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(54) Titre : STABILISATION D'ENZYMES PAR DES COENZYMES STABLES
(54) Title: STABILIZATION OF ENZYMES WITH STABLE COENZYMES

(57) **Abrégé/Abstract:**

The present invention concerns a method for stabilizing an enzyme by storing the enzyme in the presence of a stabilized coenzyme. In addition the present invention concerns an enzyme stabilized with a stabilized coenzyme as well as the use thereof in test elements for detecting analytes.



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Abstract

The present invention concerns a method for stabilizing an enzyme by storing the enzyme in the presence of a stabilized coenzyme. In addition the present invention concerns an enzyme stabilized with a stabilized coenzyme as well as the use thereof in test elements for detecting analytes.

Stabilization of enzymes with stable coenzymes

Description

The present invention concerns a method for stabilizing enzymes by storing the enzyme in the presence of a stabilized coenzyme. In addition the present invention
5 concerns an enzyme stabilized with a stabilized coenzyme as well as the use thereof in test elements for detecting analytes.

Diagnostic test elements are important components of clinically relevant analytical methods. In this connection the focus is on the measurement of analytes, e.g. metabolites or substrates, which for example can be determined directly or
10 indirectly with the aid of an enzyme that is specific for the analyte. In this case the analytes are converted with the aid of an enzyme-coenzyme complex and subsequently quantified. This entails the analyte to be determined being brought into contact with a suitable enzyme, a coenzyme and optionally a mediator, whereby the coenzyme is physicochemically changed e.g. oxidized or reduced by the enzymatic
15 reaction. If a mediator is additionally used, it usually transfers electrons from the reduced coenzyme released during the conversion of the analyte onto an optical indicator or the conductive components of an electrode so that the process can be for example detected photometrically or electrochemically. A calibration provides a direct relationship between the measured value and the concentration of the analyte
20 to be determined.

An important criterion when providing diagnostic test elements is their long-term stability. Test elements known from the prior art which are for example used in the determination of blood glucose are generally very sensitive to moisture and heat, where usually the function of in particular the coenzyme and mediator is impaired.
25 Another problem of commercially available test elements is their sensitivity to

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ambient light where light absorption by the enzyme system can result in damage to the enzyme, coenzyme or/and mediator. In certain forms of application e.g. in the case of tests which are carried out by the end user himself, erroneous results can therefore occur due to an incorrect, unnoticed faulty storage of the measurement
5 system which can be hardly detected by the user and may result in incorrect treatment of the respective disease.

A known measure that can be used to increase the stability of diagnostic test elements is the use of stable enzymes e.g. the use of enzymes from thermophilic organisms. Furthermore, it is possible to stabilize enzymes by chemical
10 modification e.g. cross-linking, or by mutagenesis. In addition enzyme stabilizers such as e.g. trehalose, polyvinyl pyrrolidone and serum albumin can also be added or the enzymes can be enclosed in polymer networks e.g. by photopolymerization.

Attempts have also been made to improve the stability of diagnostic test elements by using stable mediators. Thus, the specificity of tests is increased and
15 interferences during the reaction are eliminated by using mediators with a redox potential that is as low as possible. However, the redox potential of the enzyme / coenzyme complexes form a lower limit for the redox potential of mediators. If the redox potential is lower than this limit, the reaction with the mediators is slowed down or even stopped.

20 Alternatively it is also possible to use diagnostic test elements without mediators in which for example coenzymes such as the coenzyme NADH are detected directly. A disadvantage of such measurement systems is, however, that native coenzymes such as NAD and NADP are unstable.

NAD and NADP are base-labile molecules whose degradation pathways are
25 described in the literature (N.J. Oppenheimer, in "The Pyridine Nucleotide Coenzyme", Academic Press New York, London 1982, editor J. Everese, B.

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Anderson, K. You, chapter 3, pages 56-65). ADP-ribose is mainly formed when NAD or NADP are degraded by cleavage of the glycosyl linkages between the ribose and the pyridine unit. In contrast the reduced forms NADH and NADPH are acid-labile: e.g. epimerization is a known degradation pathway. In both cases the

5 instability of NAD/NADP and NADH/NADPH is due to the lability of the glycosyl linkage between the ribose unit and the pyridine unit. However, under conditions that are not drastic such as e.g. an aqueous solution, the coenzymes NAD and NADP are already hydrolyzed solely due to the ambient moisture. This instability may lead to inaccuracies in the measurement of analytes.

10 A number of NAD/NADP derivatives is described for example by B.M. Anderson in "The Pyridine Nucleotide Coenzymes", Academic Press New York, London 1982, editor J. Everese, B. Anderson, K. You, chapter 4. However, most of these derivatives are not well accepted by enzymes. The only derivative that has therefore been used up to now for diagnostic tests is 3-acetylpyridine adenine dinucleotide

15 (acetyl-NAD) which was described for the first time in 1965 (N.O. Kaplan, J. Biol. Chem. (1956), 221, 823). This coenzyme also 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. However, modifications of the nicotinamide group generally have a

20 direct influence on the catalytic reaction. In most cases this influence is negative.

In another stabilization concept the ribose unit was altered in order to thus influence the stability of the glycosyl linkage. This procedure does not directly interfere with the catalytic reaction of the nicotinamide group. However, it may have an indirect influence as soon as the enzyme exhibits a strong and specific binding to the ribose

25 unit. Kaufmann et al. disclose in this connection a number of thioribose-NAD derivatives in WO 98/33936 and US 5,801,006 and in WO 01/49247. However, a

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relationship between the modification of the nicotinamide ribose unit and the activity of the derivatives in enzymatic reactions has not been shown to date.

carbaNAD, a derivative without a glycosyl linkage was described for the first time in 1988 (J.T. Slama, Biochemistry (1988), 27, 183, and Biochemistry (1989), 28, 5 7688). The ribose therein is substituted by a carbacyclic sugar unit. Although carbaNAD was described as a substrate for dehydrogenases, its activity has previously not 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 methylene bisphosphonate compound 10 instead of the natural pyrophosphate. The methylene bisphosphonate shows an increased stability towards phosphatases and was used as an inhibitor for 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 finally disclose stabilized NAD/NADH and 15 NADP/NADPH derivatives, enzyme complexes of these derivatives and their use in biochemical detection methods and reagent kits.

The underlying object of the present invention was to provide a method for stabilizing enzymes, especially for the long-term stabilization of enzymes which at least partially eliminates the above-mentioned disadvantages.

20 This object is achieved according to the invention by a method for stabilizing an enzyme, wherein the enzyme is stored in the presence of a stabilized coenzyme. It was surprisingly found that a long-term stabilization of several weeks or months at high relative humidity or even in a liquid phase, at elevated temperatures and in ambient light is possible with the aid of a stabilized coenzyme. In this connection

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the term "storage" means that the enzyme is kept for any time period, preferably for a time period of at least 2 weeks, more preferably for a time period of at least 3 months, even more preferably for a time period of at least 6 months, and most preferably for a time period of at least 12 months in the presence of the stabilized
5 coenzyme where the storage preferably takes place at atmospheric pressure, room temperature (25°C) and a relative air humidity of at least 50 %.

This finding is surprising because it is known that although enzymes exhibit an increased short-term stability of several hours in the presence of the native coenzyme (Bertoldi et al., *Biochem. J.* (2005), 389, 885; van den Heuvel et al., *J. Biol. Chem.* (2005), 280, 32115; and Pan et al., *J. Chin. Biochem. Soc.* (1974), 3, 1), they have a lower stability over a longer period (*Nutrition Reviews* (1978), 36, 251). The long-term stability of diagnostic test elements which comprise an enzyme and a stabilized coenzyme which has now been observed towards moisture or/and heat is all the more surprising because stabilized coenzymes have lower binding
15 constants with the enzyme than the corresponding native coenzymes.

The enzyme stabilized by the method according to the invention is a coenzyme-dependent enzyme. Suitable enzymes are for example dehydrogenases, in particular dehydrogenases selected from the group consisting of an alcohol dehydrogenase (EC 1.1.1.1.; EC 1.1.1.2), an L-amino acid dehydrogenase (EC 1.4.1.5), a glucose
20 dehydrogenase (EC 1.1.1.47), a glucose-6-phosphate dehydrogenase (EC 1.1.1.49), a glycerol dehydrogenase (EC 1.1.1.6), a 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30), a lactate dehydrogenase (EC 1.1.1.27; EC 1.1.1.28), a malate dehydrogenase (EC 1.1.1.37) and a sorbitol dehydrogenase. Further suitable enzymes are oxidases such as e.g. glucose oxidase (EC 1.1.3.4) or cholesterol
25 oxidase (EC 1.1.3.6), aminotransferases such as e.g. aspartate aminotransferase or alanine aminotransferase, 5'-nucleotidase, creatine kinase and diaphorase (EC 1.6.99.2). The enzyme is preferably an alcohol dehydrogenase (EC 1.1.1.1; EC

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1.1.1.2), a glucose dehydrogenase (EC 1.1.1.47), a glucose-6-phosphate dehydrogenase (EC 1.1.1.49) or a diaphorase (EC 1.6.99.2).

If a glucose dehydrogenase (EC 1.1.1.47) is used as the enzyme, then a mutated glucose dehydrogenase can for example be used within the scope of the method
5 according to the invention. The term "mutant" as used within the scope of the present application refers to a genetically modified variant of a native enzyme which, while having the same number of amino acids, has an amino acid sequence which is modified compared to 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
10 place site-specifically or non-site-specifically, preferably site-specifically using recombinant methods as known in the specialized field resulting in at least one amino acid substitution within the amino acid sequence of the native enzyme depending on the respective requirements and conditions. The mutant particularly preferably has an increased thermal or hydrolytic stability compared to the wild-type
15 enzyme. Examples of such mutants are described by Baik (Appl. Environ. Microbiol. (2005), 71, 3285), Vásquez-Figueroa (ChemBioChem. (2007), 8, 2295) as well as in WO 2005/045016 A2 to the disclosure of which reference is herewith explicitly made.

The mutated glucose dehydrogenase can in principle contain the amino acid(s)
20 which is(are) modified compared to the corresponding wild-type glucose dehydrogenase at any position in its amino acid sequence. The mutated glucose dehydrogenase preferably contains a mutation in at least one of the positions 96, 170 and 252 of the amino acid sequence of the wild-type glucose dehydrogenase, where mutants having mutations at position 96 and position 170 or mutations at position
25 170 and position 252 are particularly preferred. It has proven to be advantageous when the mutated glucose dehydrogenase contains no further mutations apart from these mutations.

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The mutation at positions 96, 170 and 252 can in principle comprise any amino acid substitution which results in a stabilization, e.g. an increase in the thermal or hydrolytic stability, of the wild-type enzyme. The mutation at position 96 preferably comprises an amino acid substitution of glutamic acid by glycine, whereas in
5 relation to position 170 an amino acid substitution of glutamic acid by arginine or lysine, in particular an amino acid substitution of glutamic acid by lysine, is preferred. With regard to the mutation at position 252 it preferably comprises an amino acid substitution of lysine by leucine.

The mutated glucose dehydrogenase can be obtained by mutation of a wild-type
10 glucose dehydrogenase derived from any biological source, where the term "biological source" in the context of this invention includes 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 and it is particularly preferably a glucose dehydrogenase from *Bacillus*
15 *megaterium*, *Bacillus subtilis* or *Bacillus thuringiensis*, in particular from *Bacillus subtilis*.

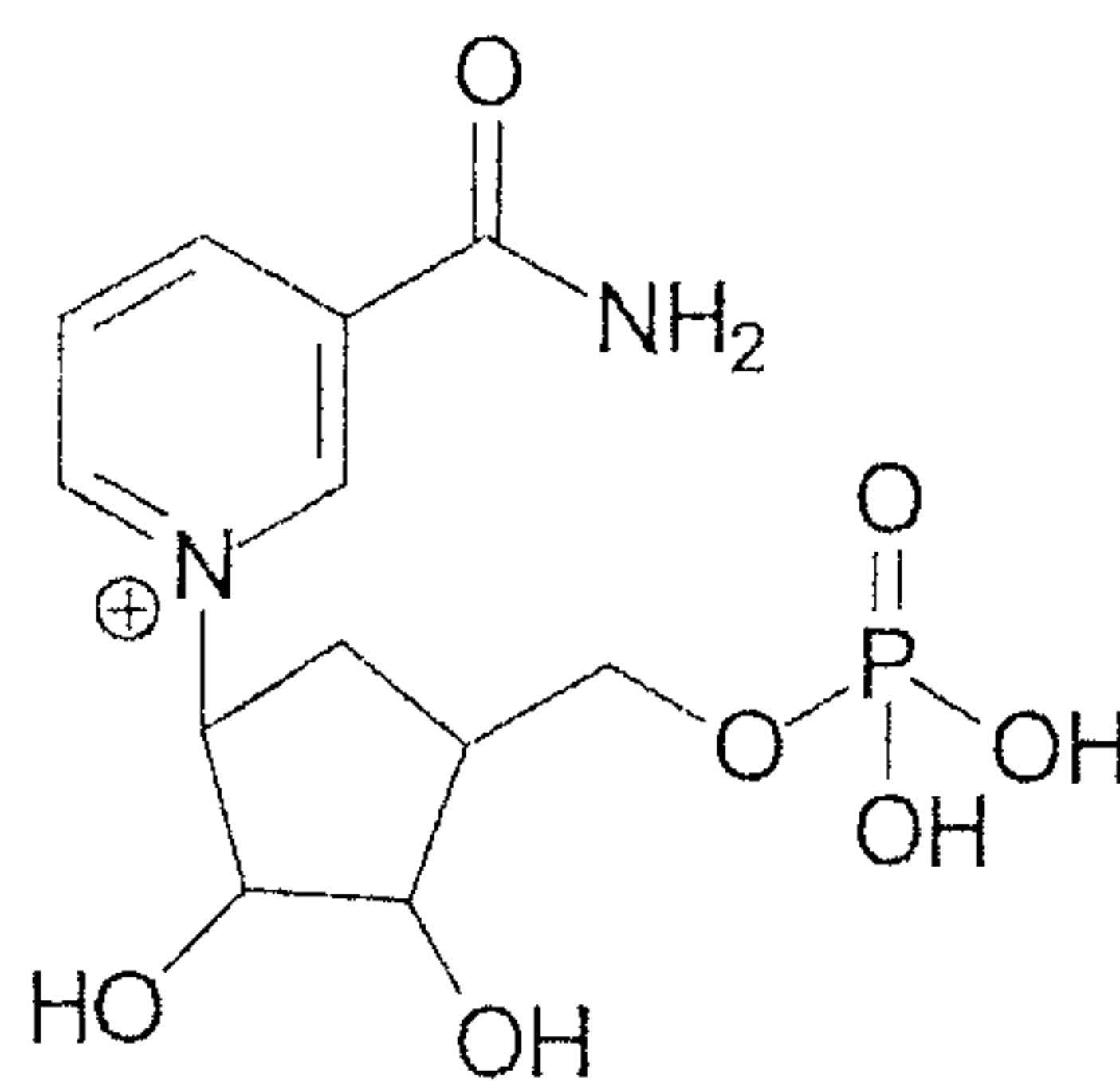
In a particularly preferred embodiment the mutated glucose dehydrogenase is a glucose dehydrogenase obtained by mutation of wild-type glucose dehydrogenase from *Bacillus subtilis* which has the amino acid sequence shown in SEQ ID NO:1
20 (GlucDH_E96G_E170K) or that shown in SEQ ID NO:2 (GlucDH_E170K_K252L).

A stabilized coenzyme within the scope of the present invention is a coenzyme which has been chemically modified compared to the native coenzyme and which at atmospheric pressure has a higher stability compared to the native coenzyme
25 towards moisture, temperatures especially in the range from 0°C to 50°C, acids and bases especially in the range from pH 4 to pH 10, or/and nucleophiles such as for example alcohols or amines and in this respect can exhibit its activity under

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identical environmental conditions over a longer time period than the native coenzyme. The stabilized coenzyme preferably has a higher hydrolytic stability compared to the native coenzyme where a complete hydrolytic stability under the test conditions is particularly preferred. In comparison with the native coenzyme,
 5 the stabilized coenzyme may have a reduced binding constant for the enzyme, for example a binding constant reduced by a factor of two or more.

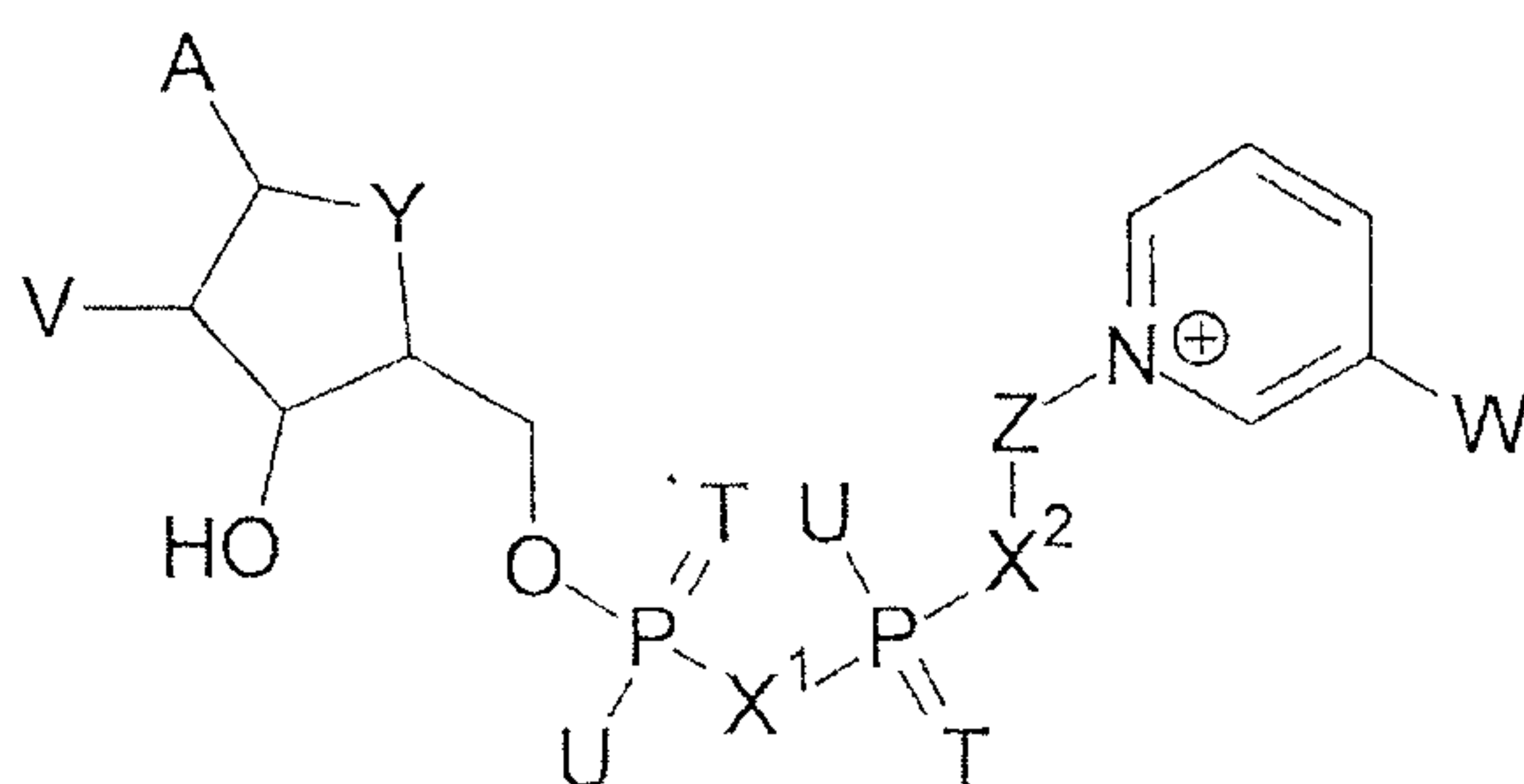
Preferred examples of stabilized coenzymes are stabilized derivatives of nicotinamide adenine dinucleotide (NAD/NADH) or nicotinamide adenine dinucleotide phosphate (NADP/NADPH) or shortened NAD derivatives e.g.
 10 without the AMP moiety or with non-nucleoside residues e.g. hydrophobic residues. The compound of formula (I) is likewise preferred as a stabilized coenzyme in the sense of the present invention.



(I).

Preferred stabilized derivatives of NAD/NADH and NADP/NADPH are described
 15 in the aforementioned references to the disclosure of which reference is herewith explicitly made. Particularly preferred stabilized coenzymes are described in WO 2007/012494 and US 11/460,366 to the disclosure of which reference is herewith explicitly made. The stabilized coenzyme is particularly preferably selected from compounds of the general formula (II):

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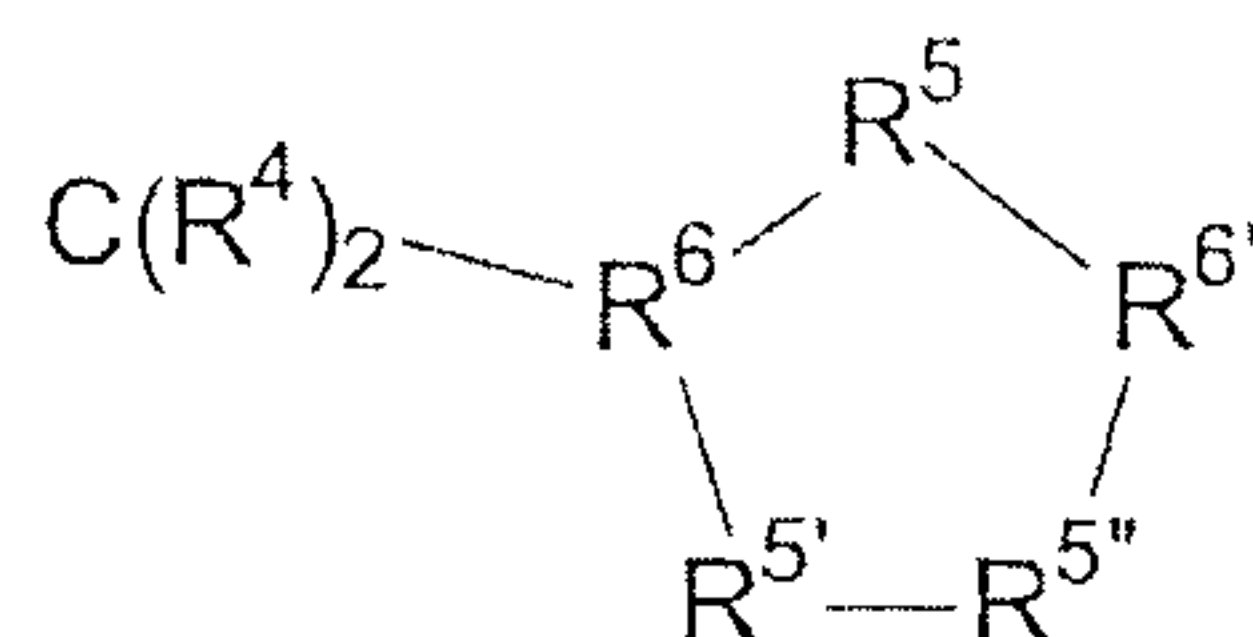
(II)

in which

- A = adenine or an analogue thereof,
- T = in each case independently O, S,
- 5 U = in each case independently OH, SH, BH_3^- , BCNH_2^- ,
- V = in each case independently OH or a phosphate group, or two groups which form a cyclic phosphate group,
- W = COOR , $\text{CON}(\text{R})_2$, COR , $\text{CSN}(\text{R})_2$ in which R = in each case independently H or $\text{C}_1\text{-C}_2$ alkyl,
- 10 X^1, X^2 = in each case independently O, CH_2 , CHCH_3 , $\text{C}(\text{CH}_3)_2$, NH, NCH_3
- Y = NH, S, O, CH_2 ,
- Z = a linear or cyclic organic residue
- provided that Z and the pyridine residue are not linked by a glycosidic bond, or a salt or optionally a reduced form thereof.
- 15 Z in the compounds of formula (II) is preferably a linear residue with 4-6 C atoms, preferably with 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 residue comprising a cyclic group with 5 or 6 C atoms which optionally contains a heteroatom selected from O, S and N as well as optionally one or more substituents, and a residue CR^4_2 , where
- 20 CR^4_2 is bound to the cyclic group and to X^2 , where R^4 is in each case independently H, F, Cl, CH_3 .

