

The invention concerns a process for the colorimetric determination of an analyte by means of enzymatic oxidation of the analyte in the presence of an electron acceptor and determination of the reduced electron acceptor by colour formation as measure for the amount of the analyte.

Furthermore, the invention concerns an agent for the colorimetric determination of an analyte by enzymatic oxidation of the analyte, containing an oxidoreductase and a colour-forming electron acceptor.

The invention especially extends to the use of an aromatic nitroso compound as direct colour-forming electron acceptor of an analyte/oxidase system or of an analyte/PQQ-dependent dehydrogenase system.

In analyses, enzymatic oxidations make possible the detection and the determination of substances in the most varied sample materials. An oxidising enzyme hereby acts on a corresponding enzyme substrate in the presence of an acceptor accepting the electrons of the oxidation reaction. The reduction of the electron acceptor shows the presence of the enzyme substrate. Hitherto, it has hereby proved to be especially advantageous when the reduced electron acceptor can be detected by colour formation since this is possible not necessarily only by means of expensive measurement apparatus but can possibly also take place visually.

Known methods for the colorimetric determination of substances by means of oxidising-acting enzymes use oxidases or dehydrogenases. Both enzyme groups belong to the the main group of the oxidoreductases (Römpps Chemie-Lexikon, Franckhsche Verlagshandlung, Stuttgart, 8th edition, 1985, Volume 4, page 2952; Lexikon Biochemie, pub. H.D. Jakubke, Verlag Chemie, Weinheim, 2nd edition, 1981, page 194), the members of which can be differentiated according to their natural electron acceptors.

The natural electron acceptor for oxidases is molecular oxygen (Römpps Chemie-Lexikon, Franckhsche Verlagshandlung, Stuttgart, 8th edition, 1985, Volume 4, page 2946). As representative for the state of the art
5 of the use of oxidases for the colorimetric determination of analytes, there is cited A. Kunst et al. in "Methods in enzymatic analysis", pub. H.U. Bergmeyer, Verlag Chemie, Weinheim, 3rd edition, 1984, Volume 6, page 178-185. Glucose is there detected in serum, plasma or
10 deproteinised blood by reaction with glucose oxidase and atmospheric oxygen in aqueous solution in that the hydrogen peroxide formed in the case of the reaction by reduction of the oxygen acts oxidisingly and thus forms colour with phenol and 4-aminophenazone also present in
15 the reaction mixture. As source of error, in this literature reference is even mentioned the presence of reducing-acting substances, such as ascorbic acid, uric acid or glutathione. Furthermore, transition metal ions or haem and haem proteins, which can easily occur in
20 samples derived from blood, act disturbingly because they decompose hydrogen peroxide. Protein component materials which lead to a colour formation with hydrogen peroxide and peroxidase and possibly further substances contained in the detection agent, such as e.g. phenol
25 or 4-aminophenazone, can lead to false results. Such materials can be e.g. bilirubin or also medicaments, such as e.g. α -methyldopa, which can well be present in samples derived from blood or in urine.

In J. Siedel et al., in "Methods in enzymatic
30 analysis", pub. H.U. Bergmeyer, Verlag Chemie, Weinheim, 3rd edition, 1984, Volume 8, page 139-148, is described the colorimetric determination of total cholesterol in serum or plasma in such a manner that ester-bound
cholesterol is first liberated with cholesterol esterase.
35 Cholesterol is then determined by reaction with cholesterol oxidase and atmospheric oxygen in aqueous solution, whereby the hydrogen peroxide formed in the case of this reaction oxidises in the presence of peroxidase and thus

acts colour-formingly on phenol and 4-aminoantipyrine also present in the reaction mixture. The colour formation is a measure for the amount of total cholesterol in the sample.

- 5 All disadvantages mentioned for the above-mentioned processes for the determination of glucose by means of oxidase apply to the same extent to the described cholesterol determination method. These disadvantages are independent of whether the detection reaction is
- 10 carried out e.g. in a cuvette or on a dry reagent carrier such as are known e.g. from EP-A-0 016 387, DE-A-32 47 608, EP-A-0 262 445 or EP-A-0 256 806. Especially in the case of carrying out of the above-mentioned determination methods on solid carriers in
- 15 so-called dry tests, the oxygen requirement has additionally proved to be disadvantageous. In particular, when much oxygen is utilised for the oxidation of high concentrations of enzyme substrate, the diffusion of oxygen from the air into the reaction medium can become
- 20 the velocity-determining step and lead to long reaction times or, especially in the case of kinetic determination methods, to false results.

In US-A-4,105,800, xanthine is detected by oxidation with xanthine oxidase in the presence of resazurin, an

25 aromatic N-oxide as electron acceptor by colour formation.

Dehydrogenases can, quite generally, be subdivided into those which, for the oxidation of enzyme substrates, need nicotinamide-adenine-dinucleotide (NAD) or nicotinamide-adenine-dinucleotide phosphate (NADP) as natural

30 direct electron acceptor and into those which are not NAD- or NADP-dependent and which thus use other substances as natural direct electron acceptors in the case of enzymatic oxidation reactions.

