

[54] **ENZYMATIC ETHANOL ASSAY**
[75] Inventor: **Giovanni Bucolo**, Irvington, N.Y.
[73] Assignee: **Calbiochem**, La Jolla, Calif.
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3,493,467 2/1970 Drell et al. 195/103.5 R
3,764,478 10/1973 Bergmeyer et al. 195/103.5 R

Primary Examiner—A. Louis Monacell
Assistant Examiner—C. A. Fan
Attorney, Agent, or Firm—Delmar H. Larsen

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[51] **Int. Cl.²**..... **C12K 1/04**
[58] **Field of Search** 195/103.5 R; 23/230 B

[56] **References Cited**

UNITED STATES PATENTS

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[57] **ABSTRACT**

This invention provides an improved enzymatic assay for ethanol in body fluids, whereby a single substance acts simultaneously as a buffer and as a trapping agent for one of the products of the enzymatic reaction.

7 Claims, No Drawings

ENZYMATIC ETHANOL ASSAY

This invention relates to an improvement in the enzymatic assay of ethanol, particularly in body fluids such as saliva, serum and urine, wherein a single chemical compound serves the dual function of a buffer and a trapping agent by which one of the products of the reaction involved is removed.

A useful method of determining the ethanol content of fluids, and particularly of body fluids when used as a test of recent alcohol ingestion, utilizes the enzymatic reaction whereby ethanol is transformed to acetaldehyde by the action of alcohol dehydrogenase in which nicotinamide adenine dinucleotide (NAD) serves as a coenzyme and in turn is reduced to its reduced form (NADH). The course and extent of the reaction are determined by ultraviolet colorimetry at 340 nanometers, at which wave length NAD does not absorb the ultraviolet light but NADH does.

In order for the reaction to be utilized in a practical assay it is necessary for the reaction to go to completion, and this, of course, is not insured by the presence of the enzyme and coenzyme alone. Rather, it is necessary to sequester or to trap in some fashion one of the reaction products, of which the most practical one is the acetaldehyde. Also, as indeed in most enzymatic reactions, the presence of a buffer is necessary to insure that a pH is maintained within the optimum range for that particular enzymatic reaction concerned.

A useful trapping agent has been found to be aminooxyacetic acid, described in connection with an ethanol assay in U.S. Pat. No. 3,493,467, of William Drell et al. and in *Clinical Chemistry* 16 402-407 (1970), in an article by Jones, Gerber and Drell.

Subsequent to the appearance of the Drell et al. publications just noted, it was found that aminooxyacetic acid apparently underwent a reaction with the NAD, leading to a blank absorbance which was subject to random variations. This difficulty was the subject of correspondence published in *Clinical Chemistry* 17, [5] 458-460 (1971). I have now discovered that two closely related compounds, as well as mixtures of the two in any proportion, not only furnish the desired and requisite buffer capacity for an assay mixture based upon the enzymatic reaction set forth hereinabove, but enable the complete elimination of the aminooxyacetic acid, these compounds or mixtures of the two quite surprisingly serving as a trapping agent for the acetaldehyde generated by the reaction. Elimination of the aminooxyacetic acid leads to a stable assay mixture in which the blank absorbance is substantially constant and predictable and also enables a considerable simplification to be made in formulating the assay mixture since a single substance now serves two separate functions.

The two compounds are 2-amino-2-hydroxymethyl-1,3-propanediol; and 2-amino-2-methyl-1,3-propanediol. The first of these is commonly known as "Trisamino"; and may also be named as tris(hydroxymethyl)aminomethane. It is entered on page 1083 of the Merck Index, 8th edition 1968, under the "generic" name of tromethamine. The second compound recited appears on page 57 of the Merck Index under the Geneva nomenclature given above. Both of these compounds, which as will be observed, differ only by one hydroxyl radical, give a pH somewhat in excess of 10 in diluted aqueous solution. When partially neutral-

ized with acid, such as hydrochloric, to a pH within the general range of 8.9 to 9.2, then they behave as buffers with a good buffer capacity, and likewise exhibit the trapping effect already mentioned.