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



(III)

in which a single or double bond may be present between $R^{5'}$ and $R^{5''}$, where

5 $R^4 =$ in each case independently H, F, Cl, CH_3 ,

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

$R^{5'} =$ O, S, NH, $\text{NC}_1\text{-C}_2\text{-alkyl}$, CR^4_2 , CHOH , CHOCH_3 , and $R^{5''} = \text{CR}^4_2$, CHOH , CHOCH_3 if there is a single bond between $R^{5'}$ and $R^{5''}$,

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

10 and

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

In a preferred embodiment the compounds according to the invention contain adenine or adenine analogues such as e.g. C_8 -substituted and N_6 -substituted adenine, deaza variants such as 7-deaza, aza variants such as 8-aza or combinations
 15 such as 7-deaza or 8-aza or carbocyclic analogues such as formycin where the 7-deaza variants can be substituted in the 7 position by halogen, $\text{C}_1\text{-C}_6$ alkynyl, $\text{C}_1\text{-C}_6$ alkenyl or $\text{C}_1\text{-C}_6$ alkyl.

In a further preferred embodiment the compounds of formula (II) contain adenosine analogues which contain for example 2-methoxydeoxyribose, 2'-fluorodeoxyribose,
 20 hexitol, altritol or polycyclic analogues such as bicyclo, LNA and tricyclo sugars instead of ribose.

10a

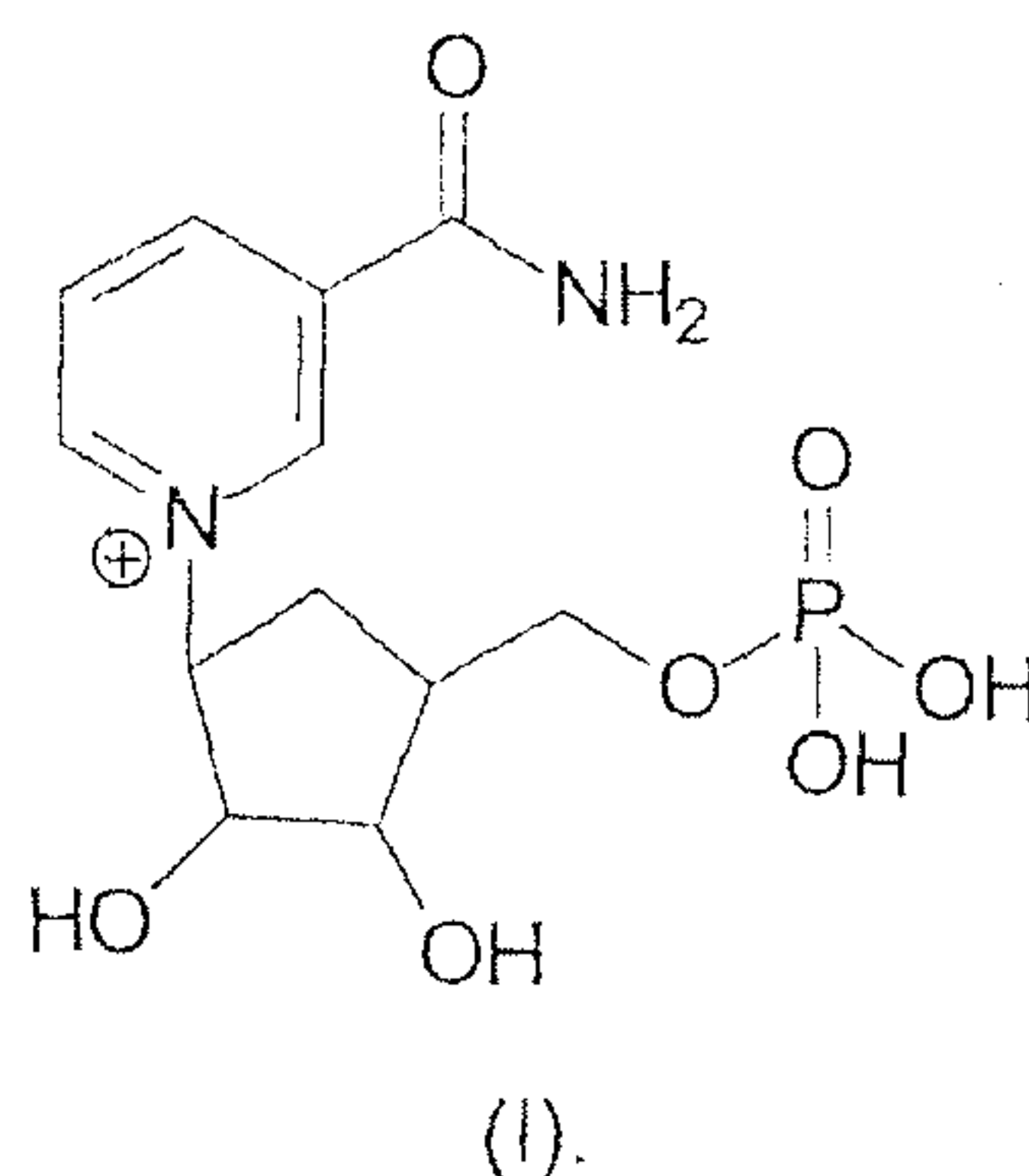
In accordance with one aspect of the present invention there is provided a method for stabilizing an enzyme

wherein

the enzyme is stored in the presence of a stabilized coenzyme, and an alcohol dehydrogenase having the number EC 1.1.1.2, a glucose-6-phosphate dehydrogenase having the number EC 1.1.1.49 or a diaphorase having the number EC 1.6.99.2 is used as the enzyme,

wherein

the stabilized coenzyme is a stabilized nicotinamide adenine dinucleotide (NAD/NADH) compound, a stabilized nicotinamide adenine dinucleotide phosphate (NADP/NADPH) compound or a compound of formula (I)



In accordance with another aspect of the present invention there is provided a enzyme which is stabilized with a stabilized coenzyme

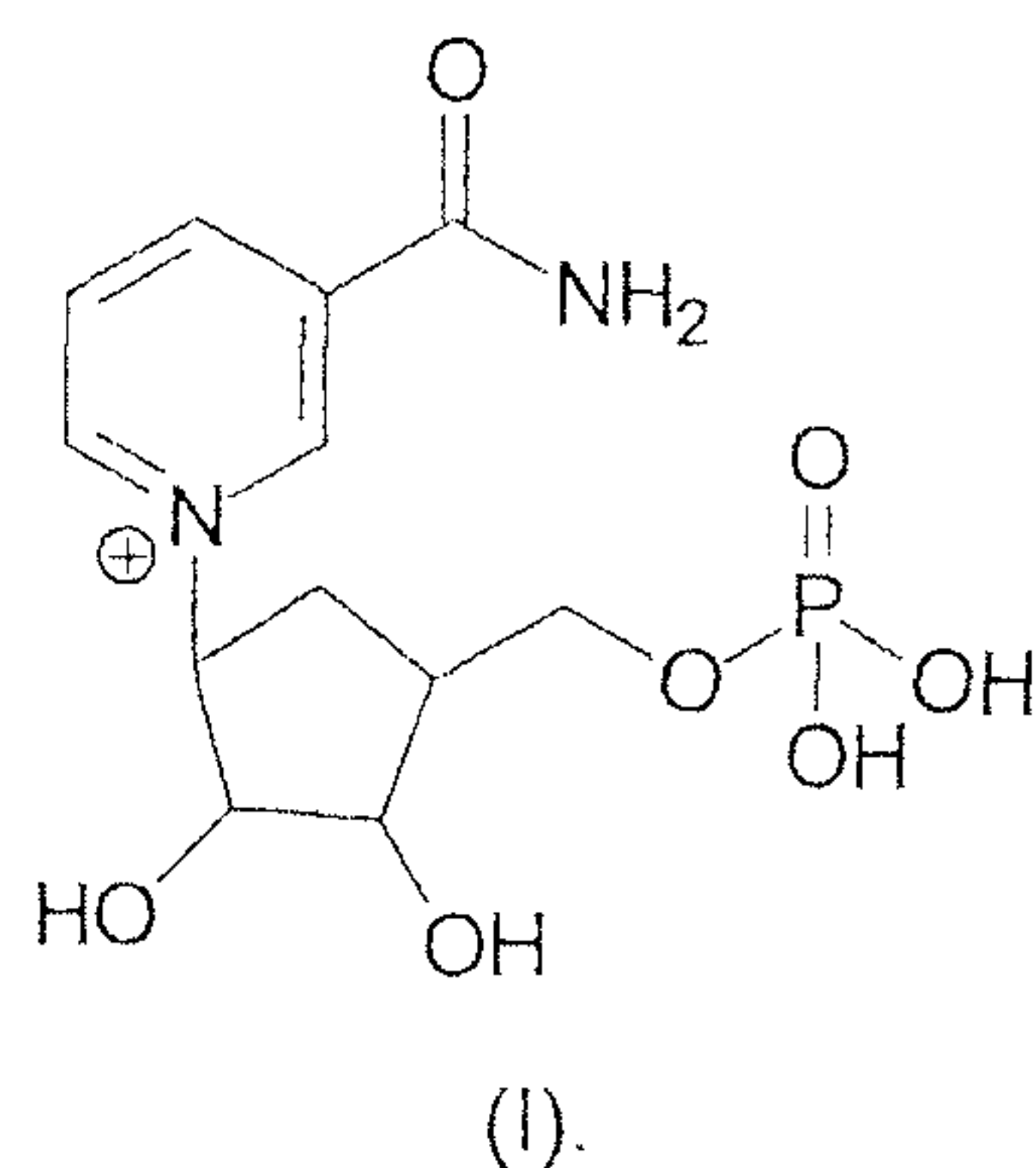
wherein

the enzyme exhibits a decrease in enzyme activity of less than 50 %, based on the initial value of enzyme activity when stored for at least 2 weeks, at a temperature of at least 20°C, or in the presence of light at a wavelength of ≥ 300 nm, optionally at high air humidity or in the absence of desiccants, wherein the enzyme is an alcohol dehydrogenase having the number EC 1.1.1.2, a glucose-6-phosphate dehydrogenase having the number EC 1.1.1.49 or a diaphorase having the number EC 1.6.99.2,

wherein

10b

the stabilized coenzyme is a stabilized nicotinamide adenine dinucleotide (NAD/NADH) compound, a stabilized nicotinamide adenine dinucleotide phosphate (NADP/NADPH) compound or a compound of formula (I)



In accordance with a further aspect of the present invention there is provided a method for stabilizing an enzyme

wherein

the enzyme is stored in the presence of a native coenzyme, where a dehydrogenase selected from the group consisting of an alcohol dehydrogenase having the number EC 1.1.1.1 or EC 1.1.1.2, an L-amino acid dehydrogenase having the number EC 1.4.1.5, a glucose dehydrogenase having the number EC 1.1.1.47, a glucose-6-phosphate dehydrogenase having the number EC 1.1.1.49, a glycerol dehydrogenase having the number EC 1.1.1.6, a 3-hydroxybutyrate dehydrogenase having the number EC 1.1.1.30, a lactate dehydrogenase having the number EC 1.1.1.27 or EC 1.1.1.28, and a malate dehydrogenase having the number EC 1.1.1.37, or a diaphorase having the number EC 1.6.99.2 is used as the enzyme

wherein

the native coenzyme is a native nicotinamide adenine dinucleotide (NAD/NADH) compound or a native nicotinamide adenine dinucleotide phosphate (NADP/NADPH) compound.

In particular (di)-phosphate oxygens can also be isotronically replaced in the compounds of formula (II) such as for example O^- by S^- or BH_3^- , O by NH, NCH_3 or CH_2 and $=O$ by $=S$. W in the compounds of formula (II) according to the invention is preferably $CONH_2$ or $COCH_3$.

- 5 R^5 in the groups of formula (III) is preferably CH_2 . Furthermore, it is preferred that $R^{5'}$ is selected from CH_2 , $CHOH$ and NH . In a particularly preferred embodiment $R^{5'}$ and $R^{5''}$ are each $CHOH$. In yet a further preferred embodiment $R^{5'}$ is NH and $R^{5''}$ is CH_2 . A compound of formula (III) in which $R^4 = H$, $R^5 = CH_2$, $R^{5'} = R^{5''} = CHOH$ and $R^6 = R^{6'} = CH$ is more preferred. Specific examples of preferred stabilized
- 10 coenzymes are shown in figures 1A and 1B. In the most preferred embodiment the stabilized coenzyme is the compound carbaNAD

The method according to the invention is especially suitable for the long-term stabilization of enzymes. This means that the stabilized enzyme is stored e.g. as a dry substance or in a liquid phase, for example over a period of at least 2 weeks,

15 preferably of at least 4 weeks and most preferably of at least 8 weeks during which the enzyme activity decreases preferably by less than 50 %, more preferably by less than 30 %, and most preferably by less than 20 % based on the initial value of the enzyme activity.

The method according to the invention additionally comprises a storage of the

20 stabilized enzyme at elevated temperatures, for example at a temperature of at least $20^\circ C$, preferably of at least $25^\circ C$ and most preferably of at least $30^\circ C$ during which the enzyme activity decreases preferably by less than 50 %, more preferably by less than 30 % and most preferably by less than 20 % based on the initial value of the enzyme activity. The storage can, if necessary, be carried out for a longer period as

25 stated above.

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Moreover, the method according to the invention envisages a storage of the stabilized enzyme in the presence of ambient light i.e. in the presence of light at a wavelength of ≥ 300 nm, during which the enzyme activity preferably decreases by less than 50 %, more preferably by less than 30 % and most preferably by less than 20 % based on the initial value of the enzyme activity. In this case the storage can, if necessary, be carried out for a longer period or/and at elevated temperatures as stated above. Due to the stability of the enzyme system towards ambient light, the stabilized enzyme can in addition also be exposed to direct sunlight shortly before use or/and after removal from a package.

10 The stabilization according to the invention also enables the stabilized enzyme to be stored without a desiccant or/and at a high relative air humidity e.g. at a relative air humidity of at least 50 %, during which the enzyme activity decreases by less than 50 %, more preferably by less than 30 % and most preferably by less than 20 % based on the initial value of the enzyme activity. In this case the storage can, if
15 necessary, be carried out for a longer period, at elevated temperatures or/and in the presence of ambient light as stated above. Methods or tests for determining the activity of enzymes are widely known in the prior art and, if required, can be adapted by a person skilled in the art to the respective requirements where the same test conditions are used in each case to compare the enzyme activity before and after
20 storage.

The stabilized enzyme can be stored on the one hand as a dry substance and, on the other hand, in a liquid phase. The stabilized enzyme is preferably stored on or in a test element that is suitable for determining an analyte. In this case the stabilized enzyme is preferably a component of a detection reagent which can optionally
25 contain other components such as mediators, optical indicators, salts, buffers etc.

The stabilized enzyme can be used to detect analytes for example parameters in body fluids, such as blood, serum, plasma or urine or in samples of sewage or in

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foods. Any biological or chemical substances that can be detected by a redox reaction can be determined as analytes e.g. substances that are substrates of a coenzyme-dependent enzyme or coenzyme-dependent enzymes themselves. 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 subject matter of the present invention is the use of a compound according to the invention or of an enzyme stabilized according to the invention to detect an analyte in a sample by means of an enzymatic reaction. This is particularly preferably the detection of glucose with the aid of a glucose dehydrogenase (EC 1.1.1.47) or a glucose-6-phosphate dehydrogenase (EC 1.1.1.49) using suitable coenzymes.

The change in the stabilized coenzyme caused by reaction with the analyte can in principle be detected in any way. In this case it is in principle possible to utilize all methods known from the prior art for detecting enzymatic reactions. However, the change in the coenzyme is preferably detected by optical methods. Optical detection methods comprise for example the measurement of absorption, fluorescence, circular dichroism (CD), optical rotation dispersion (ORD), refractometry.

Optical detection methods which are preferably used within the scope of the present application are photometry and fluorimetry. In order to photometrically measure a change in the coenzyme due to reaction with the analyte it is, however, additionally necessary for at least one mediator to be present which increases the reactivity of the reduced coenzyme and enables electrons to be transferred to a suitable optical indicator or an optical indicator system.

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Mediators which are suitable for the purposes of the present invention include among others nitrosoanilines such as for example [(4-nitrosophenyl)imino]-dimethanol hydrochloride, quinones such as for example phenanthrene quinones, phenanthroline quinones or benzo[h]-quinoline quinones, phenazines such as 1-(3-
5 carboxypropoxy)-5-ethylene phenazinium trifluoromethane sulfonate or/and diaphorase (EC 1.6.99.2).

Diaphorases, especially when compared with phenazines, have the advantage of a higher stability, but their function can be impaired by degradation products of native coenzymes e.g. by degradation products of NAD or NADP as known for example
10 from DE 2 061 984 A.

Preferred examples of phenanthroline quinones within the sense of the present invention comprise 1,10-phenanthroline-5,6-quinones, 1,7-phenanthroline-5,6-quinones, 4,7-phenanthroline-5,6-quinones as well as N-alkylated and N,N'-dialkylated salts thereof, where in the case of N-alkylated or N,N'-dialkylated salts,
15 halogenides, trifluoromethane sulfonate or other anions that increase the solubility are preferred as the counterion. Diaphorases which are particularly suitable for the purposes of the present invention comprise for example diaphorase from porcine heart, Clostridium kluyverii and Bacillus stearothermophilus as well as the diaphorase mutant described in US 2007/0196899A1, which have an improved
20 catalytic function and thermostability compared to native diaphorases. Reference is herewith explicitly made to the disclosure of the above US application.

Any substance which is reducible and undergoes a detectable change in its optical properties such as for example colour, fluorescence, reflectance, transmission, polarization or/and refractive index can be used as an optical indicator or as an
25 optical indicator system. The determination of the presence or/and the amount of the analyte in the sample can be carried out with the naked eye or/and by means of a detection device using a photometric method that appears suitable to a person

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skilled in the art. Heteropoly acids and in particular 2,18-phosphomolybdic acid are preferably used as optical indicators which are reduced to the corresponding heteropoly blue. Alternatively it is also possible to use quinones such as for example resazurine, dichlorophenol indophenol or/and tetrazolium salts as optical indicators.

- 5 Tetrazolium salts which are particularly suitable for the purpose of the present invention comprise for example the commercially available products WST-3, WST-4 and WST-5 (all from the Dojindo Company) but are, however, not limited to these.

10 The change in the coenzyme is particularly preferably detected by measuring the fluorescence. Fluorescence measurement is highly sensitive and enables even low concentrations of the analyte to be detected in miniaturized systems. Alternatively the change in the coenzyme can also be detected 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

15 reduced coenzyme into a reduced form by transfer of electrons. The analyte is determined by measuring the current which is needed to reoxidize the reduced mediator which correlates with the concentration of the analyte in the sample. Examples of mediators which can be used for electrochemical measurements comprise in particular the aforementioned mediators used for photometric

20 measurements.

It is possible to use a liquid test to detect an analyte where the reagent is for example present in the form of a solution or suspension in an aqueous or non-aqueous liquid, or as a powder or lyophilisate. However, it is also possible to use a dry test where the reagent is applied to a support. The support can for example be a

25 test strip comprising an absorbent or/and swellable material which is wetted by the sample liquid to be investigated.

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A particularly preferred test format comprises the use of the enzyme glucose-6-phosphate dehydrogenase with a stabilized NAD derivative for the detection of glucose especially in a wet test in which case a derivative of the reduced coenzyme NADH is formed. NADH is detected by optical methods e.g. by photometric or
5 fluorometric determination after UV excitation. A particularly preferred test system is described in US 2005/0214891 to which reference is herewith explicitly made.

A further aspect of the invention concerns an enzyme stabilized with a stabilized coenzyme where the stabilized enzyme exhibits a decrease in enzyme activity of less than 50 %, preferably of less than 30 % and most preferably of less than 20 %
10 based on the initial value of enzyme activity when stored for at least 2 weeks, preferably for at least 4 weeks and most preferably for at least 8 weeks at a temperature of at least 20°C, preferably of at least 25°C and most preferably of at least 30°C or/and in the presence of light at a wavelength of ≥ 300 nm, where appropriate at high air humidity or/and in the absence of desiccants. In this case an
15 enzyme is used as stated above.

Yet a further aspect of the invention concerns a detection reagent for determining an analyte which contains a stabilized enzyme as stated above. In addition the invention concerns a test element which contains an enzyme stabilized according to the invention or a detection reagent according to the invention. The detection
20 reagent and the test element may be suitable for carrying out dry tests or liquid tests. The test element is preferably a test strip for the fluorometric or photometric detection of an analyte. Such a test strip contains the stabilized enzyme in an immobilized form on an absorbent or/and swellable material such as cellulose, plastic etc.

25 Yet a further aspect of the invention concerns a method for stabilizing an enzyme especially towards ambient light, wherein the enzyme as previously specified, is stored in the presence of a native coenzyme. In a preferred variant an alcohol

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dehydrogenase (EC 1.1.1.1; EC 1.1.1.2), a glucose dehydrogenase (EC 1.1.1.47), a glucose-6-phosphate dehydrogenase (EC 1.1.1.49) or a diaphorase (EC 1.6.99.2) is used as the enzyme, where glucose dehydrogenases (EC 1.1.1.47) comprising native and mutated glucose dehydrogenases, and glucose-6-phosphate dehydrogenases (EC
5 1.1.1.49) are particularly preferred. A native nicotinamide adenine dinucleotide (NAD/NADH) or a native nicotinamide adenine dinucleotide phosphate (NADP/NADPH) compound and in particular native NAD or NADP is preferably used as the native coenzyme.

It is intended to elucidate the invention in more detail on the basis of the following
10 figures and examples.

Description of the figures

Figure 1A: Diagram of the stabilized coenzyme carbaNAD (cNAD).

Figure 1B: Diagram of the stabilized coenzyme pyrrolidinyl NAD.

Figure 2: Diagram of the results of the enzyme kinetics of glucose dehydrogenase
15 in the presence of NAD and of glucose dehydrogenase in the presence of cNAD 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 5 weeks storage at 32°C and
20 85 % relative air humidity.

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

Figure 3: Comparison of the blank values of glucose dehydrogenase in the presence of NAD or of GlucDH in the presence of cNAD over a period of up to 5 weeks at 32°C and 85 % air humidity.

Figure 4: Diagram of various function curves of glucose dehydrogenase after storage of glucose dehydrogenase in the presence of NAD at 32°C and 85 % air humidity. The storage period varied between 1 day and 5 weeks.

Figure 5: Diagram of various function curves of glucose dehydrogenase after storage of glucose dehydrogenase in the presence of cDNA at 32°C and 85 % air humidity. The storage period varied between 1 day and 5 weeks (figure 5A) and between 1 day and 24 weeks (figure 5B).

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

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

Figure 8: Diagram 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 (GlucDG-Mut2) for a period of 25 weeks in the presence of NAD or cNAD at 32°C and 83 % relative air humidity.

Figure 9: Diagram of the stability of glucose dehydrogenase in the presence of NAD or cNAD in a liquid phase at 50°C for a period of 4 days (figure 9A) or 14

days (figure 9B). Test conditions: 10 mg/ml GlucDH; 12 mg/ml NAD or cNAD; buffer: 0.1 M Tris, 1.2 M NaCl, pH 8.5; temperature 50°C.

Figure 10: Diagram of various function curves of alcohol dehydrogenase in the presence of cNAD. The concentration of cNAD varied between 25 % and 150 %
5 based on the initial concentration of NAD in the liquid test.

Figure 11: Diagram of the results of the enzyme kinetics of alcohol dehydrogenase in the presence of cNAD at various ethanol concentrations.

Figure 12: Diagram of the stability of alcohol dehydrogenase from yeast in the presence of NAD or cNAD in a liquid phase at 35°C for a period of 65 hours. Test
10 conditions: 5 mg/ml ADH; 50 mg/ml NAD or cNAD; buffer: 75 mM Na₄P₂O₇; glycine, pH 9.0; temperature 35°C.