The use of dehydrogenases for colorimetric measurements is known e.g. from DE-A-21 47 466. It is there

35 described that lactate, by catalysis of lactate dehydrogenase, is reacted with nicotinamide-adenine-

dinucleotide to give pyruvate and reduced nicotinamide-adenine-dinucleotide. The NADH formed then reacts, for example in the presence of the enzyme diaphorase, with tetrazolium salts with formation of NAD and coloured formazans, the concentration of which can be determined photometrically. Instead of diaphorase, there is also mentioned N-methylphenazinium methosulphate as a reduction catalyst for the transfer of electrons from NADH to the tetrazolium salt.

- 10 Disadvantages of this process are to be seen in the fact that, instead of NADH, other reducingly-acting substances possibly present in biological samples, such as e.g. blood, serum, plasma or urine, such as glutathione or medicaments, such as methyldopa or dobesilate, 15 in the presence of reduction catalyst, such as diaphorase or N-methylphenazinium methosulphate, convert tetrazolium salts into corresponding formazans and thus lead to falsely positive results.

- Furthermore, the necessity of reduction catalysts, 20 such as diaphorase or phenazinium sulphate, for the transfer of the electrons liberated by the oxidation of an analyte from analyte/enzyme system to an electron acceptor serving for the colour formation involve the disadvantage that other sample accompanying materials 25 can thereby also be reduced which under otherwise identical conditions, do not enter into a reduction but thus lead to falsely negative results.

- In EP-A- 0 120 440, glycerol is detected by an NAD-independent dehydrogenase in the presence of various 30 electron acceptors. In particular, phenazine methosulphate is here also used, preferably as electron transfer reagent to a further electron acceptor, such as e.g. a tetrazolium salt.

- The invention intends to provide help for the 35 above-mentioned disadvantages of the prior art. In particular, the task forming the basis of the invention is to make available a process for the analysis especially of biological fluids for the colorimetric deter-

mination of an analyte by means of enzymatic oxidation of the analyte in the presence of an electron acceptor and determination of the electron acceptor by colour formation as measure of the amount of the analyte in
5 which reducing-acting, especially hydrogen peroxide-decomposing accompanying materials in samples, especially biological samples, such as liquid derived from blood, e.g. serum, or in urine, do not disturb, which avoids the use of reduction catalysts, such as
10 diaphorase or N-methylphenazinium methosulphate, for which no oxygen is necessary and which thereby also make possible rapid colour end values even in the case of high substrate concentrations.

Furthermore, there is to be made available an agent
15 for the colorimetric determination of an analyte by enzymatic oxidation of the analyte, containing an oxidoreductase and a colour-forming electron acceptor which is suitable for the carrying out of the above-mentioned process.

20 In particular, the task extends the choice of those substances which, as colour-forming electron acceptors, can be used for the improved process and agent as stated above.

The task is solved by the invention as it is
25 characterised in the claims.

Accordingly, there was found a process for the colorimetric determination of an analyte by means of enzymatic oxidation of the analyte in the presence of an electron acceptor and determination of the reduced
30 electron acceptor by colour formation as measure for the amount of the analyte, which is characterised in that the analyte is oxidised with a flavine-dependent oxidase or a PQQ-dependent dehydrogenase in the presence of an aromatic nitroso compound or of a tautomeric
35 equivalent oxime as direct electron acceptor.

Furthermore, an agent has been found for the colorimetric determination of an analyte by enzymatic oxidation of the analyte, which contains a flavine-

dependent oxidase or a PQQ-dependent dehydrogenase and a colour-forming electron acceptor, which is characterised in that the colour-forming electron acceptor is an aromatic nitroso compound reacting directly with
5 the analyte/enzyme system.

According to the invention, by "analyte" is understood such a substance which is enzymatically oxidised. In many cases, the analyte will be that substance which is to be directly detected or quantitatively determined
10 in the sample to be investigated. For example, glucose can be oxidised directly with glucose oxidase and determined colorimetrically. However, it is also possible that the analyte is first formed from another substance by one or more preceding reactions and thus, from the
15 presence and concentration of the analyte, conclusions can be made indirectly regarding the presence and concentration of the starting substance. Thus, e.g. glycerol can be so detected and determined that, in a first reaction, glycerol is converted by means of glycerol
20 kinase and adenosine triphosphate into glycerol-3-phosphate and adenosine diphosphate and then subsequently, in a second reaction, glycerol-3-phosphate is oxidised with glycerol-3-phosphate oxidase. In this case, glycerol-3-phosphate is the analyte, the concentration
25 of which corresponds to that of the substance to be determined, namely glycerol. However, here, too, the analyte is that compound which is determined colorimetrically.

In the present invention, the analyte is that
30 substance which is accepted as substrate of the particular oxidising enzyme. In order that the analyte is oxidised, an electron acceptor must be simultaneously present which takes over the electrons from the analyte with participation of the enzyme.