Any of the usual methods common in this art for putting together the individual components so as to form the inventive combination may be used. For example, all of the components may be put into a single solution, which may conveniently be water, and this may then be freeze dried followed by grinding or sieving so as to produce a dry powdered composition. Alternately, the components may be intimately mixed together in their dry state, for which ball milling is convenient. This brings about a homogeneous composition in the form of a fine powder. Still further, enhanced stability may be given to the combination by supplying it in two moieties. This may conveniently comprise the NAD as one moiety and the balance of the components as the second. In order to carry out the assay, preselected quantities of each are dissolved in water and mixed together. The fluid to be assayed for its ethanol content is added in a preselected volume, and the absorbance of the solution before and after incubation under selected conditions, typically for 8 to 10 minutes at 30° C. is determined. A blank is carried through the procedure in the usual fashion so that appropriate correction may be automatically made. The details of the actual absorbance procedure follow the customary sequence, and need not be set forth in detail here. A typical and recommendable protocol may be found in the Jones et al. 1970 article and in the Drell et al. patent cited here and above, both of which are included herein by reference. Of course, when the components are not provided as two moieties, but rather as a single mixture, then a preselected quantity of the single mixture is dissolved in water and the assay carried out as already described.

Some working examples will now be given.

EXAMPLE 1.

A dry blend is made of the following substances in the quantities named:

2-amino-2-hydroxymethyl-1,3-propanediol	842 mg.*
Succinic acid	104 mg.
Ethylene diamine tetra-acetic acid, tetrasodium salt	26 mg.
Alcohol dehydrogenase	216 I.U.**
NAD	17.2 mg.

*Milligrams

**International Units

The foregoing ingredients are first separately dried by desiccation at room temperature, conveniently over phosphorus pentoxide, and then dry blended to form the inventive composition. For use, 13.5 milliliters (ml) of water are added and the composition is permitted to dissolve. For carrying out the assay, a suitable quantity of body fluid such as 0.1 ml. of serum, saliva, or the like is diluted with 4.9 ml. of water or if preferred of isotonic saline (0.9% aqueous sodium chloride). This is mixed with the assay solution in the proportion of 0.1 ml. of the diluted serum or the like as already described and 2.6 ml of the assay solution. A blank is conveniently carried out in parallel fashion in which 0.1 ml of the saline solution alone is added to 2.6 ml of the assay solution. These operations are conveniently carried out by adding the solutions to a cuvet adapted to photomet-

ric measurements. The two cuvetts are held at 30° C for 8 to 10 minutes, whereupon the ultraviolet absorbance of both is determined at 340 nm. The amount of alcohol present in the body fluid may be readily calculated by anyone skilled in the art and indeed the particular apparatus used may conveniently be calibrated by carrying out a series of tests using diluted alcohol of varying concentrations.

EXAMPLE 2.

The composition of Example 1 and the procedures set forth therein are carried out as stated, except that in place of 842 mg of 2-amino-2-hydroxymethyl-1,3-propanediol there are used 733 mg of 2-amino-2-methyl-1,3-propanediol.

EXAMPLE 3.

The composition and procedures of Example 1 are carried out as stated except that in place of 842 mg of 2-amino-2-hydroxymethyl-1,3-propanediol there are used 421 mg of 2-amino-2-hydroxymethyl-1,3-propanediol together with 369 mg of 2-amino-2-methyl-1,3-propanediol.

EXAMPLE 4.

The composition and procedures of Example 1 are carried out as stated with the exception that the ethylene diamine tetra-acetic acid, tetrasodium salt, is omitted.

EXAMPLE 5.

The composition and procedures of Example 2 are carried out as stated with the exception that the ethylene diamine tetra-acetic acid, tetrasodium salt, is omitted.

EXAMPLE 6.

The composition and procedures of Example 3 are carried out as stated with the exception that the ethylene diamine tetra-acetic acid, tetrasodium salt, is omitted.

EXAMPLE 7.

The composition of Example 1 is prepared and dissolved in 13.5 ml of distilled water. The solution is then freeze-dried to yield a dry, homogeneous mixture, which is used for carrying out the assay as already described in Example 1.

EXAMPLE 8.

The procedure of Example 7 is carried out as stated therein except that the composition of Example 4 instead of the composition of Example 1 is used.

As regards relative proportion of the several components, typical quantities appear in the examples hereinabove. It is convenient to consider the quantities needed for a single assay, for which for purposes of discussion, one may assume 2.6 ml of assay solution per test, as shown in Example 1. The quantities shown in Example 1 are sufficient for five assays, as will be clear from the details given therein. Thus, I prefer the following quantities, per five-test batch of dry components:

1. buffer/trapping agent 6.99 millimoles
2. acid - sufficient to give pH in range of 8.8 - 9.2
3. alcohol dehydrogenase 200 - 220 I.U.

4. NAD 16 - 18 mg.
5. heavy metal sequestrant 10 - 50 mg.

It will be readily appreciated that when the composition is varied within the limits set forth above then the assay solution should be calibrated by the use of a known quantity of dilute alcohol.