Figure 13: Diagram of various function curves of glucose dehydrogenase after 11 weeks storage in the presence of NAD and different mediators at room temperature.

Figure 14: Diagram of the results of the enzyme kinetics of glucose dehydrogenase
15 in the presence of NAD and 1-(3-carboxypropoxy)-5-ethylphenazinium-trifluoromethane sulfonate at various glucose concentrations.

Figure 15: Schematic representation of the glucose detection using GlucDH as the enzyme and diaphorase as the mediator.

Figure 16: Diagram of the function curves of glucose-dye-oxidoreductase
20 (GlucDOR) in the presence of pyrroloquinoline quinone (PQQ) and [(4-nitrosophenyl)imino]dimethanol hydrochloride as the mediator and of glucose

dehydrogenase in the presence of NAD and diaphorase / [(4-nitrosophenyl)imino]-dimethanol hydrochloride as the mediator.

Figure 17: Diagram of the results of the enzyme kinetics of glucose dehydrogenase in the presence of NAD and diaphorase at various glucose concentrations.

- 5 **Figure 18:** Diagram 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 or cNAD. Test conditions: 25 mM NAD or cNAD; 2.5 seconds delay; 5 seconds measurement time.

- 10 **Figure 19:** Diagram of various function curves of glucose dehydrogenase in the presence of NAD after irradiation by UV light at a wavelength of 360 nm.

Figure 20: Diagram of the absorption spectrum of cNAD and cNADH in the wavelength range of 250-450 nm.

Figure 21: Diagram of the amino acid sequences of the glucose dehydrogenase double mutants GlucDH_E96G_E170K and GlucDH_E170K_K252L.

15 **Example 1**

- carbaNAD (fig. 1A) or NAD were added to the glucose-specific GlucDH. These formulations were each applied to Pokalon foils (Lonza) and, after drying, stored under warm and humid conditions (32°C, 85 % relative air humidity). Subsequently the reaction kinetics and the function curve were determined at regular intervals. In parallel at each of the measurement times a cNAD/NAD analysis and a determination of the residual activity of the enzyme were carried out.
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The kinetics curves for NAD (figure 2A) and cNAD (figure 2B) determined on the first day are comparable and also show a similar increase in the glucose dependency. However, a significant difference in the kinetics curves can be seen after 5 weeks. Whereas there is a major decrease in the dynamic range of the kinetics for NAD (figure 2C), the kinetics of the enzyme stabilized with cNAD remains virtually unchanged (figure 2D).

There is also a considerable difference in the blank values (dry blank value before application of a blood sample) as shown in figure 3. The increase in the dry blank value for NAD is due to the formation of fluorescent particles (Oppenheimer (1982), Supra). Surprisingly this does not occur with cNAD.

The different stability of glucose dehydrogenase in the presence of NAD or cNAD is also evident from a comparison of figures 4 and 5. After 5 weeks the function curve for the enzyme stabilized with cNAD still lies within the set of curves of the previous measurements (figure 5A), whereas the curve for the enzyme treated with NAD (figure 4) shows a fall-off at higher concentrations which is a typical sign for inadequate amounts of enzyme / coenzyme. Figure 5B shows various function curves of the glucose dehydrogenase stabilized with cNAD over a period of 24 weeks. In this connection it is clear that the function of the enzyme is only slightly changed at high glucose concentrations over the entire period and approximately corresponds after 24 weeks to the value obtained after 5 weeks.

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

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The result of the determination of residual activity of the active enzyme GlucDH after 5 weeks at 32°C and 85 % relative air humidity (figure 7A) is completely surprising. The enzyme stabilized with NAD now only has an extremely low enzyme activity (0.5 %), whereas the enzyme stabilized with cNAD still has a
5 residual activity of 70 % (in each case compared to the samples stored in a refrigerator (KS) with desiccant (TM)). After 24 weeks at 32°C and 85 % relative air humidity (figure 7B) the residual activity of the enzyme when stabilized with cNAD is still about 25 %.

If a mutant is used instead of the wild-type enzyme (from *Bacillus subtilis*), it is
10 possible to further increase the residual activity of GlucDH. After 24 weeks storage at 32°C and 85 % relative air humidity in the presence of cNAD the residual activity of a mutant GlucDH_E96G_E170K with the amino acid substitutions 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
15 mutant GlucDH_E170K_K252L with the amino acid substitutions glutamic acid → lysine at position 170 and lysine → leucine at position 252 (GlucDH-mut2) is about 50 % (figure 8).

Storage of glucose dehydrogenase in a liquid phase also clearly shows the difference between NAD and cNAD (figures 9A and 9B). After 95 hours at 50°C the residual
20 activity of glucose dehydrogenase in the presence of the native coenzyme NAD is » 5 %, whereas the residual activity of GlucDH in the presence of the artificial coenzyme cNAD is 75 % (figure 9A). After 336 hours storage at 50°C the residual activity of the enzyme stabilized with NAD is now only about 1 %; a residual activity of still about 70 % is observed for the enzyme stored in the presence of
25 cNAD. The corresponding SDS gels also show a change in the GlucDH bands in the presence of the native coenzyme NAD: new bands are seen at higher molar masses and there is a shift in the 30 kDa band.

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Overall it is an extremely surprising result that the 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 an adverse effect on the stability of the enzyme GlucDH and even
5 accelerates its inactivation. Replacement of native NAD by artificial analogues allows GlucDH to be stored under stress conditions (e.g. elevated temperatures) 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
10 desiccant is possible.

Example 2

cNAD or NAD was added to a detection solution containing alcohol dehydrogenase. These mixtures were stored at 2 - 8°C and at 35°C. Subsequently the stability of alcohol dehydrogenase (ADH) was checked at regular intervals and the residual
15 activity of the enzyme was determined.

Figure 10 shows the linearity of the conversion of ethanol by means of alcohol dehydrogenase (ADH) in the presence of various concentrations of cNAD which demonstrates the practical usability of the enzyme system ADH/cNAD to determine ethanol. Moreover, the kinetics curves of the conversion of ethanol by means of a
20 combination of alcohol dehydrogenase and cNAD show that there is a considerable dependency on the concentration of the substrate where the rate of conversion increases with increasing ethanol concentrations (figure 11).

Again storage in a liquid phase shows the difference between storage in the presence of NAD or cNAD (figure 12). The residual activity of alcohol

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dehydrogenase in the presence of the native coenzyme NAD is at about 6 % after 65 hours at 35°C, whereas the residual activity of the enzyme in the presence of the artificial coenzyme cNAD is still at about 60 %.

If alcohol dehydrogenase is stored together with native NAD or with cNAD for several months at 2 - 8°C in a refrigerator, then one observes in the case of cNAD a considerable decrease in enzyme activity over the entire storage period. Whereas the difference after 2 weeks storage is still slight, there is an approximately 20 % higher residual activity of alcohol dehydrogenase after 12 months storage in the presence of 16 mM cNAD compared to a corresponding solution containing 16 mM NAD as the coenzyme. The results are shown in table 1.

Table 1

ingredient	concentr. (mmol/l)	U/ml (initial value)	% of weighed amount (initial value)	2 weeks at 2- 8°C (% of initial value)	3 months at 2- 8°C (% of initial value)	7.5 months at 2- 8°C (% of initial value)	10 months at 2- 8°C (% of initial value)	12 months at 2- 8°C (% of initial value)
comparable mixture	16 (NAD)	142.2	85.2	96.9	92.1	80.2	70.3	60.5
exchange NAD/cNAD	16	120.2	71.9	100.7	91.3	95.6	88.2	83.8

The extent of the stabilization in relation to the amount of cNAD used is shown in table 2. Accordingly the residual activity of alcohol dehydrogenase can be increased somewhat in samples that have been stored for 2 weeks at 2 - 8°C by increasing the concentration of cNAD. In the stress model, which envisages a storage of the enzyme for 2 weeks at 35°C, the decrease of the enzyme activity of alcohol dehydrogenase can, however, be significantly increased by increasing concentrations of cNAD and in the case of a concentration of 15 mM to cNAD an

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approximately 45 % higher residual activity is observed compared to a solution of the enzyme in the presence of 0.5 mM cNAD.

Table 2

Sample	pH /additive	U/ml (initial value)	% of weighed amount (initial value)	2 weeks at 2- 8°C (% of initial value)	2 weeks at 35°C (% of initial value)
1	pH 7.85 0.5 mM cNAD	123.1	73.7	93.8	31.1
2	pH 7.85 1 mM cNAD	125.8	75.3	97.7	45.5
3	pH 7.85 5 mM cNAD	125.1	74.9	101.1	74.1
4	pH 7.85 15 mM cNAD	123.7	74.1	104.0	77.6

5 Example 3

Various test systems which each contained glucose dehydrogenase, NAD, a mediator and, where appropriate, an optical indicator were measured photometrically and electrochemically in order to determine glucose.

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

As shown in figure 13, a decrease in the reflectance was observed with increasing glucose concentrations for all four mediators that were used i.e. [(4-nitroso-phenyl)imino]dimethanol hydrochloride (med A), 1-methyl-5,6-dioxo-5,6-dihydro-1,10-phenanthroline-trifluoromethane sulfonate (med B), 7-methyl-5,6-dioxo-5,6-dihydro-1,7-phenanthroline-trifluoromethane sulfonate (med F) and 1-(3-

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carboxy-propoxy)-5-ethylphenazinium-trifluoromethane sulfonate (med G) and thus the above-mentioned mediators are in principle suitable for determining glucose by means of photometry.

At high glucose concentrations in the region of 800 mg/dl the reflectance of the measured sample is still about 20 % when using [(4-nitrosophenyl)imino]di-methanol hydrochloride or 1-(3-carboxypropoxy)-5-ethylphenazinium-trifluoromethane sulfonate 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 system glucose dehydrogenase, NAD, 1-(3-carboxypropoxy)-5-ethylphenazinium-trifluoromethane sulfonate and 2,18-phosphomolybdic acid at glucose concentrations in the range from 0 to 800 mg/dl is shown in figure 14.

The schematic representation in figure 15 shows that the photometric determination of glucose can also take place with the (additional) use of diaphorase as an intermediary mediator. Figure 16 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 glucose-dye-oxidoreductase, pyrroloquinoline quinone, [(4-nitrosophenyl)imino]dimethanol hydrochloride and 2,18-phosphomolybdic acid system (system 2) served as a comparison 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 is shown in figure 17 for glucose concentrations in the range from 0 to 800 mg/dl.

As an alternative to photometry, an electrochemical measurement can also be used for the purposes of determining analytes. Thus, the current required to reoxidize the reduced mediator was found to be linearly dependent on the glucose concentration

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(figure 18) both for a test element which besides glucose dehydrogenase, contained NAD as a coenzyme and 1-(3-carboxypropoxy)-5-ethylphenazinium-trifluoromethane sulfonate as a mediator, and for a corresponding system which contained the stabilized coenzyme cNAD instead of NAD.

- 5 It has thus been shown that the analyte determination can be carried out using the dehydrogenase / stabilized coenzyme system as well as by means of electrochemical detection and evaluation at another wavelength which is independent of the coenzyme. The overall formulation should also be further stabilized by the use of the stabilized enzyme / coenzyme pair.

10 **Example 4**

In order to determine their stability towards ambient light, various test systems which each contained an enzyme selected from glucose dehydrogenase (GlucDH), glucose-6-phosphate dehydrogenase (G6PDH) and glucose dehydrogenase mutant 2 (GlucDH-mut2) in combination with NAD or carbaNAD were prepared in
15 accordance with the methods described in WO 03/097859 A2 and subsequently a blood sample was added. In particular the test systems were prepared by applying a photopolymerizable liquid composition comprising an enzyme and coenzyme to a support and subsequently irradiating the composition with light at a wavelength of 360 nm and 400 W for 10 seconds to obtain reagent layers having a thickness of 12
20 - 15 µm. Detection was carried out in the fluorescence mode for a period of several minutes.

Figure 19 shows the results of the measurements for the GlucDH/NAD system. As shown by the graph no reduction of fluorescence whatsoever was observed over the entire measurement period which allows the conclusion that even irradiation of the
25 reagent layer lasting several minutes with energy-rich radiation does not impair the reagent layer due to the lack of absorption of the GlucDH/NAD system in the region

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≥ 300 nm. Taking into consideration the fact that the G6PDH/NAD system and the carbaNAD-containing systems GlucDH/carbaNAD, G6PDH/carbaNAD and GlucDH-mut2/carbaNAD do not absorb in the wavelength region ≥ 300 nm (figure 20), all these systems also have a high stability towards ambient light (results not shown).

Example 5

Specific examples of compositions that can be used to produce the diagnostic test elements described herein are specified in the following.

a) Liquid reagent for determining the activity of lactate dehydrogenase

10 A liquid reagent which, among others, contained diaphorase, carbaNAD, a tetrazolium salt and lactate was used to determine the activity of lactate dehydrogenase. The detection reagent which was stored in solution at 25°C contained the following ingredients:

3 U/ml diaphorase (from porcine heart)
15 2 mM carbaNAD or 0.2 mM NAD
2 mM tetrazolium salt WST-3
50 mM Na lactate
0.1 M tricine/HCl, pH 8.8

A measurement of the activity of diaphorase at various storage times showed a
20 considerably increased residual activity of the formulation containing carbaNAD compared to a corresponding formulation containing NAD as the coenzyme.

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b) Test strips for the determination of blood glucose

A composition which, among others, contained glucose dehydrogenase (GlucDH), carbaNAD, diaphorase, nitrosoaniline and phosphomolybdic acid was used to determine blood glucose. Test strips were obtained by applying a first formulation to a polycarbonate foil using a doctor blade (layer height 100 µm), drying the first layer, applying a second formulation to the first layer (doctor blade gap 30 µm), and drying the second layer and they were stored at 32°C and 30 - 70 % relative air humidity. The formulations used for the first and second layer are shown in table 3:

Table 3

Ingredients	1st layer	2nd layer
xanthan gum	0.09 g	---
sodium aluminium silicate	5.00 g	---
polyvinyl propionate dispersion (50 % by weight in water)	4.80 g	5.80 g
methyl vinyl ether-maleic acid copolymer	---	1.40 g
titanium dioxide	---	14.00 g
NaOH 16 %	---	2.80 g
silicic acid	---	2.50 g
phosphate buffer 0.1 M	13.80 g	4.40 g
N-octanoyl-N-methyl-glucamide	0.17 g	0.34 g
Na-methyl oleyl taurate	0.03 g	0.03 g
polyvinyl pyrrolidone (MW 25000)	0.85 g	1.80 g
tetraethylammonium chloride	0.12 g	---
Bis-(2-hydroxyethyl)-(4-hydroxyiminocyclohexa-2,5-dienylidene)- ammonium chloride	0.10 g	---
2,18-phosphomolybdic acid, hexasodium salt	0.50 g	---
NaCl	1.00 g	---
carbaNAD (or NAD)	1.00 g (0.10 g)	---
Diaphorase	1.94 g (203 KU)	---
glucose dehydrogenase	0.97 g (207 KU)	---
Hexanol	0.17 g	0.16 g
2-methoxy-propanol	4.30 g	4.30 g

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The activity of diaphorase was measured at various storage times by extracting the enzyme from the test strip and in the case of the formulation containing carbaNAD a considerably increased residual activity was observed compared to the formulation containing NAD.

5 c) Test strips for the determination of triglycerides

A composition which, among others, contained glycerol dehydrogenase, carbaNAD, diaphorase and a tetrazolium salt was used to determine triglycerides. Test strips were obtained by applying the formulation mentioned in table 4 to a polycarbonate foil by means of a doctor blade (layer height 80 µm) and subsequent drying, and they were stored at 32°C and 30 - 70 % relative air humidity.

Table 4

Ingredients	Amount
xanthan gum	0.30 g
silicic acid (Aerosil)	5.00 g
polyvinylpropionate dispersion (50 % by weight in water)	6.00 g
titanium dioxide	18.00 g
N-octanoyl-N-methyl-glucamide	0.20 g
polyvinyl pyrrolidone (MW 25000)	0.40 g
Tricine	0.30 g
HCl	ad pH 8.5
lipase (porcine pancreas), 12 KU/g	5.00 g
glycerol dehydrogenase, 100 U/mg	1.00 g
carbaNAD (or NAD)	1.60 g (0.20 g)
diaphorase, 20 U/mg	2.00 g
WST-5	2.00 g
Water	82.00 g

The activity of diaphorase was measured at various storage times by extracting the enzyme from the test strip and in the case of the formulation containing carbaNAD

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a considerably increased residual activity was observed compared to the formulation containing NAD.

Example 6

In order to evaluate the stabilization of glucose dehydrogenase and diaphorase by carbaNAD, a number of test strips were prepared in analogy to example 5. The formulations used for the first and the second layer of the test strip are shown in table 5.

Table 5

Ingredients	1st layer	1st layer	2nd layer
xanthan gum	0.29 g	0.29 g	---
Gantrez S97	---	---	1.44 g
tetraethylammonium chloride	0.07 g	0.07 g	0.45 g
Mega 8	0.17 g	0.17 g	0.36 g
Geropon T77	0.03 g	0.03 g	0.03 g
polyvinyl pyrrolidone (MW 25000)	0.87 g	0.87 g	1.99 g
Transpafill (or silicic acid)	4.82 g (4.79 g)	4.82 g (4.79 g)	---
silicic acid	---	---	16.02 g
titanium dioxide	---	---	2.18 g
Propiofan	5.04 g	5.02 g	6.14 g
N,N-bis-(2-hydroxyethyl)-4-nitrosoaniline	0.10 g	0.10 g	0.16 g
2,18-phosphomolybdic acid, hexasodium salt	0.33 g	0.33 g	1.98 g
K/Na buffer 0.1 M	10.00 g	9.96 g	---
NAD	0.58 g	---	---
carbaNAD	---	4.00 g	---
glucose dehydrogenase, mutant 2	1.06 g (324 KU)	1.06 g (324 KU)	---
Diaphorase	0.78 g (80 KU)	1.26 g (130 KU)	---
K ₃ [Fe(CN) ₆]	0.01 g	0.01 g	0.01 g
NaOH 16 %	0.25 g	0.28 g	2.69 g
tert. amyl alcohol	1.00 g	1.00 g	1.07 g

10 The test strips were stored over a period of 18 weeks at temperatures of 5°C (KS, refrigerator), 25 °C (RT), 30°C (GT), 35°C (DT) and 45°C (HT), and the enzyme

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activity in the test strips was determined at the start of storage (0 weeks), after 6 weeks, after 9 weeks, after 12 weeks and after 18 weeks. The results are shown in table 6 (glucose dehydrogenase) and in table 7 (diaphorase).

Table 6

5

Storage temperature	Coenzyme	Enzyme activity (U/cm ²)					
		0 weeks	6 weeks	9 weeks	12 weeks	18 weeks	Target
KS	NAD	14.6	16.5	16.6	14.4	13.8	25.4
RT			15.8	14.4	12.9	12.2	
GT			n.d.	n.d.	9.7	9.1	
DT			12.9	10.9	9.4	8.5	
HT			7.8	6.2	n.d.	n.d.	
KS	carbaNAD	19.8	20.4	21.2	18.5	18.8	25.8
RT			20.7	21.5	18.0	17.7	
GT			n.d.	n.d.	17.6	16.8	
DT			19.8	20.2	17.2	16.9	
HT			19.0	18.8	n.d.	n.d.	

Table 7

Storage temperature	Coenzyme	Enzyme activity (U/cm ²)					
		0 weeks	6 weeks	9 weeks	12 weeks	18 weeks	Target
KS	NAD	2.7	2.2	2.4	3.3	3.7	6.3
RT			2.3	2.2	2.8	3.4	
GT			n.d.	n.d.	2.4	2.8	
DT			1.5	1.8	2.4	2.8	
HT			1.5	1.1	n.d.	n.d.	
KS	carbaNAD	4.6	4.4	5.2	4.8	7.0	10.4
RT			4.5	5.1	4.6	6.7	
GT			n.d.	n.d.	4.3	6.2	
DT			3.4	4.4	4.0	5.3	
HT			3.9	3.8	n.d.	n.d.	

10 As shown in tables 7 and 8, storage of glucose dehydrogenase and diaphorase in the presence of carbaNAD over a period of 18 weeks allows a high enzyme activity to be maintained while the degradation rate of the enzymes in the presence of NAD is considerably more pronounced.

Example 7

In order to determine the influence of NAD and carbaNAD on the thermostability of various dehydrogenases the enzymes (1 mg/ml) were dialysed in a first step against the respective measurement buffer for 20 hours at 4°C. Subsequently 3.8 mM NAD
5 or carbaNAD was added to the samples and the samples were kept at 4°C.

In order to determine the binding of NAD or carbaNAD to the various dehydrogenases, a dynamic differential scanning calorimetry (DSC) was carried out in which the calorimetric scans were carried out at temperatures between 20°C and 100°C and at a scan rate of 120°C/h and each sample was measured in triplicate.

10 The DSC device was cleaned and calibrated according to the MicroCal handbook before carrying out the measurements in order to ensure a correct operation. Lysozyme (1 mg/ml) in 0.1 M glycine-HCl pH 2.4 was measured in duplicate as a control before and after each scan process. Measuring cells, valve and syringe were washed three times with water after every six injections. The data were analysed
15 using the MicroClas Origin software.

Glucose dehydrogenase mutant2 in Tris buffer, pH 8.0 led to well-defined peaks in the absence as well as in the presence of NAD and the melting point (T_M) was 79.1°C in the absence of NAD and 80.8°C in the presence of NAD. Thus, the binding of the glucose dehydrogenase mutant2 to NAD resulted in a T_M shift of
20 more than 1.5°C. With the exception of alcohol dehydrogenase, T_M was also increased in the case of other dehydrogenases for example in the case of wild-type glucose dehydrogenase when the enzyme was contacted with NAD or carbaNAD.

Table 8 shows the data obtained by DSC analysis for the various dehydrogenases where in each case experimental conditions were selected which resulted in a single

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peak in the DSC spectrum. The effect of binding NAD or carbaNAD to the dehydrogenase was compared in each case with scans in which no coenzyme (ligand) was present. In each case 0.1 M Tris buffer, pH 8.5 was used as the measurement buffer if not stated otherwise.