35 By "colour-forming" is understood that the electron acceptor after reduction is either itself directly present with a colour different from that before the enzymatic oxidation of the analyte or that the reduced

acceptor itself admittedly does not lead directly to a colour production but, in a following reaction, causally leads to a colour change of the reaction mixture. Colour change hereby includes not only the change from colourless to coloured but also from one colour to another. For the colour formation by subsequent reaction, many possibilities are known to the expert from which he can select, depending upon the circumstances. For example, there may here be mentioned those reactions in which the reduced electron acceptor itself becomes a part of a coloured compound, such as oxidative coupling reactions. However, colour-forming subsequent reactions can also be those in which the reduced electron acceptor, due to its reducing action on another substance, leads to a colour change thereof.

By oxidation stage is understood a numerical value which characterises the oxidation state of a particular atom in a compound, which can be a neutral molecule or a charged complex. The determination of oxidation stages is known to the expert. Instructions for the determination of this numerical value are to be found in basic chemical textbooks, such as "Anorganische Chemie", Hofmann & Rüdorff, Verlag F. Vieweg & Sohn, Braunschweig, 20th edition, 1969, page 216-219; "Lehrbuch der anorganischen Chemie", Hollemann-Wiberg, Verlag Walter de Gruyter & Co., Berlin, 71st - 80th edition, 1971, page 197-199, and "Anorganikum", ed. L. Kolditz, VEB Deutscher Verlag der Wissenschaften, Berlin, 4th edition, 1972, page 446.

Suitable nitroso compounds which are usable for the process according to the invention for the colorimetric determination of an analyte are preferably those which contain the nitroso radical bound to an aromatic group.

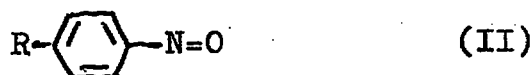
Especially preferred are carbon aromatic nitroso compounds, such as nitrosobenzene and nitrosobenzene derivatives. As nitrosobenzene derivatives are hereby quite outstandingly appropriate for the process according to the invention all those which are

converted by reduction into those compounds which can serve for the colour formation in the sense of the previously given explanation.

Nitrosobenzene and nitrosobenzene derivatives are, as a rule, weakly coloured, yellow, green or brown coloured substances which, in the reduced state, are not coloured. In order to be able colorimetrically to indicate an analyte, they must, therefore, lead to a colour formation in a subsequent reaction. This can, for example, be achieved in that the reduced nitroso compound is so reacted with another substance that a coloured substance is obtained which contains the reduced nitroso compound as partial structure.

Oxidative coupling reactions are one possibility for this type of colour formation. Therefore, those nitrosobenzene derivatives are especially preferred for the process according to the invention which act not only as electron acceptors for oxidising enzymes but which are also usable in the reduced state for oxidative coupling reactions.

Quite especially preferred are nitrosobenzene derivatives of the general formula II



in which R signifies a hydroxyl group or an amino group, whereby the amino group can be substituted once or twice by lower alkyl and the lower alkyl group can, in turn, be substituted by a hydroxyl group, an amino group substituted one or more times by lower alkyl, PO_3H_2 , SO_3H or CO_2H .

Outstandingly suitable are especially those nitrosobenzene derivatives of the general formula II which have a low volatility.

In the above definition, lower alkyl signifies an alkyl radical with 1 - 5 carbon atoms. Methyl and ethyl are especially preferred.

The acid residues PO_3H_2 , SO_3H and CO_2H can be present as such or in salt form as ammonium, alkali metal or alkaline earth metal salts. Ammonium salts are those which contain the unsubstituted ammonium cation NH_4^+ or those which contain ammonium cations substituted one or more times by lower alkyl, aryl and/or lower alkylaryl radicals. Lower alkyl means, in each case, an alkyl radical containing 1 - 5 carbon atoms. The aryl radical is an aromatic ring system having 6 - 10 carbon atoms.

Methyl and ethyl are preferred as lower alkyl. Preferred aryl radical is phenyl, whereby benzyl is a preferred lower alkylaryl radical.

As electron acceptors for the process according to the invention, there are quite outstandingly suitable p-hydroxynitrosobenzene, p-dimethylaminonitrosobenzene, p-diethylaminonitrosobenzene and p-dihydroxyethylaminonitrosobenzene.

As stated above, for the process according to the invention, nitrosobenzene or nitrosobenzene derivatives, in which nitrogen is present in the oxidation stage +1, are preferably brought into contact with the sample to be investigated and an oxidising enzyme. In the case of the presence of an analyte in the sample which is accepted as enzyme substrate, nitrosobenzene or a nitrosobenzene derivative functions as electron acceptor and is thereby reduced to the corresponding amine. As a rule, this reduction of the nitroso compounds does not suffice alone for a colorimetric determination of the analyte since either only colour decreases occur or colour changes are only weakly observable. However, the reduced electron acceptor can serve as starting material for an oxidative coupling reaction, whereby, depending upon the choice of the coupling partner, the most varied colours can be obtained.