In any case, in order to achieve efficient trapping of the acetaldehyde produced during the enzymatic reaction, the buffer/trapping agent should be present in the assay mixture solution at a concentration of at least 0.3 molar. An excess does no harm, but is of course wasteful. A practical upper limit is 3 molar, although even this may be exceeded without affecting the inventive assay.

As mentioned, both species of the buffer/trapping agent used in accordance with the invention have a natural alkalinity somewhat higher than the optimum pH for the enzymatic reaction concerned so that I include an acid in the inventive composition. Where the composition is prepared by mixing together dry ingredients then a solid acid is desirable, particularly one that does not have any water of crystallization in the molecule. Succinic acid is well adapted for this purpose and in general I find it best. Tartaric acid in any of its isomeric forms may likewise be used as well as other solid acids, such as, for example, fumaric, gluconic, citric, malic and the like. Where the assay composition is prepared by a preliminary dissolution and then freeze-dried, the acids already named may be used and non-solid acids as well, such as hydrochloric, sulphuric, phosphoric and the like. The relative proportions of acid and buffer/trapping agent are selected to give a pH in the range already stated when the solid assay mixture is dissolved in water in such proportion as to give a molarity of at least 0.3 for the buffer/trapping agent. Needless to say, the weight proportion will vary depending on the particular acid or mixture of acids selected. A typical weight ratio appears in the examples.

I prefer and find it best to select components which are entirely free of any contamination by heavy metal ions. It is sometimes difficult to find commercial sources of the buffer/trapping agents disclosed which are sufficiently pure in this regard although, of course, they may be purified by anyone wishing to practise the invention. By way of a safeguard, accordingly, I find it convenient to include a heavy metal sequestrant in the inventive composition, even when it is believed that the individual components are sufficiently pure. Indeed, in any case this guards against nickel, chromium or iron ions which might inadvertently be introduced if the user employs water which had been in contact with stainless steel. The heavy metal sequestrant which I prefer and find best is ethylene diamine tetra-acetic acid, which may be added as an acid or as any of its sodium salts. The amount used is small in any case, so that whether it is added as the acid or salt form is not critical particularly since any acid contribution therefrom will automatically be compensated for when a determination is made of the quantity of the selected acid needed to achieve a pH in the stated range. Many other heavy metal sequestrants may be employed, such as, for example, diethylene triamine penta-acetic acid; 8-hydroxyquinoline; orthophenanthroline; bipyridyl; and many others known to those skilled in the art of coordination chemistry. The sequestrant may typically

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be employed in quantities of from about 1 to 5 parts per 100 of the buffer/trapping agent.

It will be understood that while I have explained the invention with the aid of specific examples, nevertheless considerable variation is possible in choice of materials, proportions, reaction conditions, and the like, with the broad scope of the invention as set forth in the claims that follow.

Having disclosed the invention, I claim:

1. A composition of matter useful as a reagent in determining the ethanol content of body fluids consisting essentially of:

- a. the enzyme alcohol dehydrogenase;
- b. the coenzyme nicotinamide adenine dinucleotide;
- c. a buffer/acetaldehyde-trapping agent selected from the group consisting of 2-amino-2-hydroxymethyl-1,3-propanediol, 2-amino-2-methyl-1,3-propanediol and mixtures thereof;
- d. an acid in quantity sufficient to lower the pH of an aqueous solution of said mixture to within the range of about 8.8 to 9.2;

said mixture being free of any other acetaldehyde trapping agent.

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2. The composition of claim 1 wherein said composition is present in the form of two moieties, one containing said coenzyme and the other the balance of the recited components.

3. The composition of claim 1 which includes a heavy metal sequestering agent.

4. The composition of claim 3 in which said sequestering agent is ethylene diamine tetra-acetic acid, or a sodium salt thereof.

5. An assay solution useful in the determination of the ethanol content of body fluids consisting essentially of the composition of claim 1 dissolved in sufficient water to provide a concentration of said buffer/trapping agent of at least 0.3 molar.

6. A composition in accordance with claim 1 wherein, for each 6.99 millimoles of said buffer/trapping agent, 16 - 18 mg of NAD; and from 200 - 220 I.U. of alcohol dehydrogenase are present.

7. An assay solution useful in the determination of the ethanol content of body fluids consisting essentially of the composition of claim 6 dissolved in sufficient water to provide a concentration of said buffer/trapping agent of approximately at least 0.3 molar.

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