5

Table 8

dehydrogenase	ligand	ΔH_{cal} (kcal/mol)	T_M (°C)
alcohol dehydrogenase*	---	260	55.5
	NAD	300	55.5
	carbaNAD	310	55.4
glucose dehydrogenase (wild- type)	---	64	52.8
	NAD	110	63.5
	carbaNAD	84	58.5
D-lactate dehydrogenase $\neq$	---	18	58.4
	NAD	19	60.4
	carbaNAD	19	59.1
L-lactate dehydrogenase (porcine)	---	80	59.8
	NAD	85	61.8
	carbaNAD	82	61.5
L lactate dehydrogenase (from rabbit)	---	64	58.2
	NAD	72	60.1
	carbaNAD	66	60.2
malate dehydrogenase $\nmid$	---	310	42.4
	NAD	380	45.1
	carbaNAD	340	43.2

* 0.75 M glycine sodium pyrophosphate, pH 9.0

$\neq$ 0.1 M Tris buffer, pH 7.5

$\nmid$ 0.5 M carbonate buffer, pH 10.0

Claims

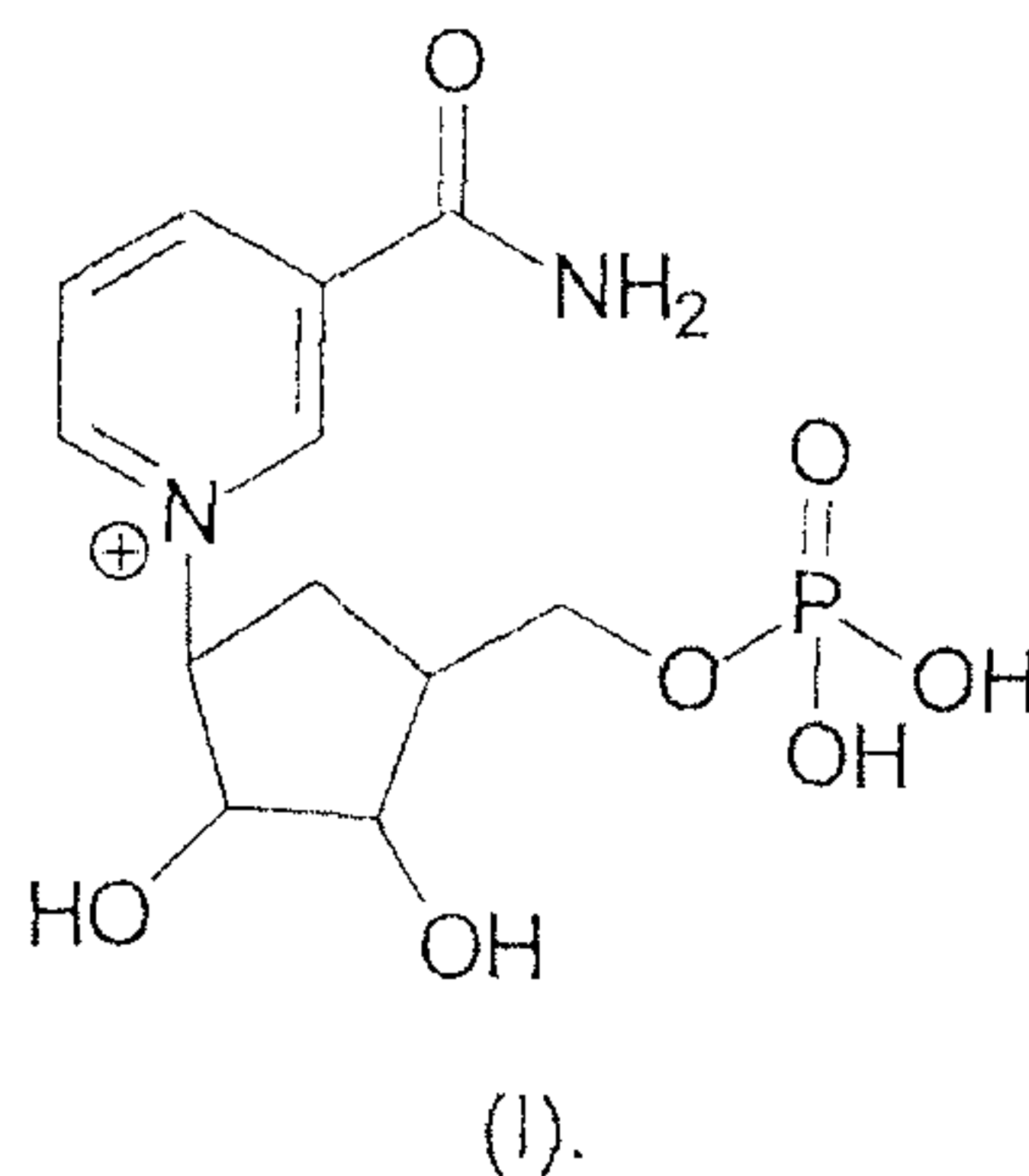
1. Method for stabilizing an enzyme

wherein

the enzyme is stored in the presence of a stabilized coenzyme, and an alcohol dehydrogenase having the number EC 1.1.1.2, a glucose-6-phosphate dehydrogenase having the number EC 1.1.1.49 or a diaphorase having the number EC 1.6.99.2 is used as the enzyme,

wherein

the stabilized coenzyme is a stabilized nicotinamide adenine dinucleotide (NAD/NADH) compound, a stabilized nicotinamide adenine dinucleotide phosphate (NADP/NADPH) compound or a compound of formula (I)

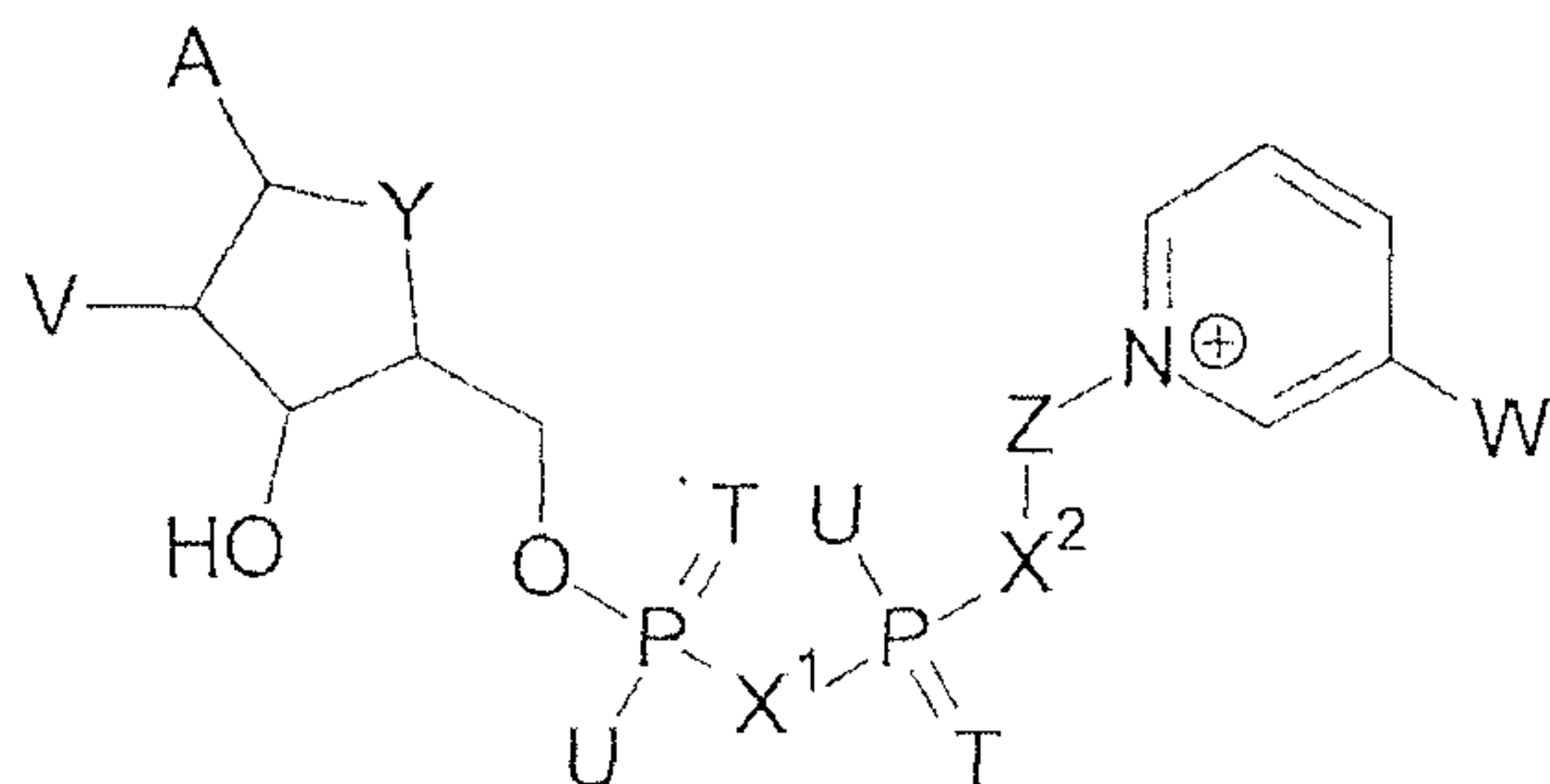


2. The method of claim 1,

wherein

a compound of the general formula (II) is used as the stabilized coenzyme:

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(II)

in which

A = adenine or an analogue thereof,

T = in each case independently O, S,

U = in each case independently OH, SH, BH_3^- , BCNH_2^- ,

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

W = COOR , $\text{CON}(\text{R})_2$, COR , $\text{CSN}(\text{R})_2$ in which R = in each case independently H or $\text{C}_1\text{-C}_2$ alkyl,

X^1 , X^2 = in each case independently O, CH_2 , CHCH_3 , $\text{C}(\text{CH}_3)_2$, NH, NCH_3

Y = NH, S, O, CH_2 , and

Z = a linear or cyclic organic residue,

provided that Z and the pyridine residue are not linked by a glycosidic bond, or a salt or optionally a reduced form thereof.

3. The method of claim 2,

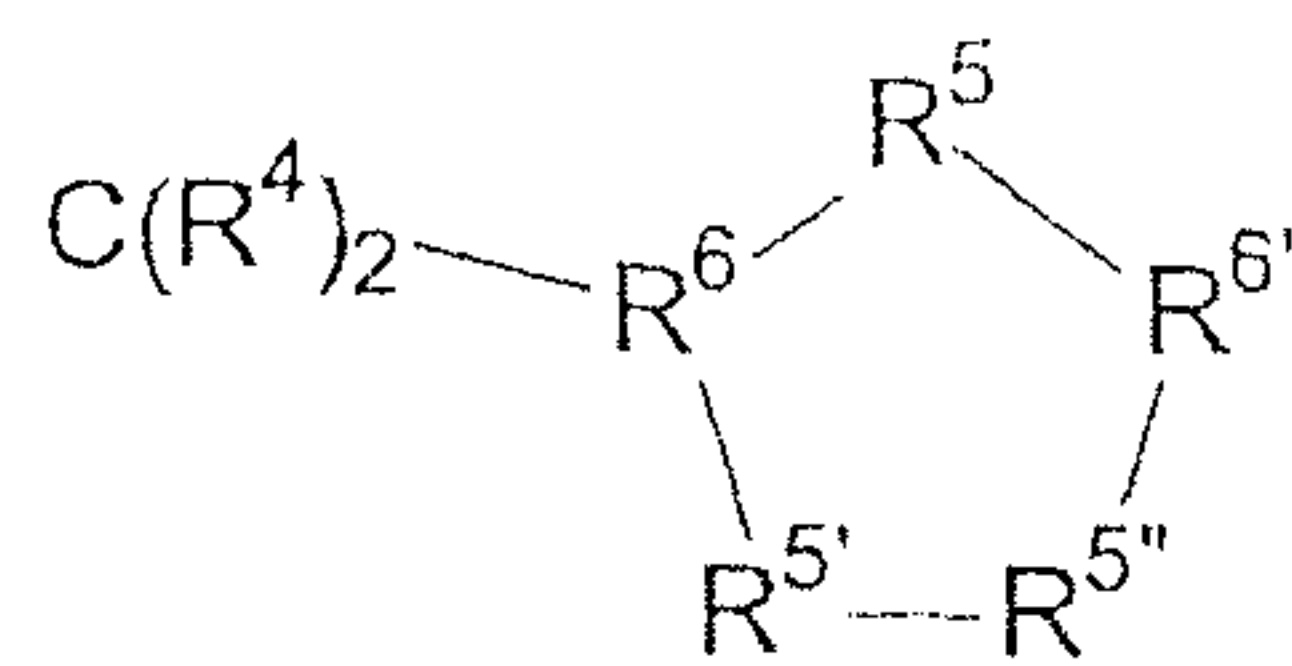
wherein

Z is a saturated or unsaturated carbocyclic or heterocyclic 5-membered ring.

4. The method of claim 3, wherein Z is, in a compound of the general formula

(III)

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(III)

in which a single or double bond may be present between $R^{5'}$ and $R^{5''}$, where

$R^4 =$ in each case independently H, F, Cl, CH_3 ,

$R^5 = CR^4_2$,

$R^{5'} =$ O, S, NH, NC_1-C_2 -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 $R^{5''}$,

$R^{5'} = R^{5''} = CR^4_2$ 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 .

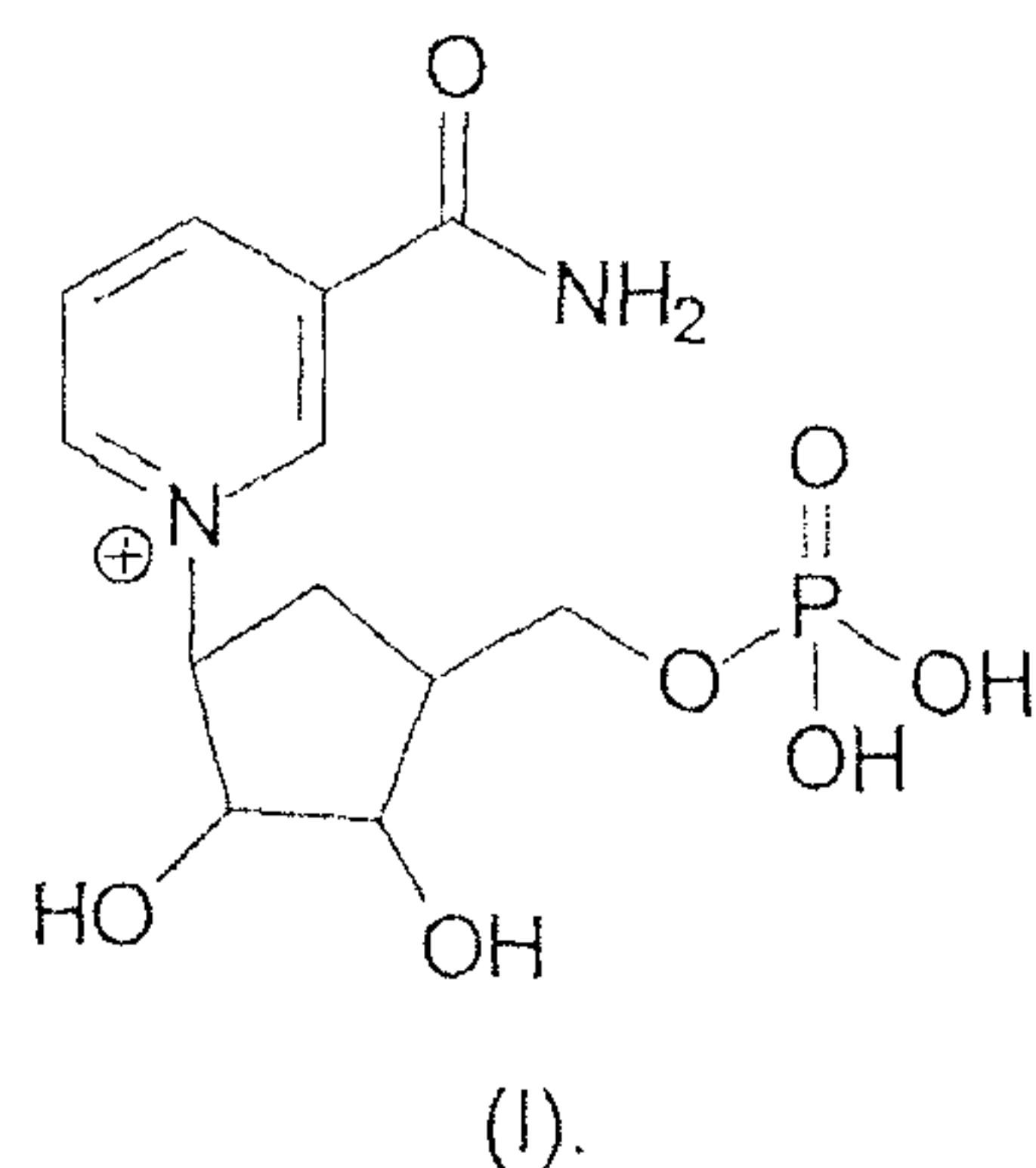
5. The method of any one of the claims 1 to 4,
wherein
carbaNAD is used as the stabilized coenzyme.
6. The method of any one of the claims 1 to 5,
wherein
the stabilized enzyme is stored for a period of at least 2 weeks.
7. The method of any one of claims 1 to 6,
wherein
the stabilized enzyme is stored for a period of at least 4 weeks.
8. The method of any one of claims 1 to 7,
wherein
the stabilized enzyme is stored for a period of at least 8 weeks.

9. The method according to one of the claims 1 to 8,
wherein
the stabilized enzyme is stored at a temperature of at least 20°C.
10. The method according to one of the claims 1 to 9,
wherein
the stabilized enzyme is stored at a temperature of at least 25°C.
11. The method according to one of the claims 1 to 10,
wherein
the stabilized enzyme is stored at a temperature of at least 30°C.
12. The method according to one of the claims 1 to 11,
wherein
the stabilized enzyme is stored at a relative air humidity of at least 50 % or in the absence of desiccants.
13. The method according to one of the claims 1 to 12,
wherein
the stabilized enzyme is stored as a dry substance or in a liquid phase.
14. Enzyme which is stabilized with a stabilized coenzyme
wherein
the enzyme exhibits a decrease in enzyme activity of less than 50 %, based on the initial value of enzyme activity when stored for at least 2 weeks, at a temperature of at least 20°C, or in the presence of light at a wavelength of $\geq$ 300 nm, optionally at high air humidity or in the absence of desiccants,
wherein the enzyme is an alcohol dehydrogenase having the number EC 1.1.1.2, a glucose-6-phosphate dehydrogenase having the number EC 1.1.1.49 or a diaphorase having the number EC 1.6.99.2,

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wherein

the stabilized coenzyme is a stabilized nicotinamide adenine dinucleotide (NAD/NADH) compound, a stabilized nicotinamide adenine dinucleotide phosphate (NADP/NADPH) compound or a compound of formula (I)



15. The enzyme of claim 14, wherein the enzyme exhibits a decrease in enzyme activity of less than 30 %.
16. The enzyme of claim 14 or 15, wherein the enzyme exhibits a decrease in enzyme activity of less than 20%.
17. The enzyme of any one of claims 14 to 16, said enzyme being stored for at least 4 weeks.
18. The enzyme of any one of claims 14 to 17, said enzyme being stored for at least 8 weeks.
19. The enzyme of any one of claims 14 to 18, said enzyme being stored at a temperature of at least 25°C.
20. The enzyme of any one of claims 14 to 19, said enzyme being stored at a

temperature of at least 30°C.

21. Detection reagent for determining an analyte wherein said detection reagent contains a stabilized enzyme according to any one of claims 14 to 20.
22. Test element,
comprising a stabilized enzyme according to any one of claims 14 to 20 or a detection reagent according to claim 21.
23. Method for stabilizing an enzyme
wherein
the enzyme is stored in the presence of a native coenzyme, where a dehydrogenase selected from the group consisting of an alcohol dehydrogenase having the number EC 1.1.1.1 or EC 1.1.1.2, an L-amino acid dehydrogenase having the number EC 1.4.1.5, a glucose dehydrogenase having the number EC 1.1.1.47, a glucose-6-phosphate dehydrogenase having the number EC 1.1.1.49, a glycerol dehydrogenase having the number EC 1.1.1.6, a 3-hydroxybutyrate dehydrogenase having the number EC 1.1.1.30, a lactate dehydrogenase having the number EC 1.1.1.27 or EC 1.1.1.28, and a malate dehydrogenase having the number EC 1.1.1.37, or a diaphorase having the number EC 1.6.99.2 is used as the enzyme
wherein
the native coenzyme is a native nicotinamide adenine dinucleotide (NAD/NADH) compound or a native nicotinamide adenine dinucleotide phosphate (NADP/NADPH) compound.
24. The method according to claim 23,
wherein
an alcohol dehydrogenase having the number EC 1.1.1.1 or EC 1.1.1.2, a glucose dehydrogenase having the number EC 1.1.1.47, a glucose-6-

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phosphate dehydrogenase having the number EC 1.1.1.49 or a diaphorase having the number EC 1.6.99.2 is used as the enzyme.

25. The method of any one of claims 1 to 13, 23 and 24,
wherein
the stabilized enzyme is stored in the presence of light at a wavelength of
 ≥ 300 nm.

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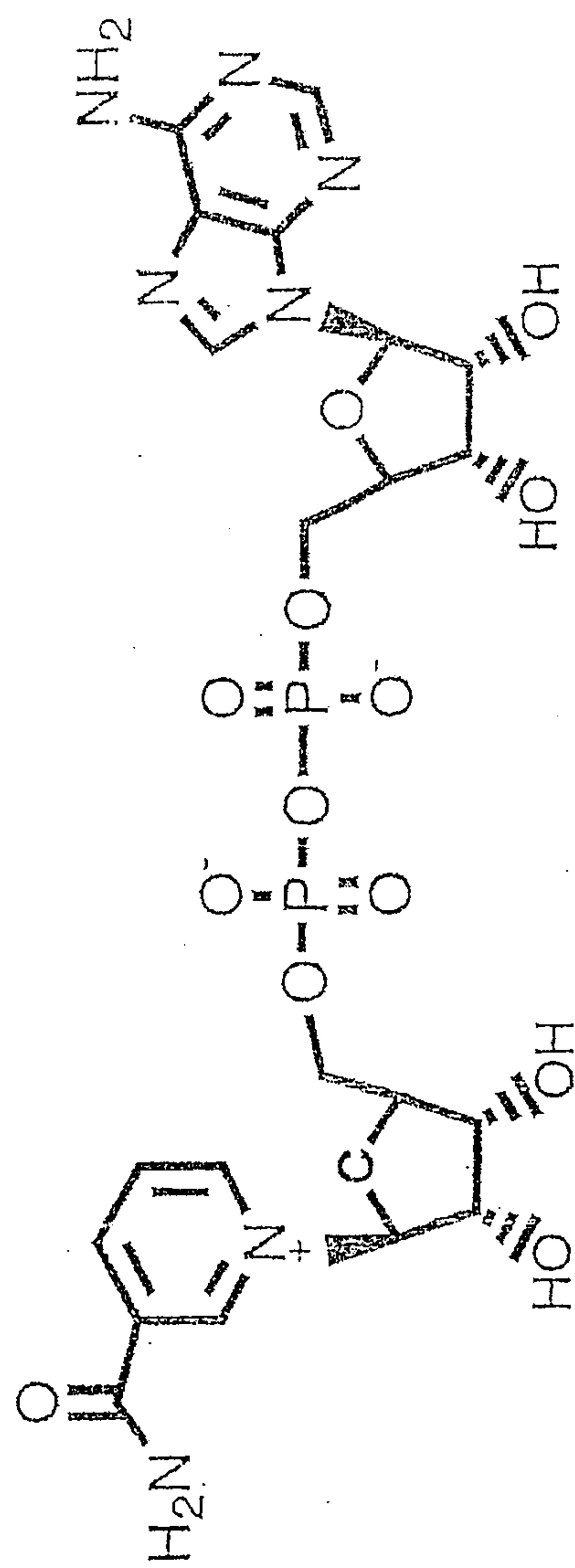