Oxidative coupling reactions are known to the expert and described, for example, in EP-A-O 175 250 or in H. Hünig et al., Angewandte Chemie, 70, 215-222

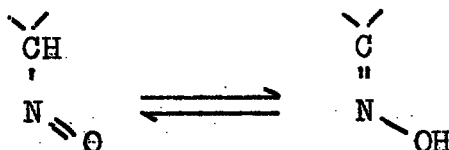
(1958). In general, oxidative couplings are frequently used for the production of dyestuffs. In this connection, they can be regarded as reaction between an electron-rich aromatic compound and an oxidisable coupling component which takes place in the presence of oxidation agents, such as e.g. sodium or potassium hexacyanoferrate (III), copper salts, mercury salts, iron (III) chloride, lead dioxide, hydrogen peroxide, lead tetraacetate, sodium or potassium salts of persulphuric acid, peracetic acid, periodic acid or chloramine T. According to DE-A-33 31 588, oxidases can also be used as oxidation agents. The above-described nitrosobenzenes represent in their reduced state, after they have acted as electron acceptors in enzymatic oxidations, compounds which, as oxidisable coupling components, are available for oxidative coupling reactions. For this purpose, the amines resulting by the reduction can be oxidised by oxidation agents, as are mentioned above by way of example, in order to form a coloured material with electron-rich coupling components simultaneously present in the reaction mixture.

A plurality of electron-rich coupling components are available to the expert. These coupling components can be chosen depending upon the desired colour of the coupling product. Examples are aromatic amines, phenols and methylene-active compounds. Especially preferred compounds can be chosen from the group of the anilines, e.g. N-methylantranilic acid, and the anilinophosphonic acids mentioned in EP-A-O 175 250.

A further possibility for the determination of the reduced electron acceptor by colour formation consists, in the case of the nitroso compounds, in oxidising the reduced nitroso compounds by other substances which, on the basis of this transition from the oxidised into the reduced form, in turn form a colour, whereby, here, too, by colour formation is to be understood not only the transitions from one colour to the other but also from the colourless into the coloured state. Examples of

such colour forming reactions are e.g. the oxidation of the reduced electron acceptor by means of metal salts which are hereby reduced to coloured metal salts with the metal in a lower oxidation stage or possibly completely reduced to the neutral metal. Copper (II) salts can, for example, be converted into red copper (I) salts and silver salts into metallic silver. The reduction of phosphomolybdate to molybdenum blue is also possible.

10 Instead of aromatic nitroso compounds, for the process according to the invention, there can also be used aromatic oximes as electron acceptors for enzymatic oxidations. Nitroso compounds can be in a tautomeric equilibrium with oximes. This is especially
15 the case when, for this purpose, a hydrogen atom is available in the α -position to the nitroso group.



or when a corresponding delocalisation of the electrons and protons is possible.

20 It was found that oximes which, with nitroso compounds which function as electron acceptors in enzymatic oxidation reactions, can stand in a tautomeric nitroso/oxime equilibrium, can also be used in the process according to the invention. This applies
25 especially to oximes of the general formula III



in which R' signifies oxygen, a further oxime group or a positively charged amino group, whereby the amino groups are substituted one or twice by lower alkyl and the lower alkyl group can, in turn, be
30 substituted one or more times by lower alkyl, PO_3H_2 , CO_2H or SO_3H .

In the above definition, lower alkyl means an alkyl radical with 1 - 5 carbon atoms. Methyl and ethyl are especially preferred.

5 The acid residues PO_3H , SO_3H and CO_2H can be present as such or in salt form as ammonium, alkali metal or alkaline earth metal salts.

Ammonium salts are those which contain the unsubstituted ammonium cation NH_4^+ or those which contain ammonium cations substituted one or more times by
10 lower alkyl, aryl and/or lower alkylaryl. Lower alkyl means, in each case, an alkyl radical containing 1 - 5 carbon atoms. The aryl radical is an aromatic ring system containing 6 -10 carbon atoms. Methyl and ethyl are preferred as lower alkyl. Preferred aryl radical
15 is phenyl, whereby benzyl is a preferred lower alkyl-aryl radical.

In such pximes, the nitrogen possesses the oxidation stage -1.

In the same way as the nitrosobenzenes, the oximes
20 of the general formula III are also colourless or coloured substances which, in the reduced state, are not coloured. As described above for nitrosobenzenes as electron acceptors in the process according to the invention, aromatic oximes, after their function as
25 electron acceptors, can be brought to colour formation. Not only nitroso compounds but also oximes give amines in the case of exhaustive reduction. For oximes as electron acceptors, in the process according to the invention, fundamentally the same subsequent reactions
30 are usable for the colour formation as for the nitroso compounds.

Surprisingly, it was found that nitroso compounds and oximes, as they are described above, can serve as colour-forming electron acceptors which are catalysed
35 as oxidoreductases. Preferably, they are there usable where, for the electron transfer from analytes to be oxidised to the colour-forming electron acceptor, no

reduction catalysts, such as diaphorase or N-methyl-phenazinium methosulphate, are desired. Surprisingly, it was found that these substances are advantageous when oxidases or PQQ-dependent dehydrogenases are used as oxidising enzymes. If such enzymes are used for the oxidation of an analyte, the electron transfer can take place directly, i.e. without cooperation of a reduction catalyst, from the analyte/enzyme system to the above-mentioned colour-forming electron acceptors. As analyte/enzyme system, in this connection, is to be understood the combination necessary for the oxidation reaction of analyte and oxidising enzyme, as well as possibly together with co-enzymes and/or co-factors, such as for example metal salts, naturally necessary for the oxidation and working together with the enzyme.

