein  $\text{R}^4 =$  in each case is independently selected from H, F, Cl and  $\text{CH}_3$ .
9. The method according to Claim 7, in which Z is a compound of the general formula (III)



(III)

where a single or double bond may be present between  $\text{R}^{6'}$  and  $\text{R}^{6''}$ , with

$\text{R}^4 =$  is independently selected from H, F, Cl and  $\text{CH}_3$ ;

$\text{R}^5 = \text{CR}^4_2$ ;

where  $R^{5'} = O, S, NH, NC_1-C_2\text{-alkyl}, CR^4_2, CHOH$  or  $CHOCH_3$ ; and

$R^{5''} = CR^4_2, CHOH$  or  $CHOCH_3$  if there is a single bond between  $R^{5'}$  or  $R^{5''}$ ;

wherein  $R^{5'} = R^{5''} = CR^4$  if there is a double bond between  $R^{5'}$  and  $R^{5''}$  and

$R^6, R^{6'}$  are independently selected from  $CH$  and  $CCH_3$ .

10. The method according to any one of Claims 7-9, wherein  $W = CONH_2$  or  $COCH_3$ .
11. The method according to Claim 9 or 10, wherein  $R^5$  is  $CH_2$ .
12. The method according to any one of Claims 9-11, wherein  $R^{5'}$  is selected from  $CH_2, CHOH$  and  $NH$ .
13. The method according to any one of Claims 9-12, wherein  $R^{5'}$  and  $R^{5''}$  are each  $CHOH$ .
14. The method according to any one of Claims 9-12, wherein  $R^{5'}$  is  $NH$  and  $R^{5''}$  is  $CH_2$ .
15. The method according to any one of Claims 1-14, wherein the stable coenzyme is carbaNAD.
16. The method according to any one of Claims 1-15, wherein the enzyme stabilized with a stable coenzyme is stored for a period of at least 2 weeks.
17. The method according to any one of Claims 1-15, wherein the enzyme stabilized with a stable coenzyme is stored at a temperature of at least  $20^\circ C$ .

18. The method according to any one of Claims 1-16, wherein the enzyme stabilized with a stable coenzyme is stored without a drying reagent.
19. The method according to any one of Claims 1-17, wherein the enzyme stabilized with a stable coenzyme is stored at a relative humidity of at least 50%.
20. The method according to any one of Claims 1-18, wherein the storage of the enzyme stabilized with a stable coenzyme takes place as dry substance.
21. The method according to any one of Claims 1-18, wherein the storage of the enzyme stabilized with a stable coenzyme takes place in liquid phase.
22. The method according to any one of Claims 1-20, wherein the storage of the enzyme stabilized with a stable coenzyme takes place on a test element.
23. An enzyme which is stabilized with a stable coenzyme, wherein it shows on storage for at least 2 weeks, at a temperature of at least 20°C, a decline in the enzymatic activity of less than 50% compared with the initial level, wherein the enzyme is a mutated glucose dehydrogenase and the mutated glucose dehydrogenase has an increased thermal or hydrolytic stability compared with the corresponding wild-type glucose dehydrogenase.
24. The enzyme according to claim 23 wherein the storage is with high humidity and without a drying reagent.
25. A detection reagent for determining an analyte, comprising a stabilized enzyme according to Claim 23, or claim 24.

26. A test element comprising a stabilized enzyme according to Claim 23 or claim 24 or a detection reagent according to Claim 25.

27. A method for stabilizing an enzyme as claimed in Claim 1, substantially as hereinbefore described with reference to the examples.

## SEQUENCE LISTING

<110> F. Hoffmann - La Roche  
 <120> Stabilization of dehydrogenases with stable coenzymes  
 <130> 40444P WO  
 <150> EP 08 003 054.7  
 <151> 2008-02-19  
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35 40 45

Lys Glu Glu Val Ile Lys Ala Gly Gly Glu Ala Val Val Val Gln Gly  
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Asp Val Thr Lys Glu Glu Asp Val Lys Asn Ile Val Gln Thr Ala Ile

|                                                                 |  |     |  |     |  |     |
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