Figure 1A

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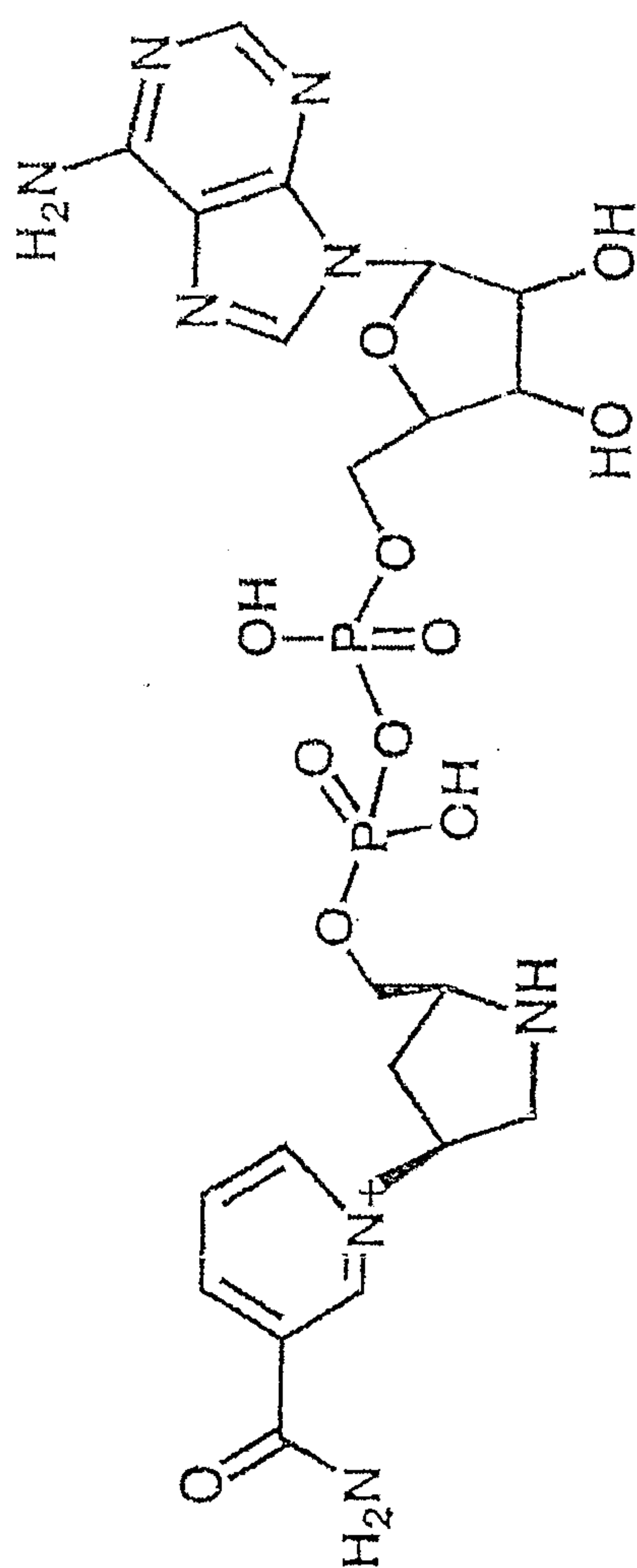
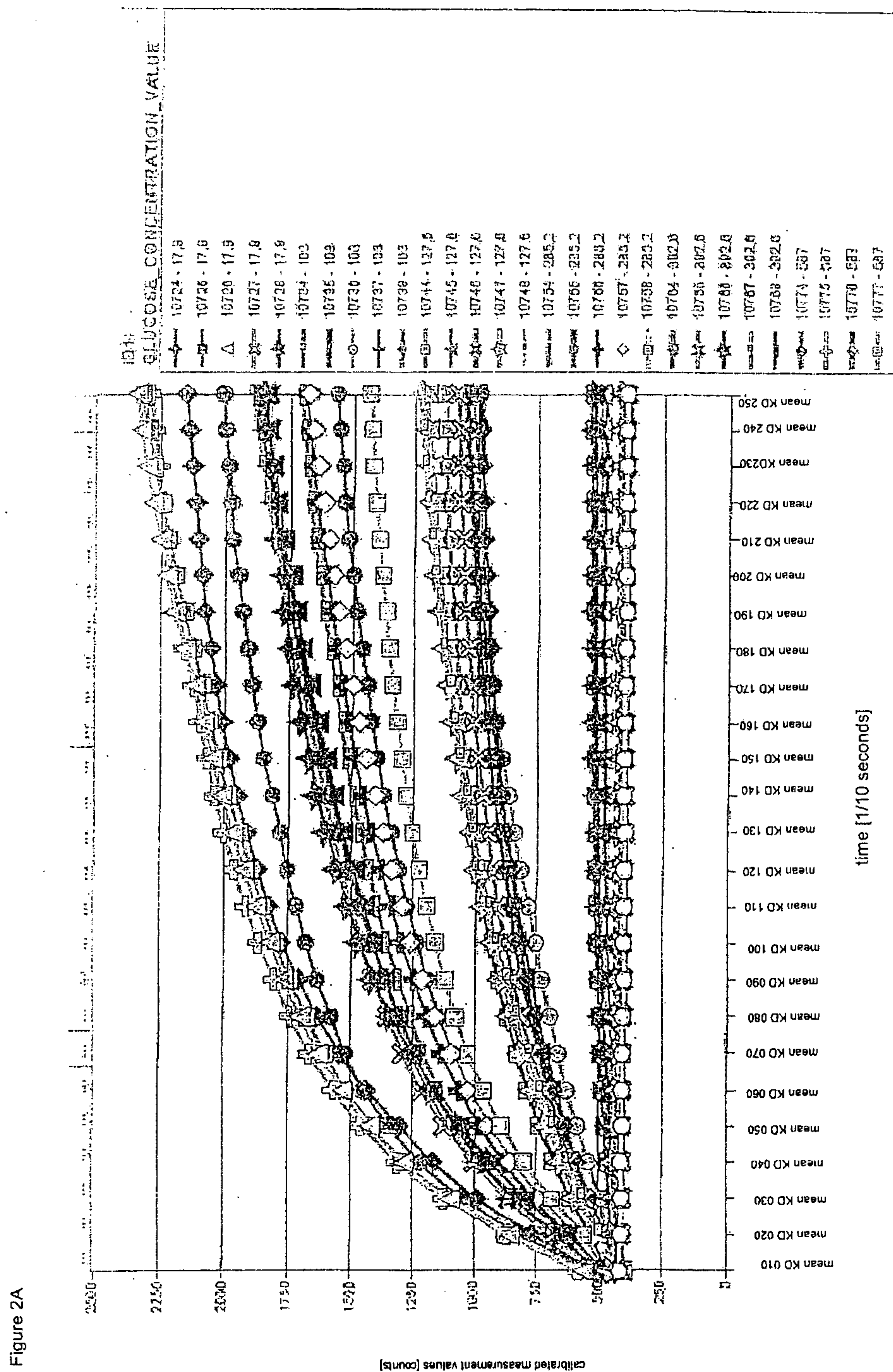
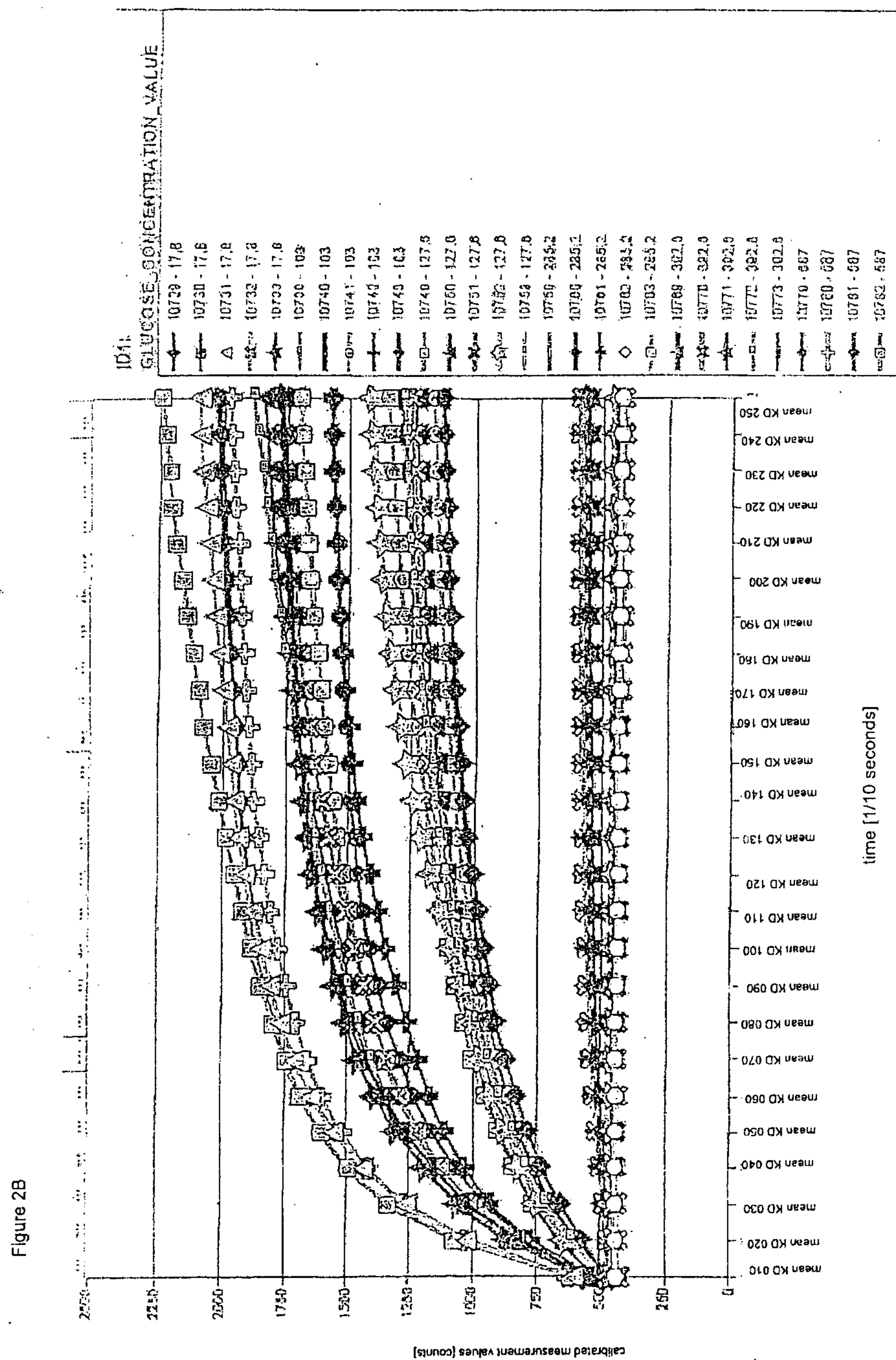


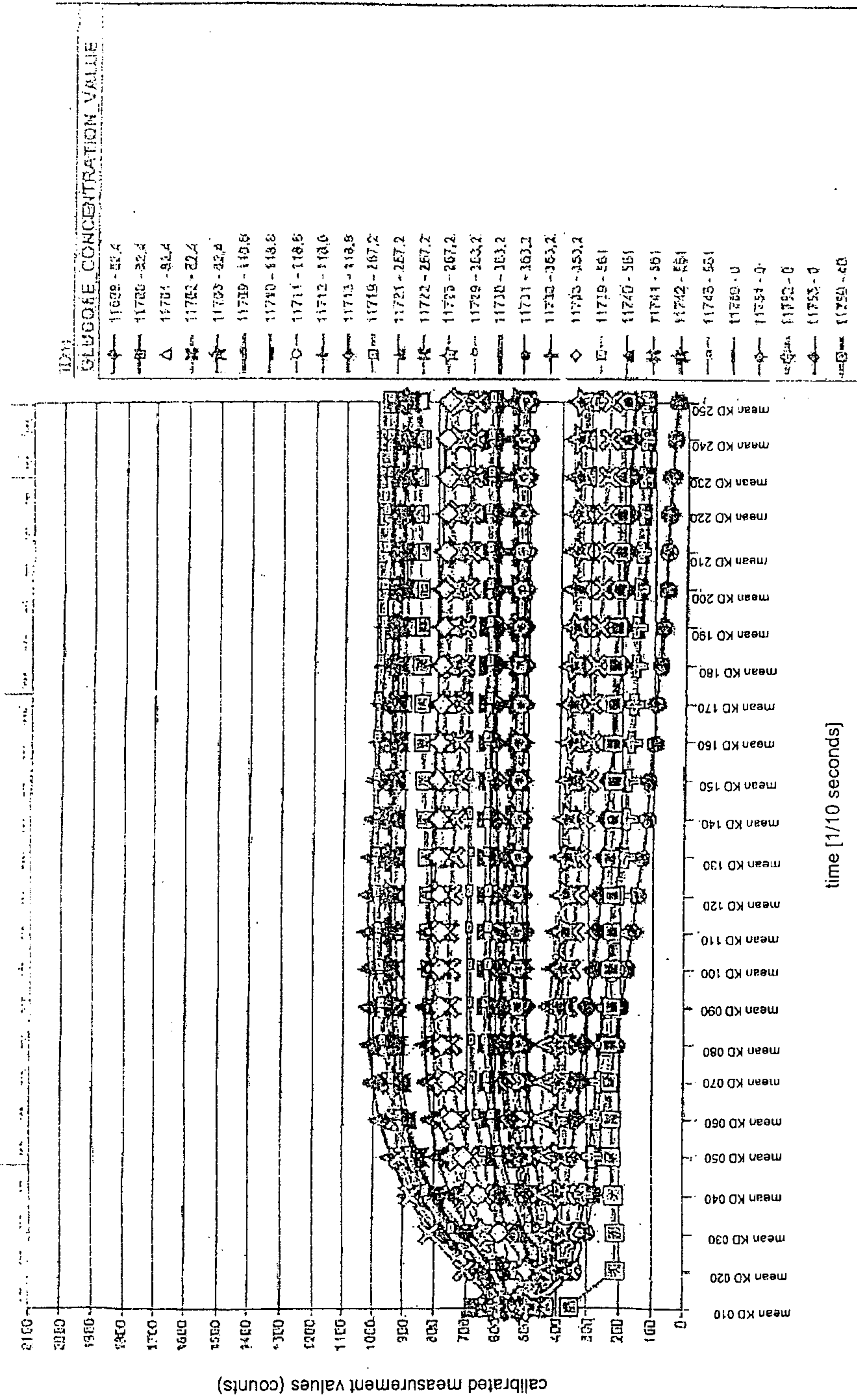
Figure 1B





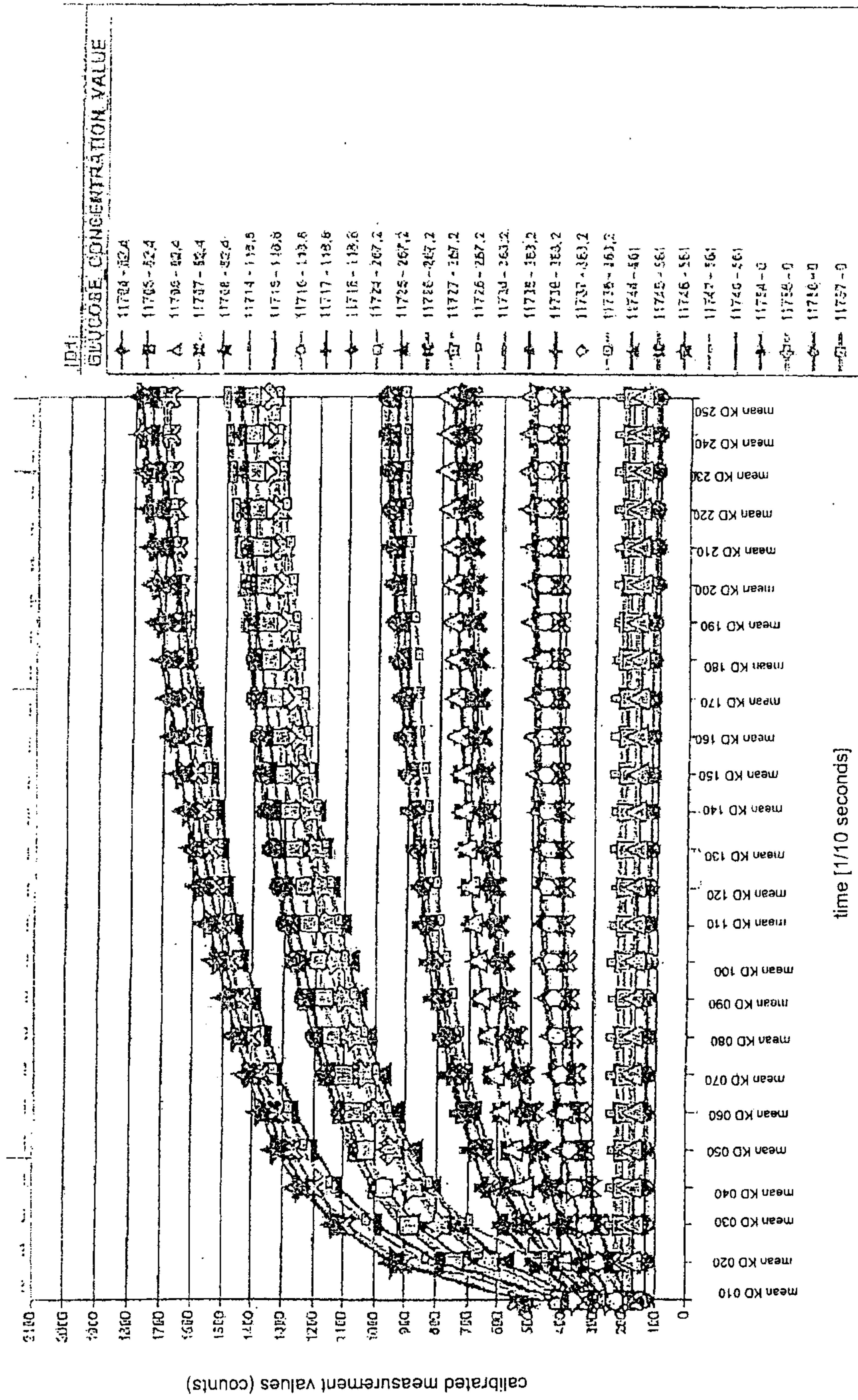
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Figure 2C

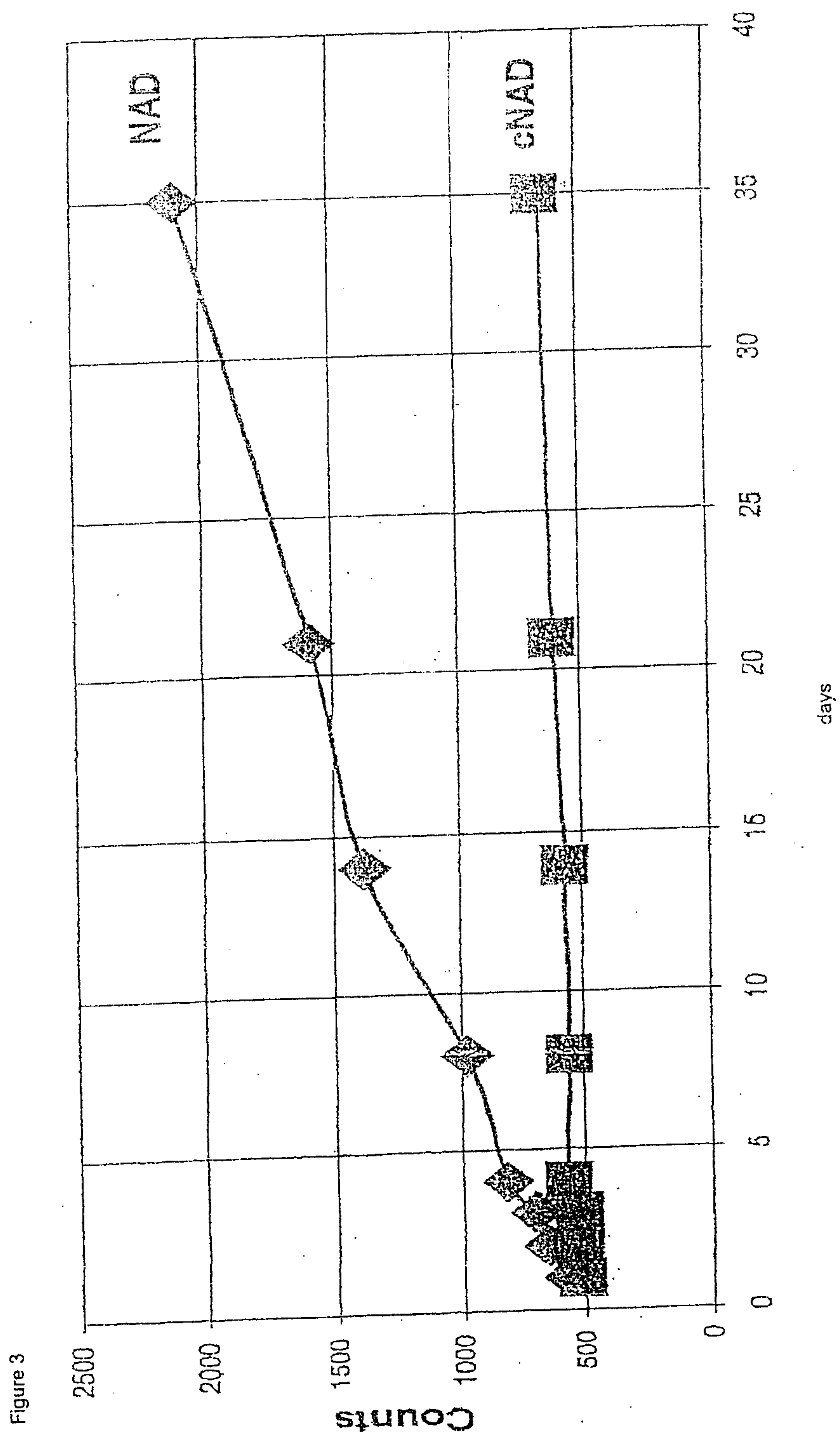


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Figure 2D



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PCT/EP2010/062045

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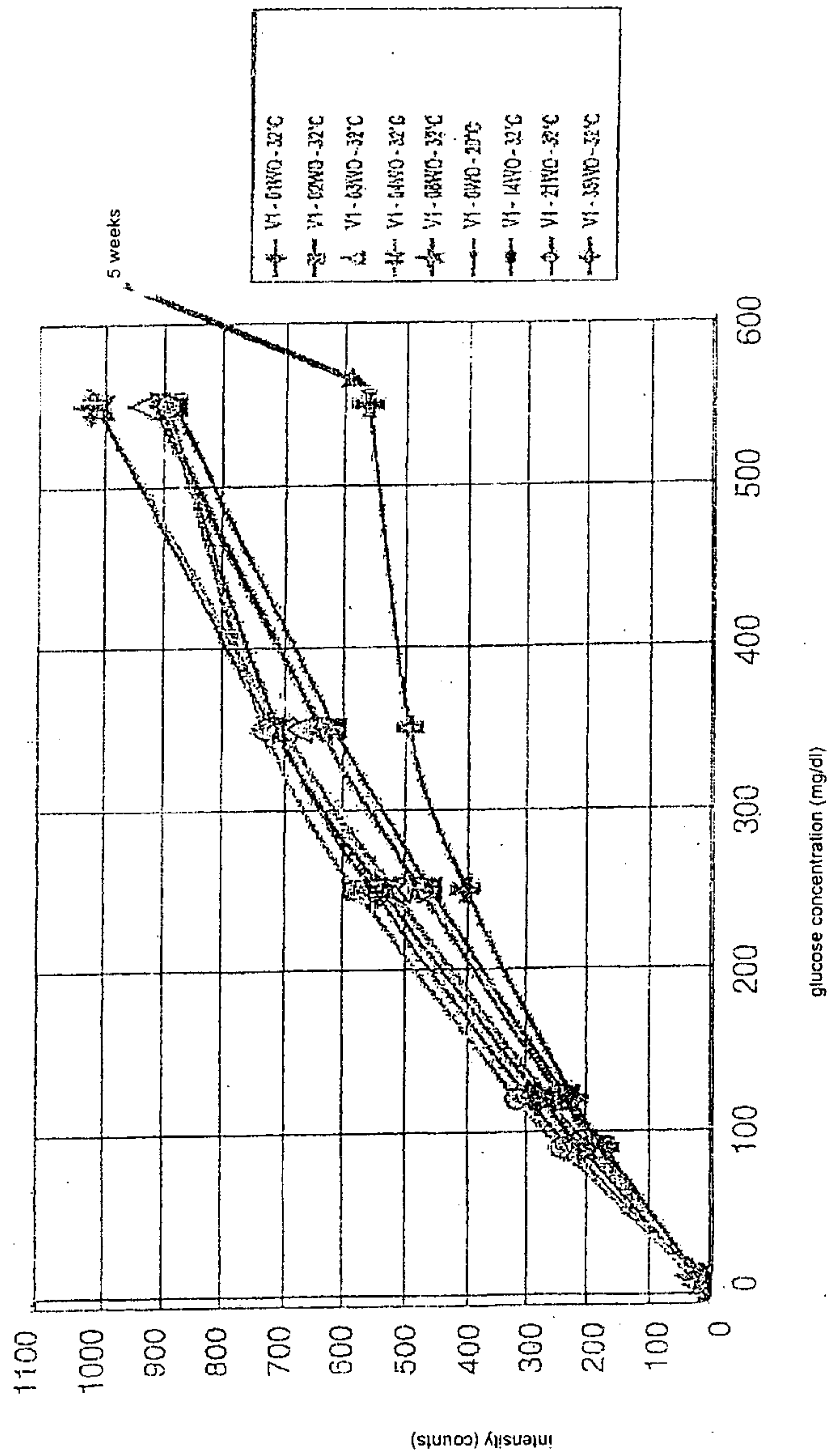