For the process according to the invention, flavine-dependent oxidases are especially preferred. Examples are L- and D-amino acid oxidases, cholesterol oxidase, glucose oxidase, glycerol-3-phosphate oxidase, lactate oxidase and pyruvate oxidase. Oxidases which, according to the invention, are quite especially preferred are glucose oxidase, glycerol-3-phosphate oxidase, lactate oxidase and pyruvate oxidase.

Furthermore, pyrroloquinoline-quinone (PQQ)-dependent dehydrogenases are especially advantageously usable for the process according to the invention. Especially glucose dehydrogenase is very suitable for the colorimetric determination of glucose in the presence of aromatic nitroso compounds as colour-forming electron acceptors. In the same way, PQQ-dependent alcohol dehydrogenase can be used for the determination of alcohols, such as ethanol.

The process according to the invention is so carried out that the sample to be investigated is contacted with a suitable oxidoreductase and one or more of the above-described colour-forming electron acceptors. If the sample contains an analyte which

is oxidised by the oxidoreductase, the colour-forming electron acceptor is reduced. If the reduced electron acceptor displays a colour other than that of the original electron acceptor in its oxidised form or if
5 the electron acceptor, due to the enzymatic oxidation procedure, passes over from the colourless state into a coloured one, the intensity of the colour formed can be correlated directly visually, possibly by comparative colours, or photometrically with the concentration of
10 the analyte in the sample. If the colour of the reduced electron acceptor is substantially the same as that of the electron acceptor originally used or if a colour lightening or complete decolorisation takes place, then a following reaction is attached to the enzymatic
15 oxidation reaction which leads to a colour from the intensity of which the concentration of the analyte in the sample can also be determined visually or photometrically.

The process can be carried out in a so-called wet
20 test, e.g. in a cuvette, or as so-called dry test on an appropriate reagent carrier, whereby the necessary test reagents are present on a solid carrier, especially an absorbent or swellable material. Such test carriers are known, e.g. from EP-A-0 016 387, DE-A-32 47 608,
25 EP-A-0 262 445 or EP-A-0 256 806.

Agents for the colorimetric determination of an analyte for the carrying out of the process according to the invention, as they are characterised in the claims, are also the subject of the invention. Such
30 an agent contains, besides the oxidoreductase necessary for the enzymatic oxidation of the analyte to be determined, at least one colour-forming electron acceptor which takes over the electrons liberated in the case of the oxidation directly from the analyte/enzyme system.
35 As oxidising enzymes and colour-forming electron acceptors, there are used the materials described above for the process according to the invention.

For the maintenance of a pH value suitable for the carrying out of the process, which depends especially upon the enzymes to be used, the agent according to the invention contains a buffer system. Furthermore, it can
5 contain further suitable additive materials usually employed for such agents, such as for example wetting agents, stabilisers etc. If the oxidation of the electron acceptor does not lead to a measurable colour change, for the colorimetric determination of an analyte, the
10 agent according to the invention naturally also includes the reagents necessary for a subsequent reaction.

The agent according to the invention can be present in the form of a solution or can be applied to an absorbent or swellable carrier. In the form of a solution,
15 the agent preferably contains all reagents needed for the process according to the invention. As solvents, there come into question water, water-soluble organic solvents, such as e.g. methanol, ethanol, acetone or dimethylformamide, or mixtures of water with such
20 organic solvents. For reasons of storage stability, it can be advantageous to divide the reagents needed for the test into two or more solutions which are first mixed in the case of the actual investigation. The
latter can then especially apply when, after oxidation
25 of the analyte, the reduced electron acceptor is further reacted in a subsequent reaction, e.g. an oxidative coupling reaction. Typical concentrations for the electron acceptors used in the agent according to the invention are: 0.01 - 100 mMol/l, preferably 0.1 - 25
30 mMol/l. Reagents for subsequent reactions are used at least in stoichiometric relationship to the electron acceptors, preferably in an excess, especially in a 2 - 10 fold excess.

The agent according to the invention can also be
35 present in the form of a test strip. Such test strips are known in many types of embodiment, for example from EP-A-0 016 387, DE-A-32 47 608, EP-A-0 262 445

or EP-A-0 256 806. It is common to all that the reagents needed for the carrying out of the determination process are present on solid carrier layers.

As carrier layers, there come into question especially
5 absorbent and/or swellable materials which are wetted by the sample to be investigated. Examples are gelatin, cellulose or synthetic fibre fleece. The reagents are present in solid form in or on these carrier materials.

In the case of application of the sample liquid to
10 the test strips or dipping of the test strips into the sample liquid, a liquid medium forms in the strips within which the detection reaction takes place. The colour formation caused by the reaction can be evaluated visually or photometrically, e.g. reflection photo-
15 metrically.

A special subject of the invention concerns the use of an aromatic nitroso compound as direct electron acceptor of an analyte/enzyme system. As analyte/enzyme system, in this connection there is to be understood
20 the combination necessary for an enzymatic oxidation reaction of analyte and flavine-dependent oxidase or PQQ-dependent dehydrogenase, as well as possibly together with co-enzymes, such as flavine or PQQ and/or co-factors, for example metal salts, naturally necessary for the
25 oxidation working together with the enzyme. It has been found that substances of the group of the nitroso compounds and oximes can be used quite generally as colour-forming electron acceptors in the case of the oxidation of an analyte by means of an oxidoreductase.

30 The present invention offers the advantage that no reduction catalysts, such as diaphorase or N-methylphenazinium methosulphate, are necessary for the reduction of a colour-forming electron acceptor. The reduction thereof can now take place directly by the
35 analyte/enzyme system. Possibly disturbing side reactions can thus be avoided.

Especially in the case of the use of oxidases for the enzymatic oxidation of analytes, by means of the use of the compounds according to the invention, the formation of hydrogen peroxide as precursor of a colorimetric determination is avoided. This removes or reduces the disturbing influence of reducing-acting compounds.

Finally, the compounds employed according to the invention offer a true alternative everywhere where the admission of atmospheric oxygen is limited or undesired. Oxygen can, as electron acceptor, be replaced by these compounds in the case of enzymatic oxidations. This offers quite special advantages in the case of agents according to the invention in the form of test strips. Whereas these hitherto, especially in the case of high analyte concentrations, had to be so constructed that, in the case of enzymatic oxidation by means of oxidase, atmospheric oxygen had admission to the reagent mixture applied to the test strips, test strips can now be constructed which operate especially quickly and dependably. Thus, whereas previously in the case of test strips, after application to the test strips the sample had often to be wiped off again after a certain time in order that oxygen could diffuse at all into the test strips, this measure is not necessary in the case of use of the electron acceptors according to the invention. Since the time-dependent diffusion of oxygen into the test strips is avoided, in the case of kinetic measurements, of the reaction speeds dependent upon the analyte concentration and, in the case of end point measurements, in the end point of the reaction speeds independent of the analyte concentration are obtained which permit determination processes which are quicker, more dependable and simpler than was previously possible.

The following Examples show some of the possible process variants for the colorimetric determination

of an analyte by means of enzymatic oxidation.

Example 1

Reduction of nitroso compounds by glucose oxidase and glucose

- 5 A) 2000 μ l 0.1M citric acid/NaOH buffer, pH 6.0
200 μ l p-nitroso-N-N-dimethylaniline, 0.1M in ethanol
100 μ l glucose oxidase (EC 1.1.3.4)(2500 IU/ml)
or glucose dehydrogenase (EC 1.1.99.10)
10 200 μ l of sample with known glucose content
a) 5 mM
b) 10 mM
c) 15 mM
d) 20 mM
15 e) 50 mM

in water were mixed and incubated at 25°C for 2 minutes. There were then added thereto 250 μ l N-methylantranilic acid, 0.1M in ethanol, 125 μ l potassium hexacyanoferrate (II), 0.2M in water and 125 μ l potassium
20 hexacyanoferrate (III), 0.2M in water. After a further minute, it was diluted 25 fold and the absorption of the reaction mixture, which is green coloured in the case of the presence of glucose, measured at 710 nm against a blank (above reaction mixture without glucose).
25 The results obtained gave a line. The extinction coefficient of $\epsilon_{710} = 24000 \text{ M}^{-1} \text{ cm}^{-1}$ can be used for the determination of unknown glucose concentrations in solutions.

- B) If, in the case of the above test, in A) p-nitroso-
30 dimethylaniline was replaced by
a) p-nitrosophenyl
b) p-nitroso-N,N-diethylaniline
c) p-nitroso-N,N-diethanolaniline (preparation according to D'Amico et al., J. Amer. Chem. Soc. 81, 5957/
35 1959))

then, in the case of the presence of glucose, due to the coupling with N-methylantranilic acid, a colour change of

- a) brown to blue
 - b) yellow to green
 - c) yellow to green
- was observed.

5 C) If, in the case of the above test, in A) N-methyl-anthranilic acid was replaced by

- a) N-methyl-N-methylenephosphonic acid aniline
- b) 1-hydroxynaphthalene-2-carboxylic acid
- c) aniline-2-sulphonic acid

10 than, in the case of the presence of glucose, due to the coupling with p-nitroso-N,N-dimethylaniline, a colour with

- a) $\lambda_{\max} = 735 \text{ nm}$
- b) $\lambda_{\max} = 590 \text{ nm}$

15 c) $\lambda_{\max} = 640 \text{ nm}$
was observed.