Figure 4

Figure 5A

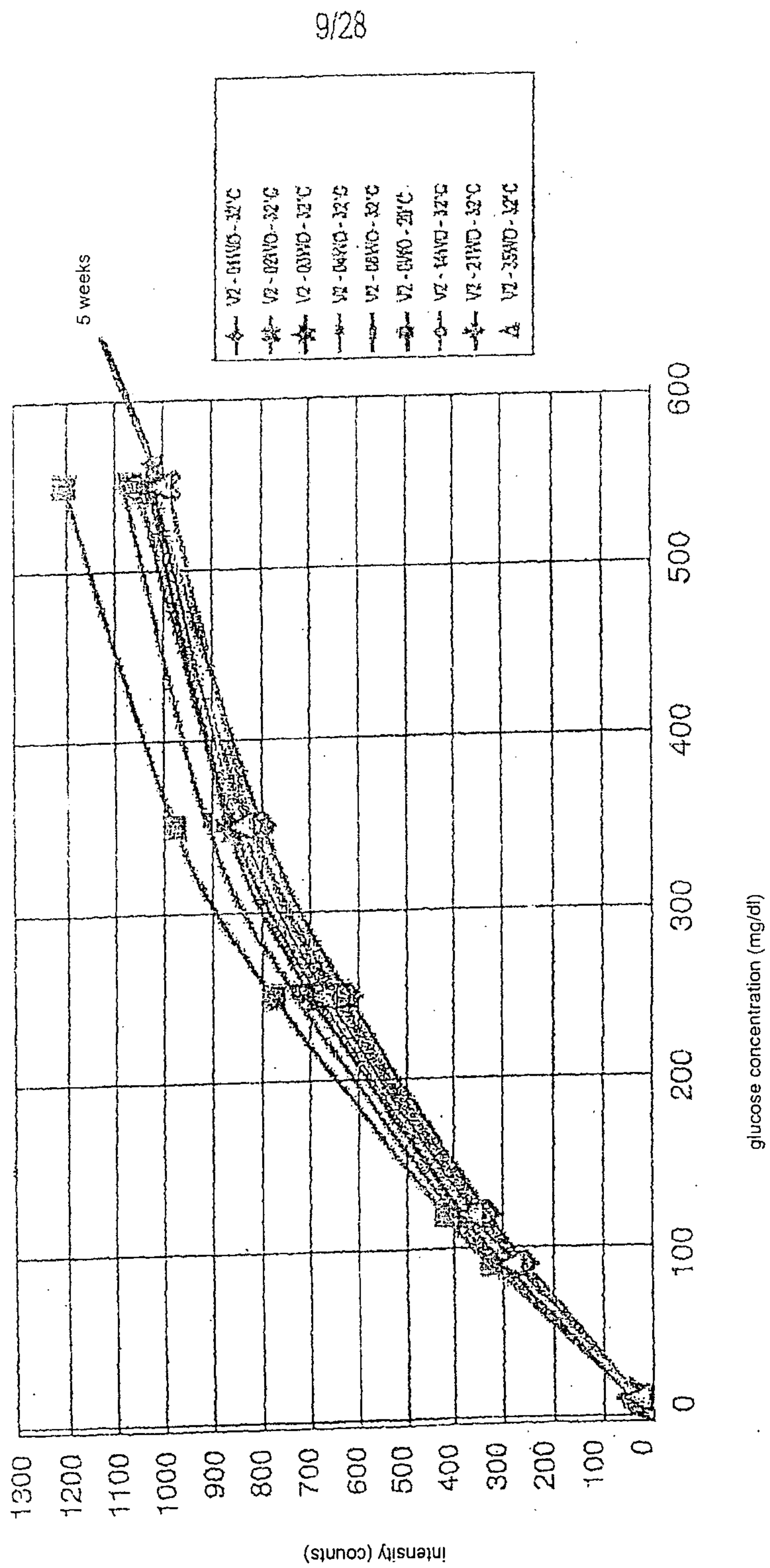
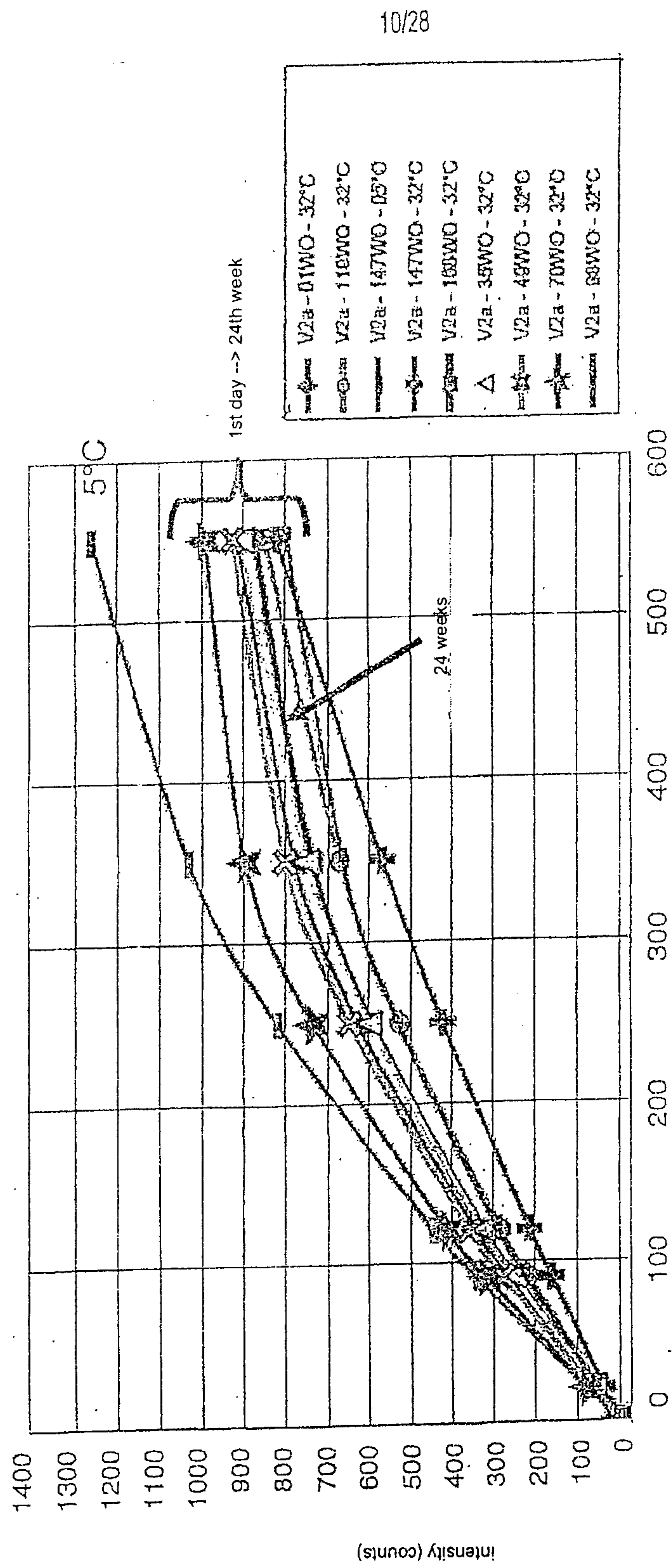
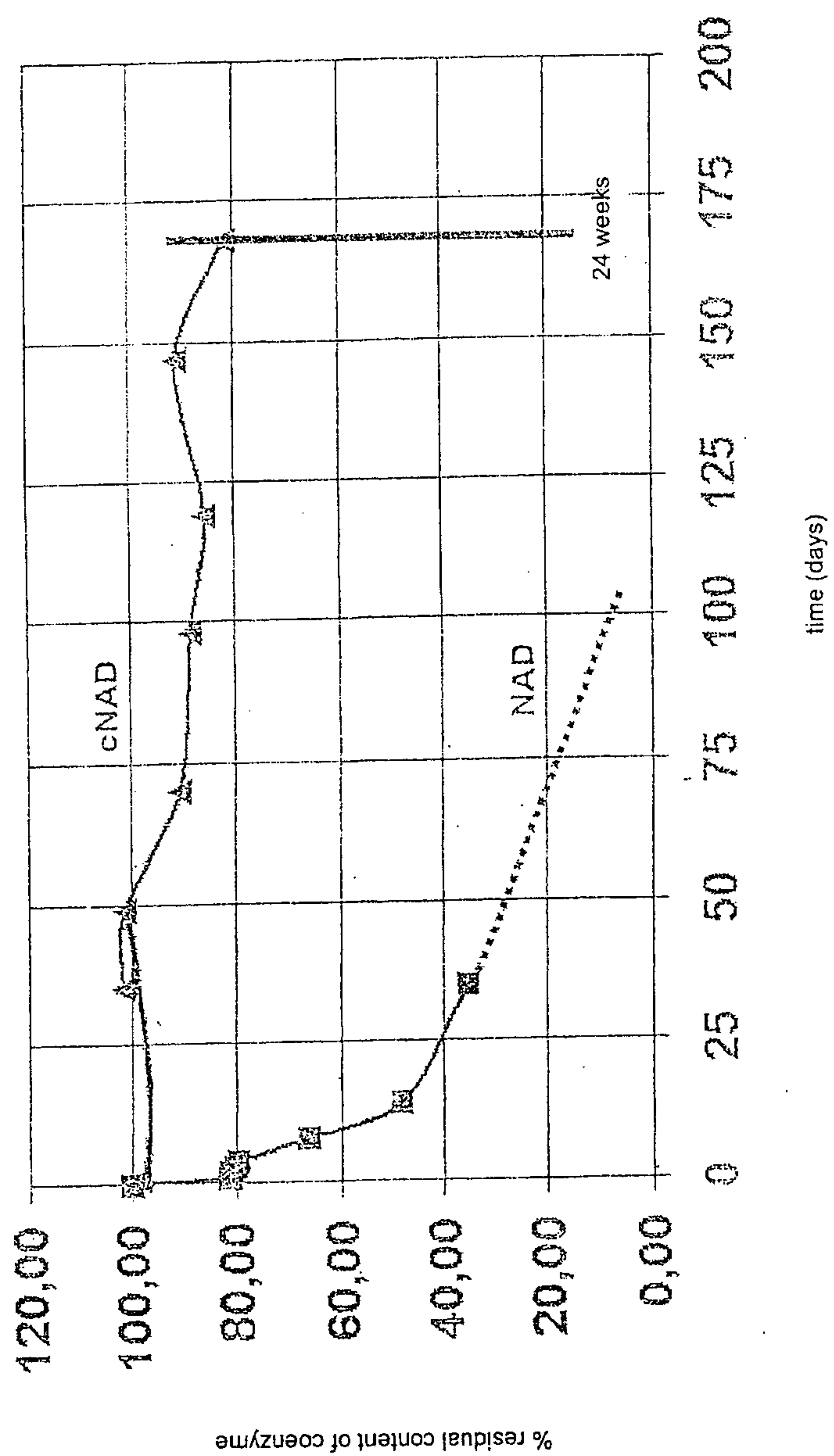


Figure 5B



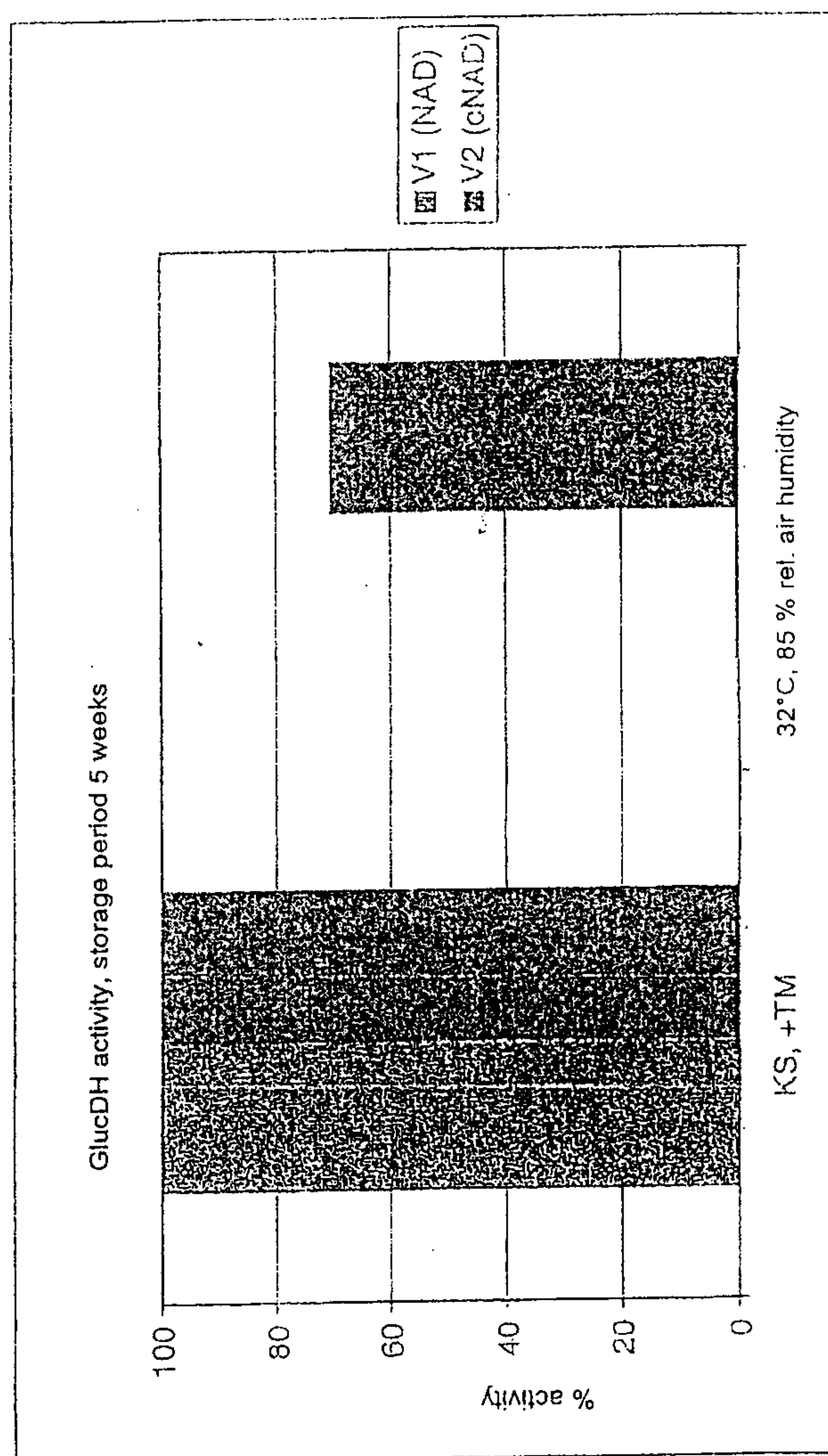
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Figure 6

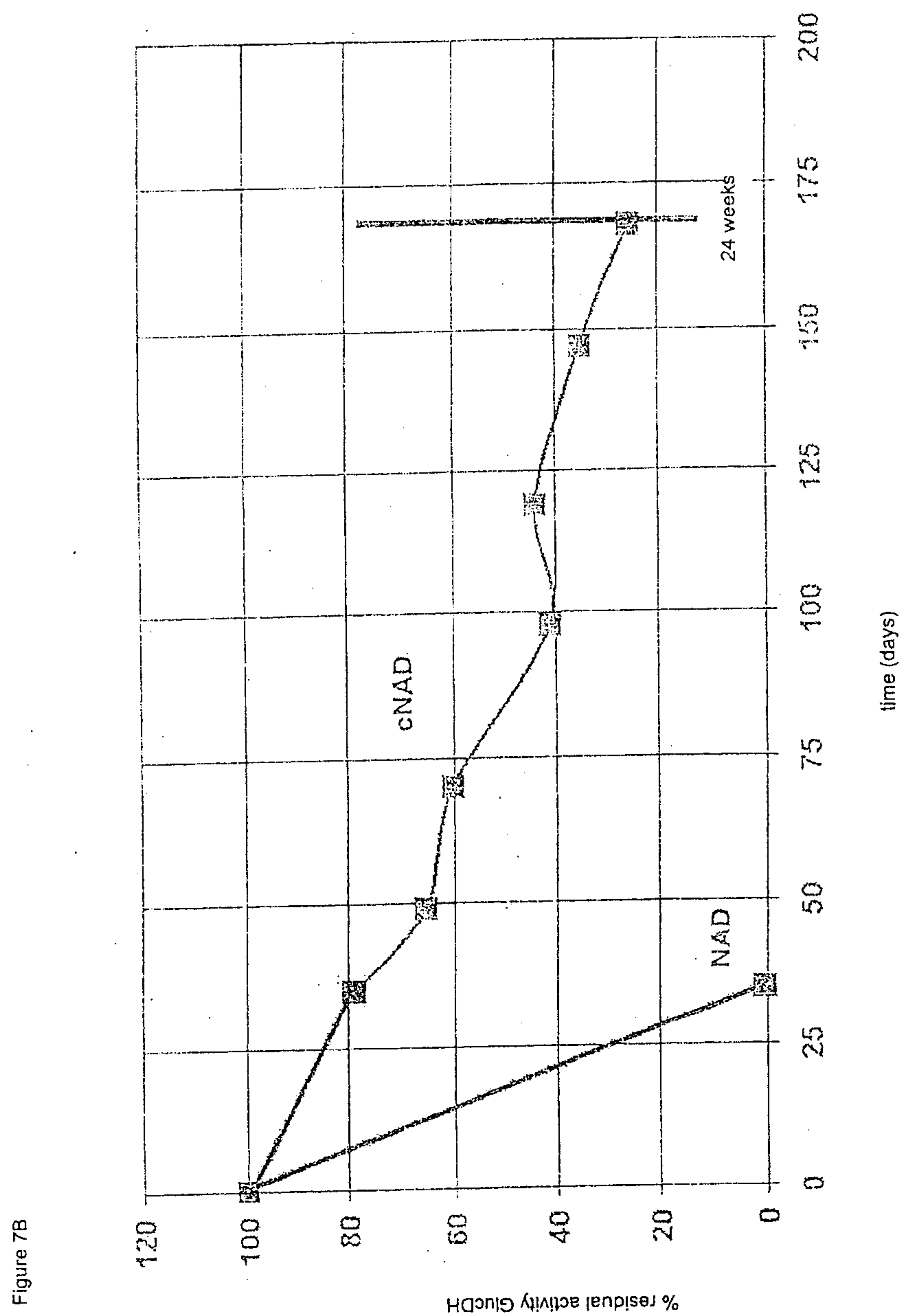


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Figure 7A

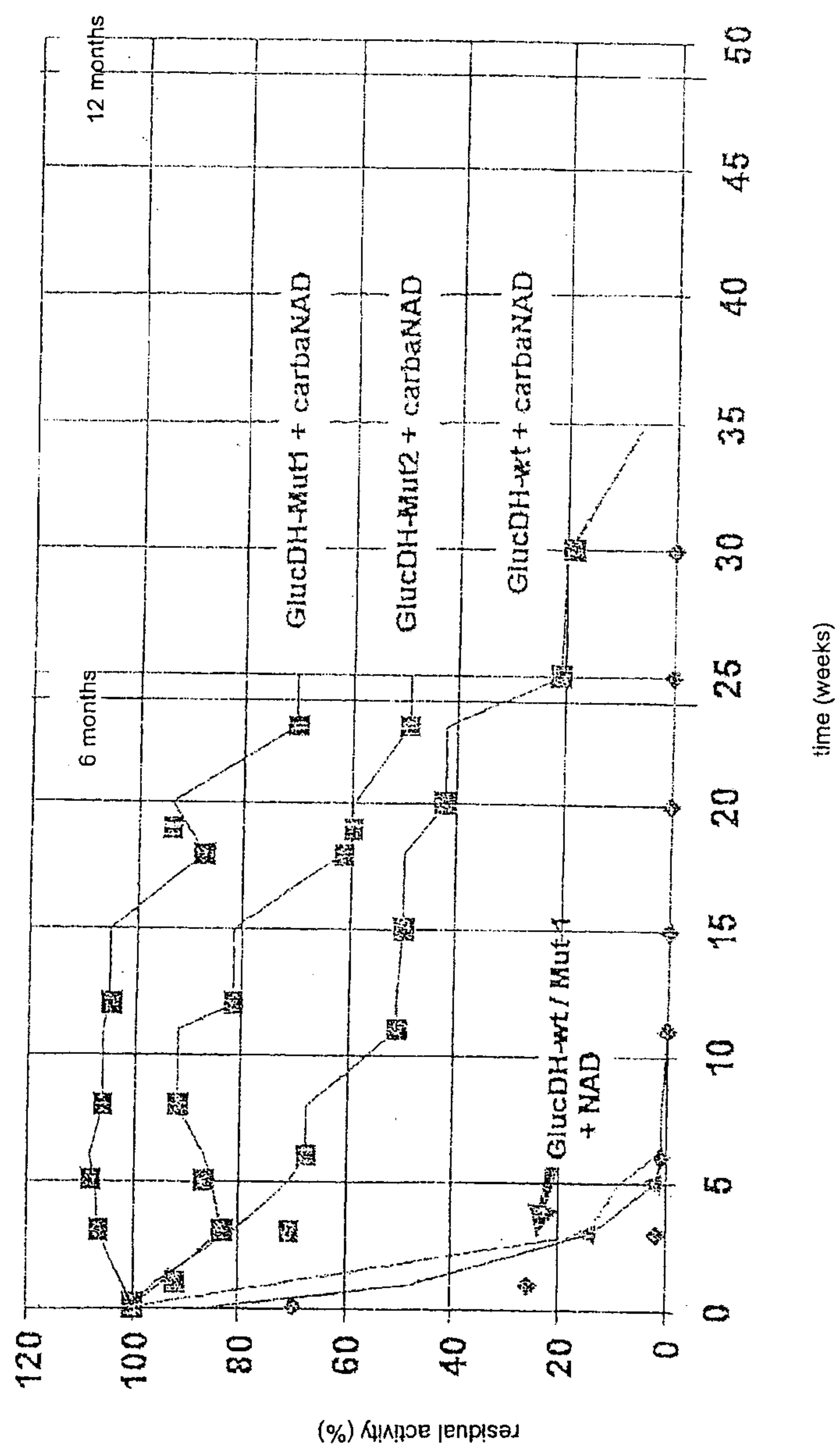


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Figure 8



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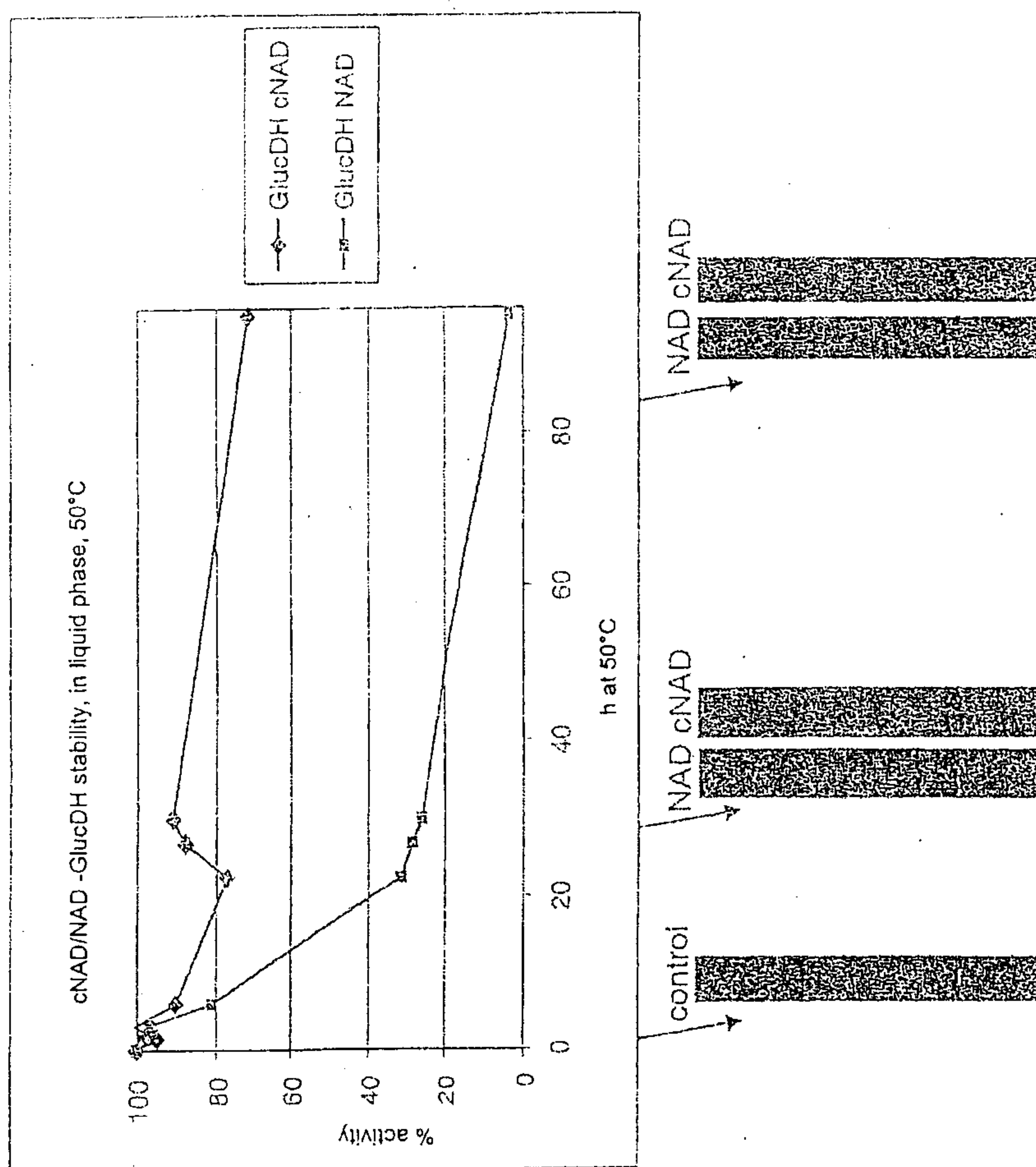