In the case of the use of p-nitroso-N,N-dimethylaniline and N-methyl-N-methylenephosphonic acid aniline, there was found the dependence shown in Fig. 1 of the
20 change of the light adsorption at 735 nm upon the glucose concentration. For this purpose, the extinction was measured after (1 + 24) dilution in citrate buffer (pH 6) and plotted against the glucose concentration in the test batch.

25 Example 2

Detection of glucose by formation of metallic silver

The batch of citrate buffer, p-nitroso-N,N-dimethylaniline, glucose oxidase and sample was incubated for 2 minutes at 25°C as in Example 1 and
30 mixed with 250 μl 100 mM silver nitrate solution in water, as well as 250 μl gold sol in water.

(The preparation of gold sol took place according to the following procedure: To 100 ml boiling distilled water are successively added 0.4 mg HAuCl_4 in 0.4 ml
35 of water; 0.2 ml 0.1M NaSCN in water and 0.5 ml 0.1M K_2CO_3 in water. After 10 minutes, it is left to cool);

With the results obtained without intermediate dilution at 700, 850 and 1300 nm, there were obtained the curves shown in Figure 2. They can serve as calibration curves for the determination of the unknown glucose content in solutions.

Example 3

Detection of glucose by molybdenum blue formation

To a solution of 200 mg 2,18-phosphomolybdic acid (preparation e.g. possible according to G. Brauer, "Handbuch der präparativen Anorganischen Chemie", Enke-Verlag, Stuttgart, p. 1278 (1954) or A. Rosenheim, A. Traube, Z. Anorg. Chemie, 65, 99 (1910) in 920 - x μ l, 0.1 M citric acid/NaOH buffer pH 5.5 were added 40 μ l 0.1M p-nitroso-N,N-dimethylaniline (in ethanol), as well as 40 μ l glucose oxidase (6250 IU/ml water). 1 Minute after addition of x μ l (x = 0, 1, 2, 3, 5, 7, 10) of a glucose-containing sample of known glucose content (1 M), it was diluted to 50 ml and the absorption change (ΔE) measured at 820 nm. As result, there was obtained the curve shown in Fig. 3. It can serve as curve for the determination of the unknown glucose content of solutions, whereby C represents the concentration in the sample before the dilution carried out for the measurement.

Example 4

Kinetic determination of glucose by means of non-NAD(P)-dependent glucose dehydrogenase

The following solutions were prepared:

test buffer: 0.1 M tris/hydrochloric acid, pH 7.5
containing 1% bovine serum albumin
electron acceptor: 0.1 M p-nitroso-N,N-dimethylaniline
in ethanol
indicator: 2,18-phosphomolybdic acid, 100 mg/ml water
enzyme: glucose dehydrogenase (EC 1.1.99.17),
50 IU/ml test buffer

glucose solution: a) 36 mg/dl human plasma
b) 72 mg glucose/dl human plasma
c) 144 mg glucose/dl human plasma
d) 360 mg glucose/dl human plasma
5 e) 720 mg glucose/dl human plasma
f) 1440 mg glucose/dl human plasma
g) 3600 mg glucose/dl human plasma

In a 1 cm cuvette were placed

1740 μ l buffer.
10 250 μ l electron acceptor
250 μ l indicator and
19 μ l glucose dehydrogenase,

the mixture thermostated at 25°C and then 250 μ l
glucose solution added thereto. With the addition of
15 the glucose as starting point, the absorption change
per minute ($\Delta E/\text{min}$) was recorded at 820 nm. The
following values were obtained:

20	glucose concentration (end concentration in the test batch)	$\Delta E/\text{min}$
	3.6 mg/dl	0.20
	7.2 mg/dl	0.38
	14.4 mg/dl	0.57
	36 mg/dl	1.00
25	72 mg/dl	1.60
	144 mg/dl	2.24
	360 mg/dl	3.13

Example 5

Test strips for glucose detection by molybdenum blue
30 formation

1 g sodium alginate (e.g. Algipon of Kelco, division
of Merck & Co., Clark, New Jersey, USA)
45 g polyvinyl propionate (e.g. Propiophan 70D of
BASF, Ludwigshafen, Federal Republic of Germany)
35 0.75 g sodium nonyl sulphate
10.15 g potassium dihydrogen phosphate and
58.5 g distilled water

4 g Aerosil COK 84 (Degussa, Hanau, Germany/FRG)
are stirred to give a homogeneous mass and adjusted
to pH 5.5 with 10 N caustic soda solution.

Subsequently, there were added thereto

- 5 65 mg glucose oxidase (250 IU/mg)
- 260 mg p-nitroso-N,N-dimethylaniline
- 1.3 g 2,18-phosphomolybdic acid.

The mass was raked out in 320 μ m layer thickness
in a 1 mm thick polystyrene foil and dried at 60°C. for
10 1 hour. In the case of applying a drop of glucose-
containing solution, a distinct green coloration
occurred within one minute. The intensity of the
coloration increased with increasing glucose concent-
ration and can be evaluated visually on the basis of a
15 comparison scale or reflection photometrically.

Instead of the electron acceptor p-nitroso-N,N-
dimethylaniline, there could also be used p-benzo-
quinone dioxime or p-nitroso-N,N-diethylaniline.

In the case of storage in the dark at room temper-
20 ature, the coloration is stable for several weeks.

If, in the above Example, one replaces p-nitroso-
N,N-dimethylaniline by peroxidase (100 mg; 200 IU/mg)
and phosphomolybdic acid by 3,3',5,5'-tetramethyl-
benzidine (300 mg), then one obtains an oxygen-
25 dependent glucose test, as is known from the prior art.
However, in contradistinction to the above Example,
the sample solution, after application to the test
strip, must be wiped off in order that oxygen can
diffuse in and for coloration to take place at all.
30 In addition to this disadvantage, the end coloration
with comparatively high glucose concentration is
achieved ever more slowly and the colour produced is
less storage stable. The advantages of the replacement
of oxygen by electron acceptors according to the
35 invention are thus:

- no wiping off of the sample after application to
the test strip necessary

- quicker reaction
- in the case of kinetic measurement, speed of reaction dependent upon the analyte concentration
- in the case of end point measurement, at the end point
- 5 speed of reaction independent of the analyte concentration
- more stable coloured material.

Example 6

Determination of lactate with lactate oxidase and
10 nitroso compounds as electron acceptor

In a cuvette, 2240 μ l of test buffer (0.2 M citric acid/sodium hydroxide, pH 6.35), 5 μ l of electron acceptor (0.1 M N,N-dimethyl-p-nitrosoaniline in ethanol) and 250 μ l of sample with known lactate
15 concentration were mixed and thermostated to 25°C. The test was started with 5 μ l of enzyme solution (lactate oxidase from *Pediococcus* sp.), 200 U/ml of test buffer) and extinction change $\Delta E/\text{min}$ recorded at 390 nm. The following results were obtained:

20

C_{lactate} : mM	$\Delta E/\text{min}$
0	0
0.1	0.093
0.2	0.182
0.3	0.250
0.5	0.343
1.0	0.443
3.0	0.508

25

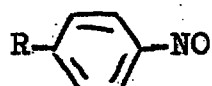
C_{lactate} is hereby the lactate concentration which is present in the cuvette in the case of carrying out of
30 the measurement.

The test could be accelerated or slowed down by changes of the electron acceptor concentration, enzyme concentration, observation wavelength and temperature. As electron acceptor, there could also be used N,N-diethyl-p-nitrosoaniline, N,N-bis-(2-hydroxyethyl)-p-nitrosoaniline, benzfuroxane and resazurin.
35

Patent Claims

1. Process for the determination of an analyte by means of enzymatic oxidation of the analyte in the presence of an electron acceptor and determination of
5 the reduced electron acceptor by colour formation as measure for the amount of analyte, characterised in that the analyte is oxidised by a flavine-dependent oxidase or by a PQQ-dependent dehydrogenase in the presence of an aromatic nitroso compound or of a
10 tautomERICALLY equivalent oxide as direct electron acceptor.
2. Process according to claim 1, characterised in that, as aromatic nitroso compound, there is used a compound of the formula I

15



(I)

- in which R signifies a hydroxyl group or an amino group, whereby the amino group can be substituted once or twice by lower alkyl and the lower alkyl group can in turn be substituted by a hydroxyl group, an amino group
20 substituted one or more times by lower alkyl, PO_3H_2 , SO_3H or CO_2H .
3. Process according to claim 1 or 2, characterised in that the aromatic nitroso compound represents p-hydroxy-nitrosobenzene, p-dimethylaminonitrosobenzene, p-diethylaminonitrosobenzene or p-dihydroxyethylamino-
25 nitrosobenzene.
4. Process according to claim 1 - 3, characterised in that the reduced electron acceptor is detected by an oxidative coupling reaction by colour formation.
- 30 5. Process according to claim 1 - 3, characterised in that the reduced electron acceptor is detected by reduction of substances which form a colour in the case of changing from the oxidised to the reduced form.



6. Agent for the colorimetric determination of an analyte by enzymatic oxidation of the analyte, containing a flavine-dependent oxidase or a PQQ-dependent dehydrogenase and a colour-forming electron acceptor, characterised in that the colour-forming electron acceptor is an aromatic nitroso compound reacting directly with the analyte/enzyme system.
7. Agent according to claim 6, characterised in that the aromatic nitroso compound represents a compound of the formula I



- in which R signifies a hydroxyl group or an amine group, whereby the amine group can be substituted once or twice by lower alkyl and the lower alkyl group can in turn be substituted by a hydroxyl group, an amine group substituted one or more times by lower alkyl, PO_3H_2 , SO_3H or CO_2H .
8. Agent according to one of claims 6 or 7, characterised in that it additionally contains an oxidative coupling reagent for the reduced electron acceptor.
9. Agent according to one of claims 6 or 7, characterised in that it additionally contains a substance which forms a colour in the case of changing from the oxidised to the reduced form.
10. Use of an aromatic nitroso compound as direct colour-forming electron acceptor of an analyte/enzyme system, whereby the enzyme represents a flavine-dependent oxidase or a PQQ-dependent dehydrogenase.