Figure 9A

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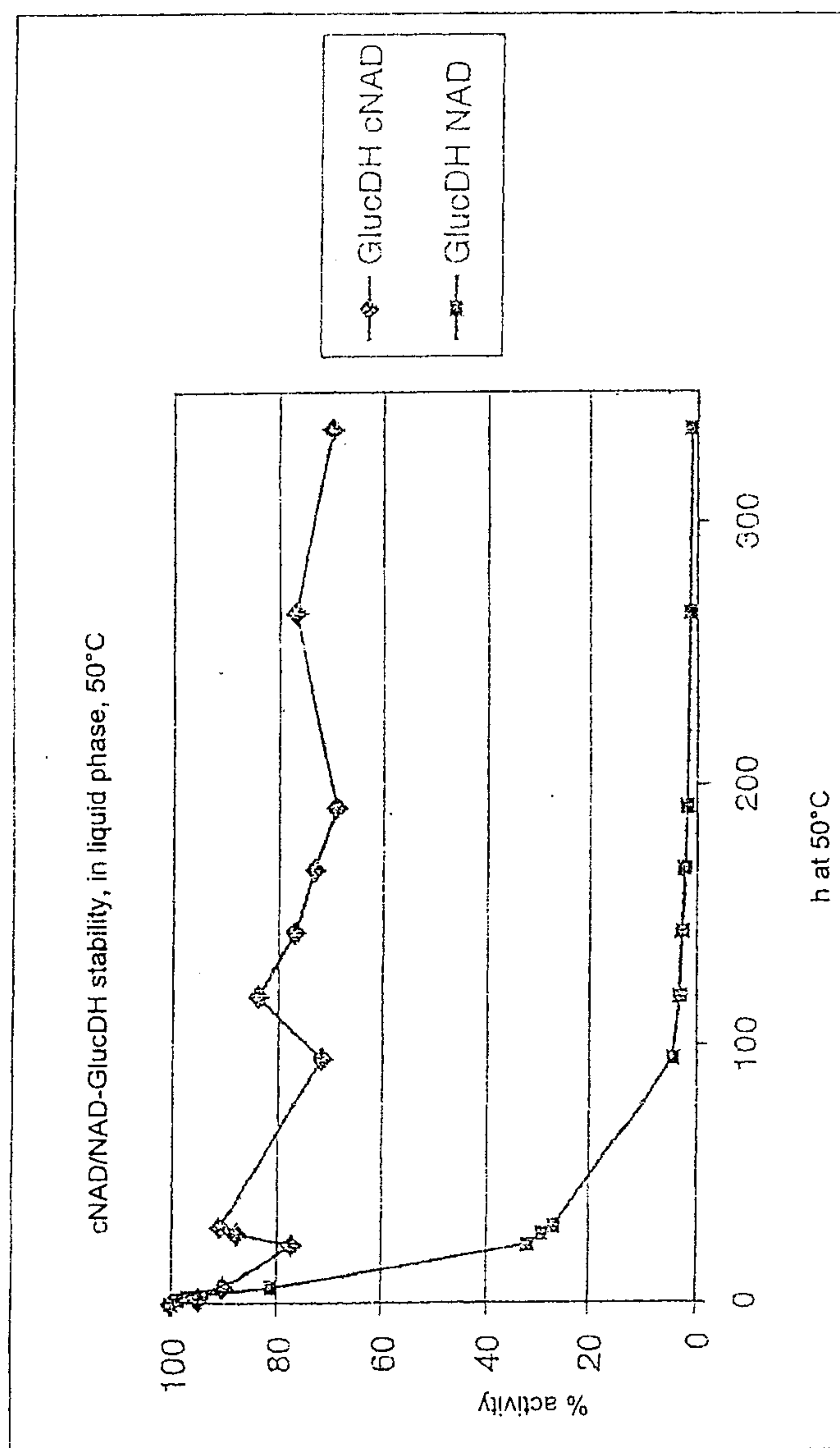


Figure 9B

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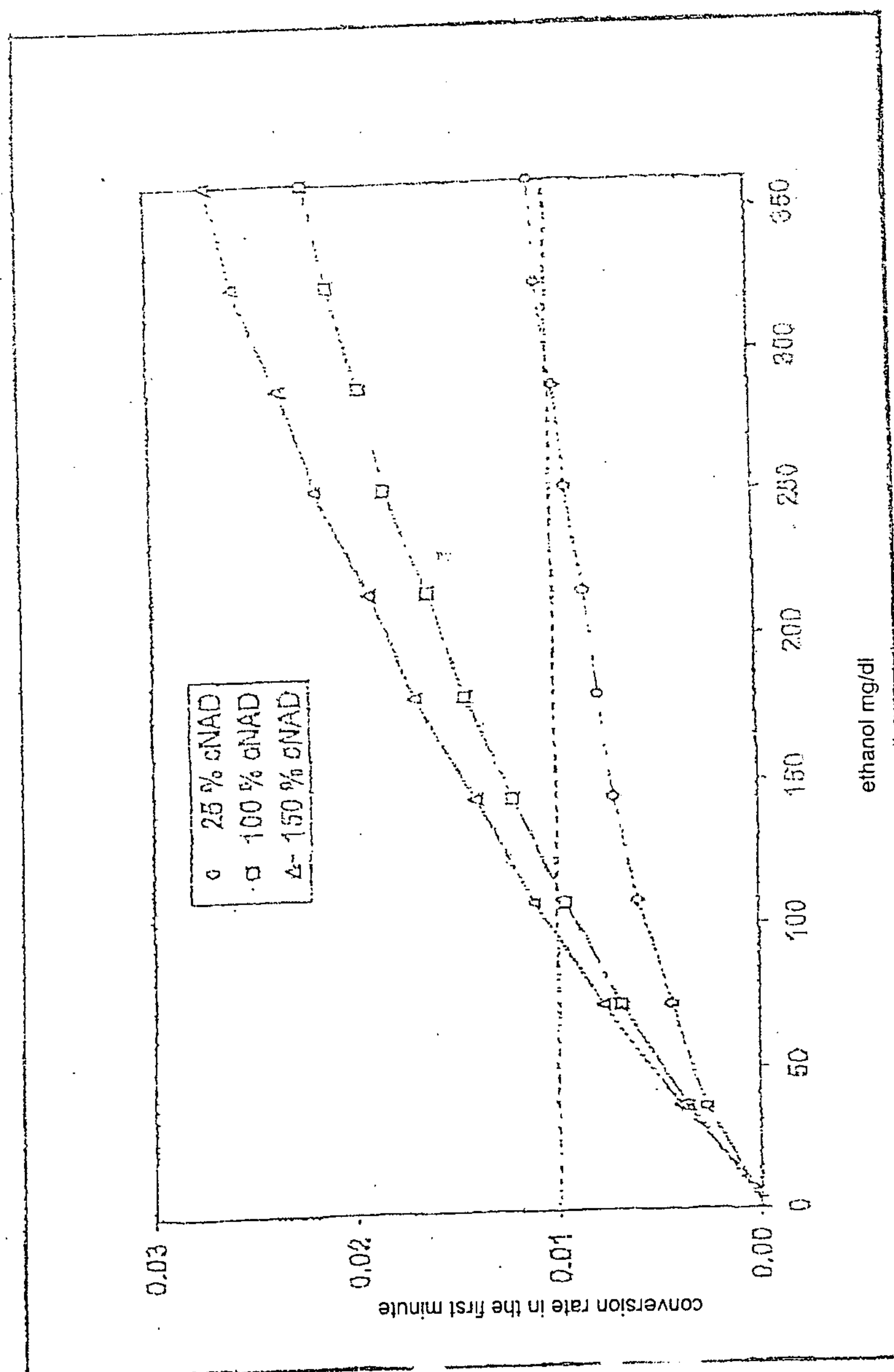
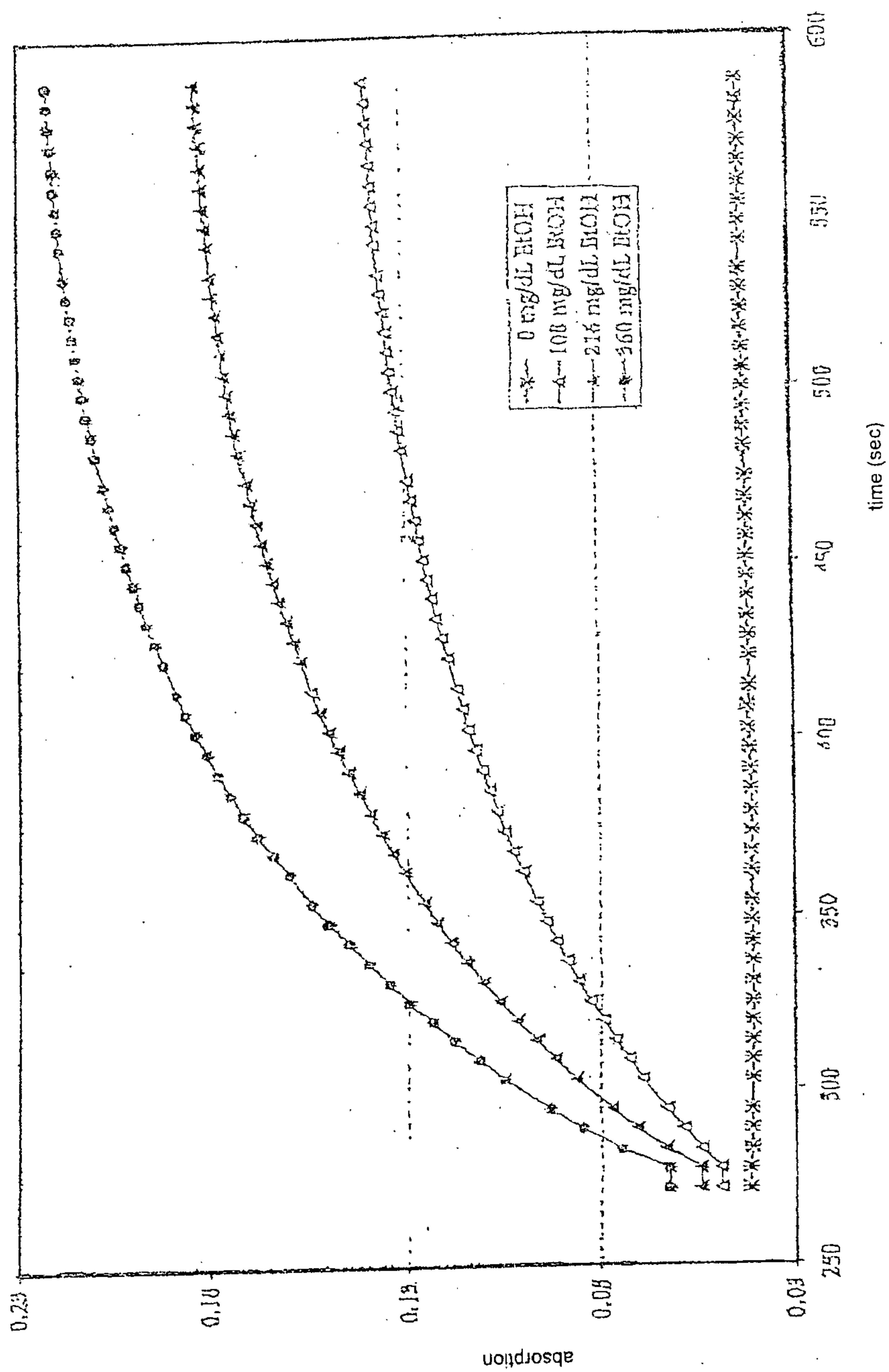


Figure 10

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Figure 11



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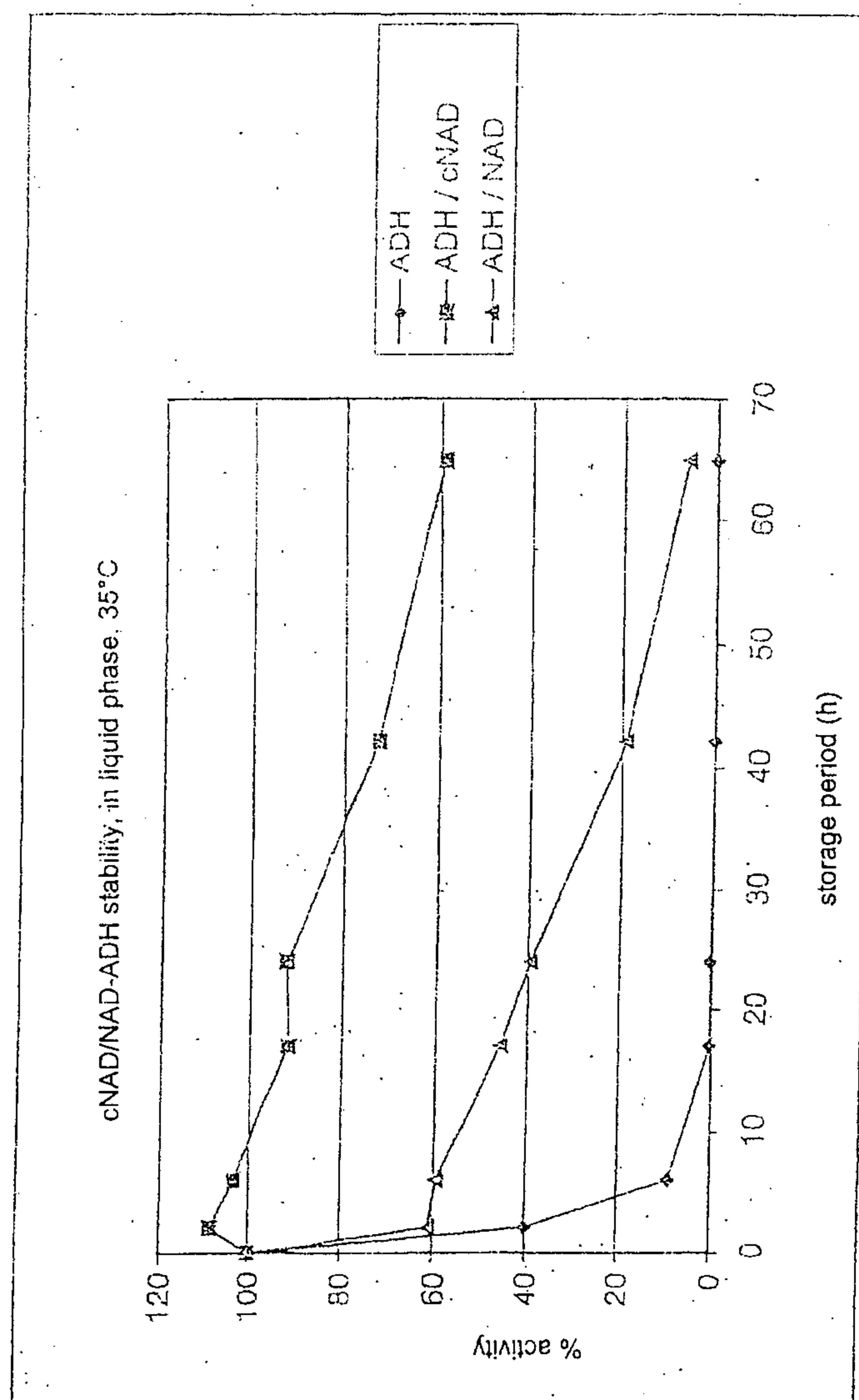
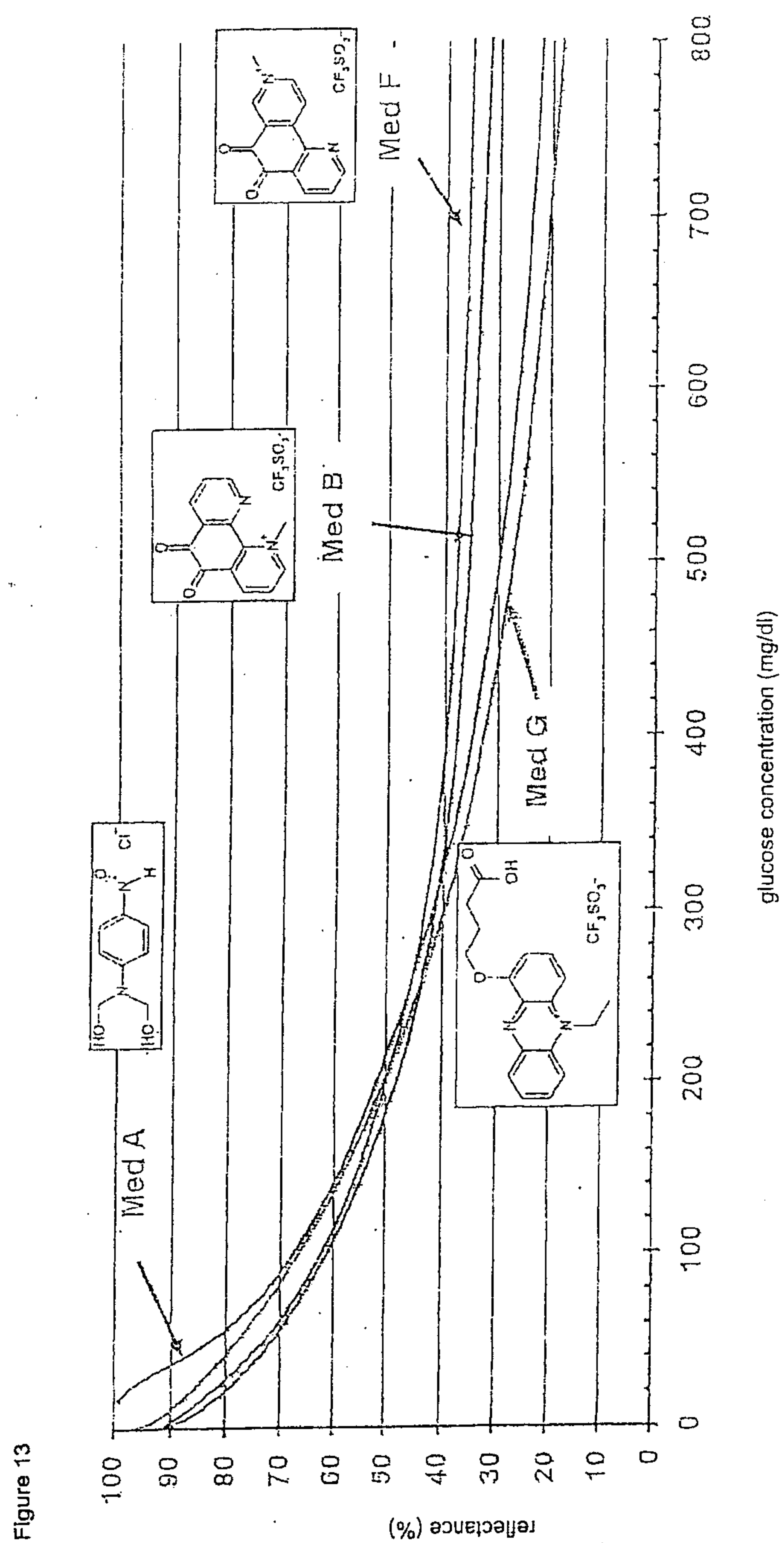


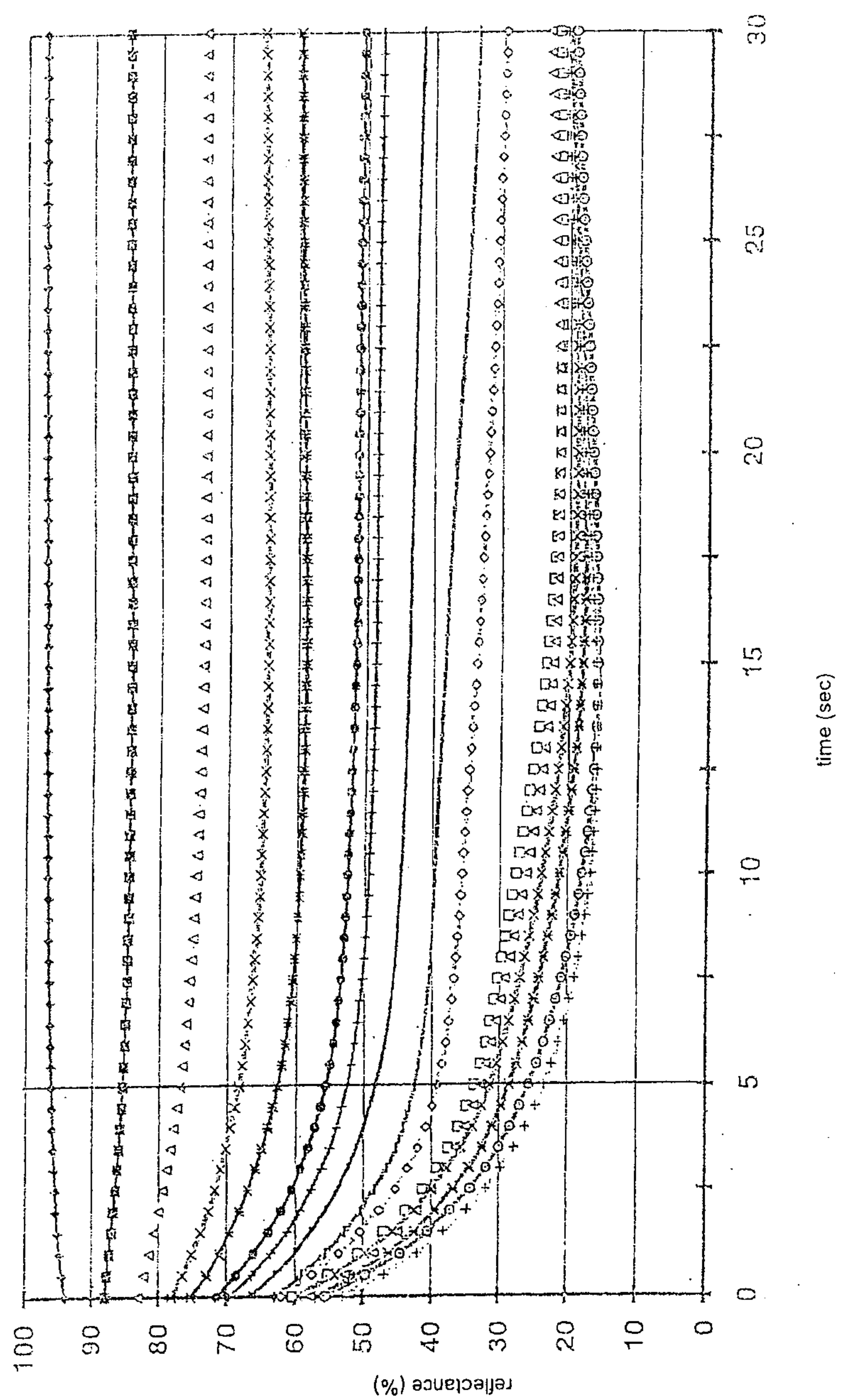
Figure 12

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Figure 14



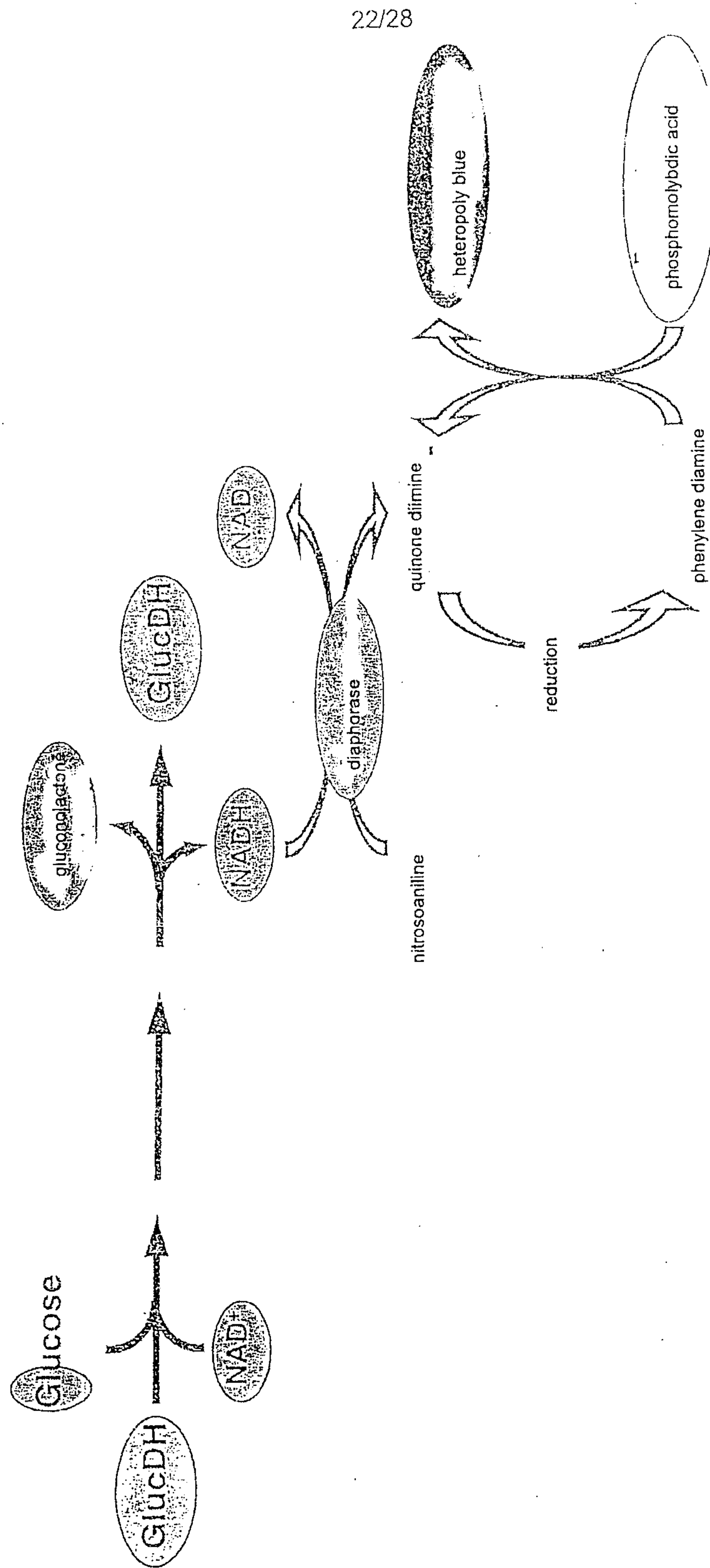
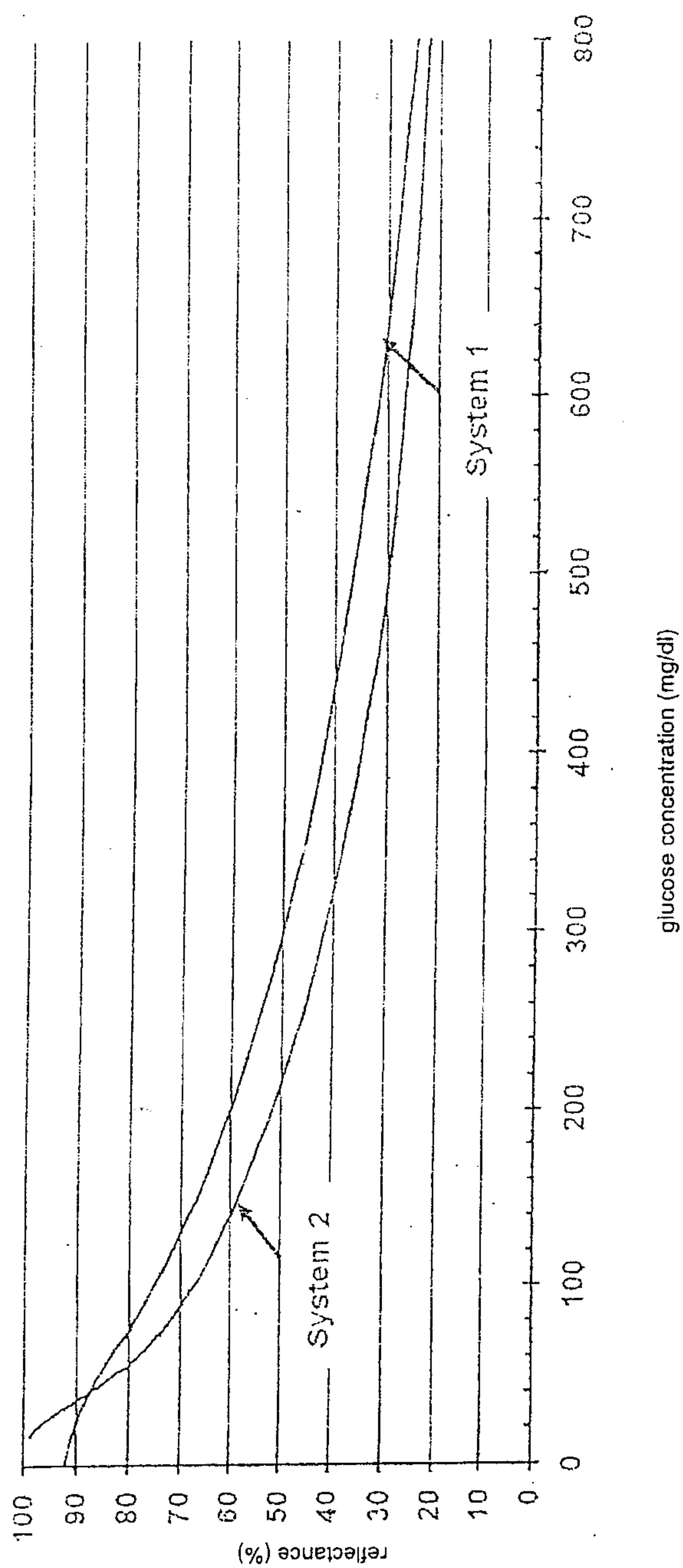


Figure 15

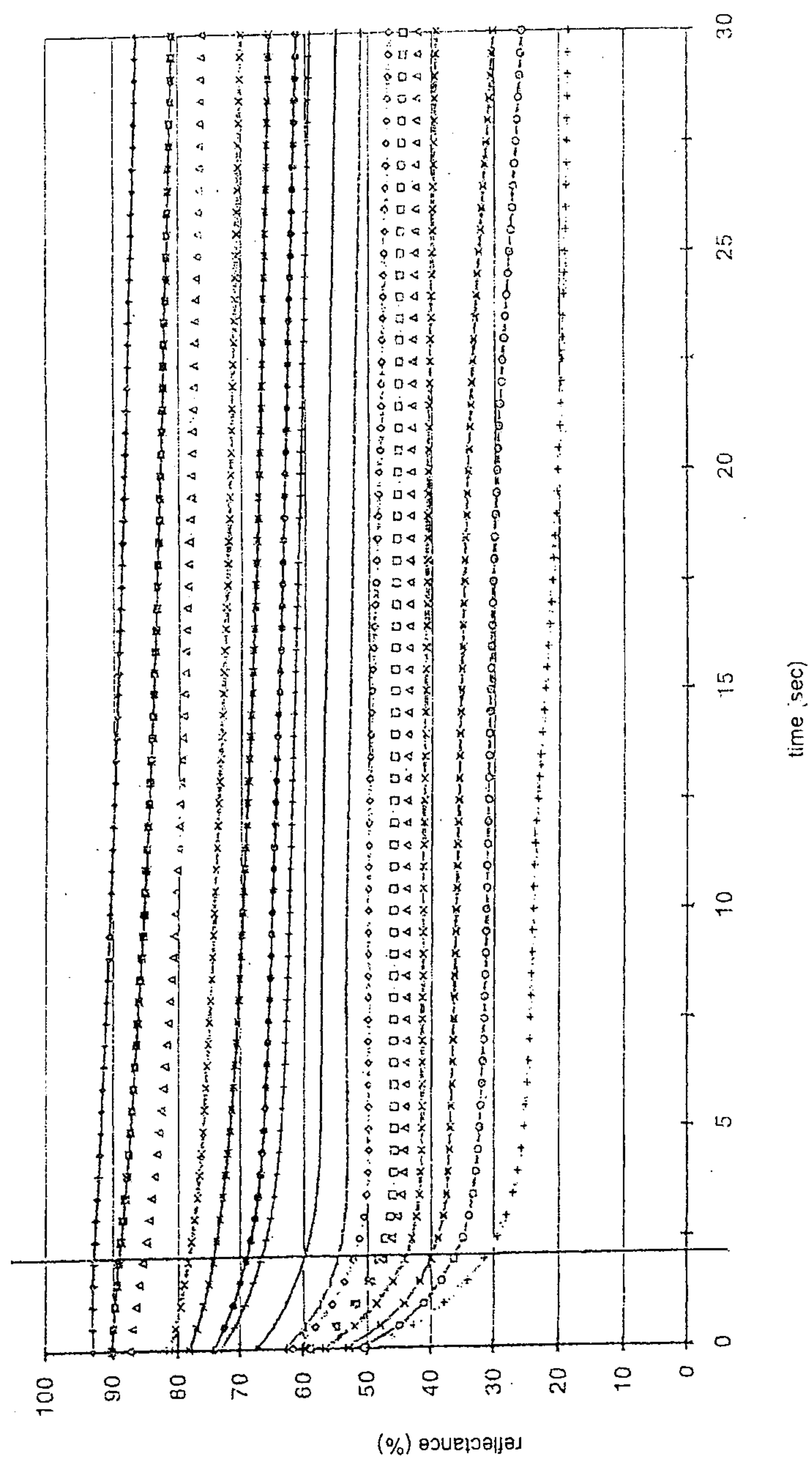
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Figure 16



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Figure 17



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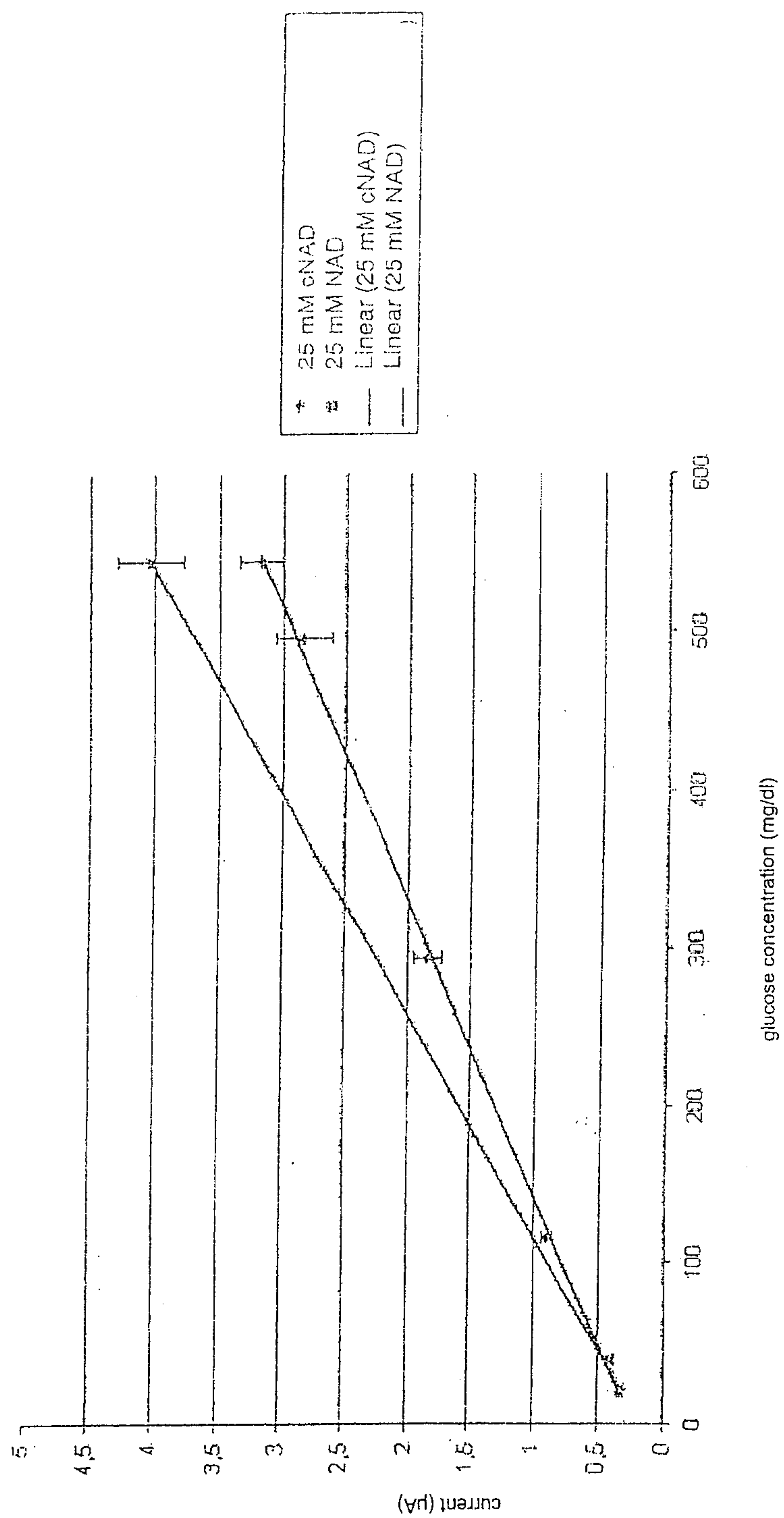
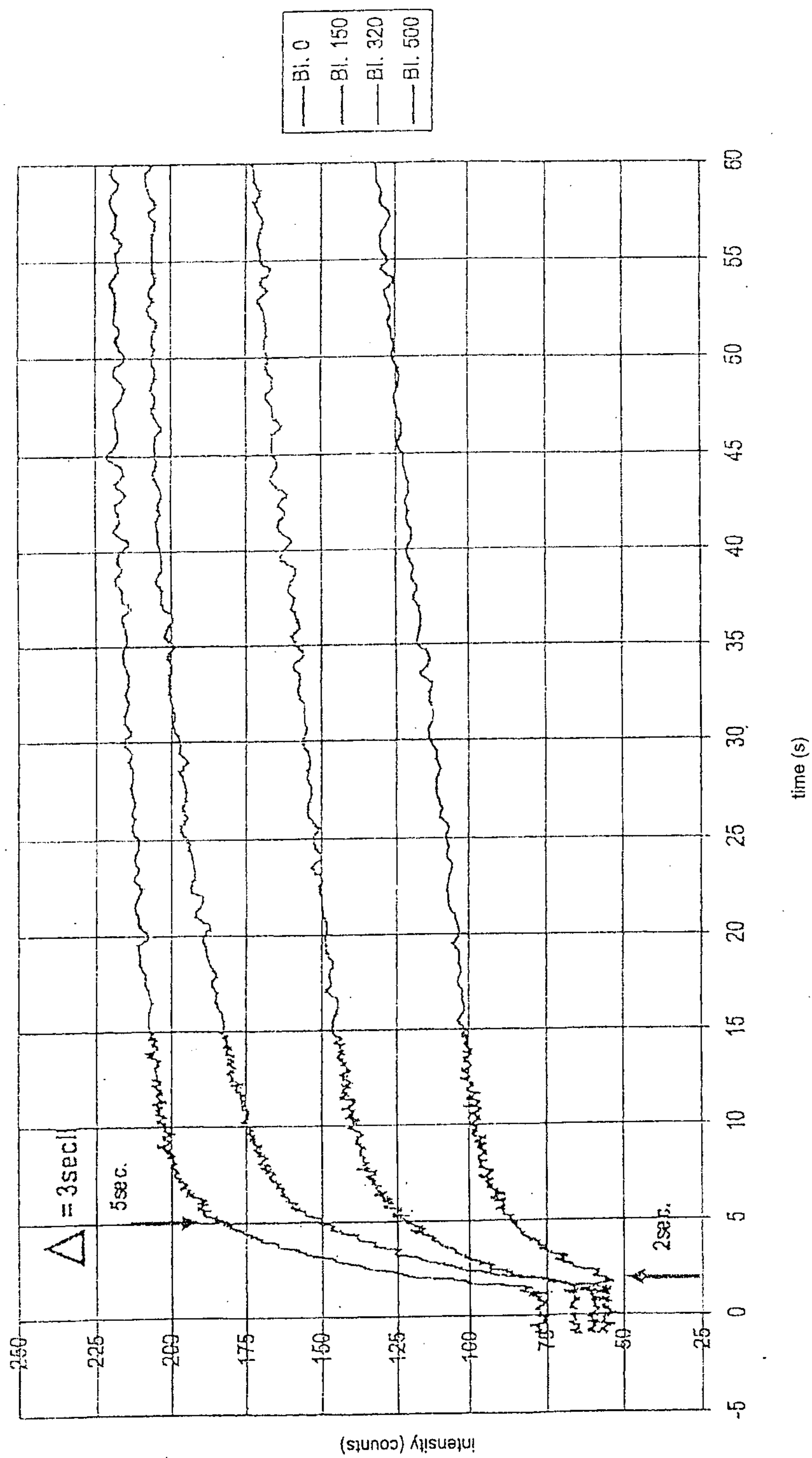


Figure 18

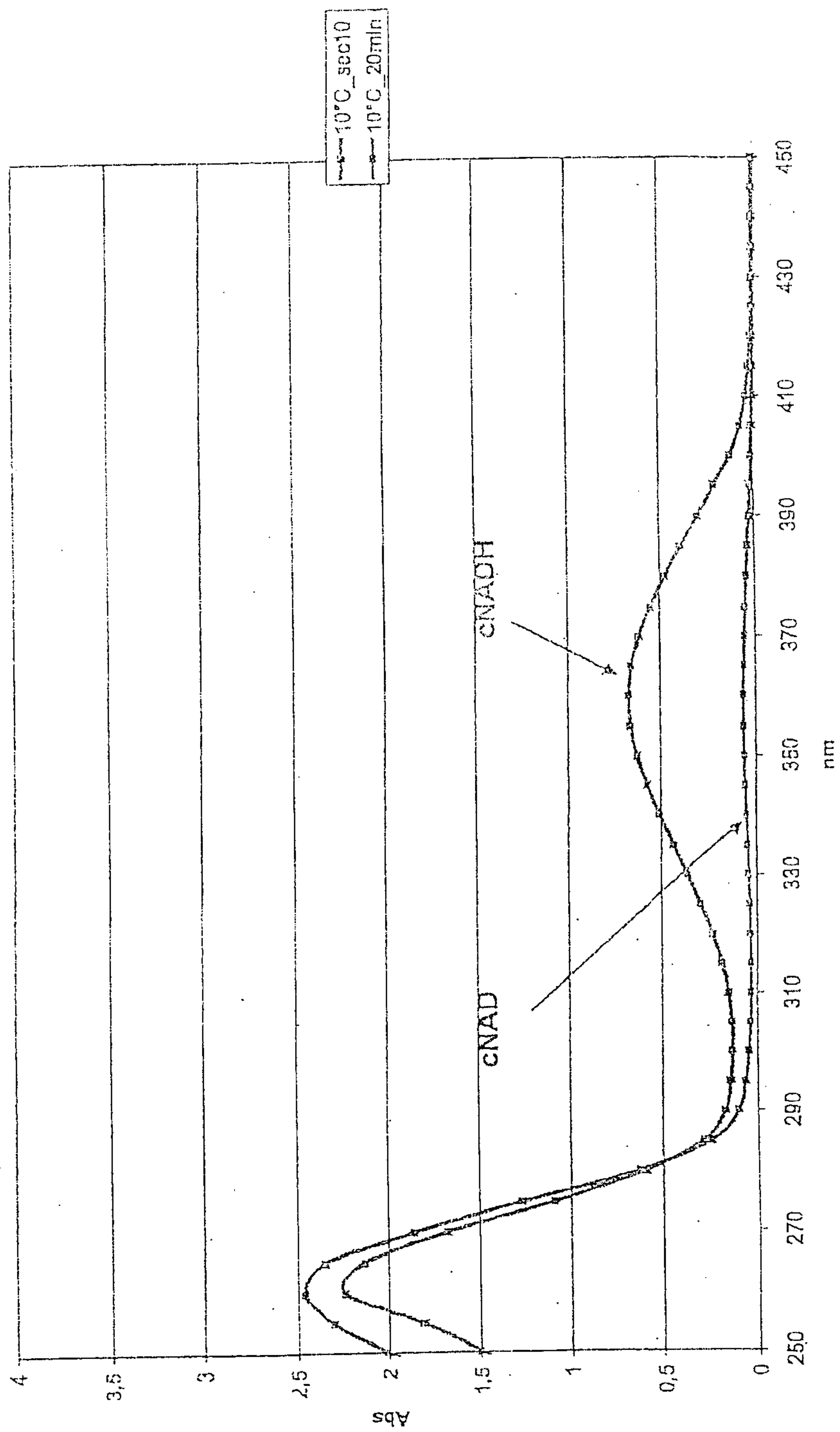
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Figure 19



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Figure 20



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Figure 21

GlucDH_E96G_E170K

M Y P D L K G K V A I T G A A S G L G K A M A I R F G K E
Q A K V V I N Y Y S N K Q D P I V Q T A I K E V I K A G G E A V
V V Q G D V T K E P V K E M P L K D G N V I N M S S V H E V I
N N A G L G N P I K Y A A S K G I K L A V A G R G
F L G S R E A I K Y A A S K G I K L A V A G R G
P W P L F V H Y A A S K G I K L A V A G R G
I R V N N I G P G A I N T P I A V A G R G
S M I P M G Y I G E P E I A V A G R G
G I T L F A D G G M T K Y P S Q A G R G

GlucDH_E170K_K252L

M Y P D L K G K V A I T G A A S G L G K A M A I R F G K E
Q A K V V I N Y Y S N K Q D P I V Q T A I K E V I K A G G E A V
V V Q G D V T K E P V K E M P L K D G N V I N M S S V H E V I
N N A G L G N P I K Y A A S K G I K L A V A G R G
F L G S R E A I K Y A A S K G I K L A V A G R G
P W P L F V H Y A A S K G I K L A V A G R G
I R V N N I G P G A I N T P I A V A G R G
S M I P M G Y I G E P E I A V A G R G
G I T L F A D G G M T K Y P S Q A G R G