Amino Acid Analysis: Aqueous Dimethyl Sulfoxide As Solvent for the Ninhydrin Reaction

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SUMMARY

Methyl Cellosolve (the monomethyl ether of ethylene glycol) has been widely used as the organic solvent in ninhydrin reagents for amino acid analysis; it has, however, properties that are disadvantageous in a reagent for everyday employment. The solvent is toxic and it is difficult to keep the ether peroxide-free. A continuing effort to arrive at a chemically preferable and relatively nontoxic substitute for methyl Cellosolve has led to experiments with dimethyl sulfoxide, which proves to be a better solvent for the reduced form of ninhydrin (hydrindantin) than is methyl Cellosolve. Dimethyl sulfoxide can replace the latter, volume for volume, in a ninhydrin reagent mixture that gives equal performance and has improved stability. The result is a ninhydrin-hydrindantin solution in 75% dimethyl sulfoxide-25% 4 M lithium acetate buffer at pH 5.2. This type of mixture, with appropriate hydrindantin concentrations, is recommended to replace methyl Cellosolve-containing reagents in the quantitative determination of amino acids by automatic analyzers and by the manual ninhydrin method.

In the development of a ninhydrin reagent (1, 2) for use in the quantitative determination of amino acids (3, 4) it was found that the presence of reduced ninhydrin in the solution is essential if the color development is to follow Beer's law. The reduced compound (in the form of hydrindantin) is almost completely insoluble in water. The preparation of a practical reagent requires the inclusion of a water-miscible organic solvent which is capable of dissolving both hydrindantin and the blue-colored compound (diketohydrindylidene-diketohydrindamine) formed by the reaction of ninhydrin with amino acids. At the time of the initial studies (1), the most effective solvent tested was methyl Cellosolve; it suffers from the serious limitation, in common with many volatile organic solvents, that it has to be handled with care1 because of its toxicity (5-8). The maximum allowable concentration in the air is estimated at 25 ppm (6, 8); the first symptom of overexposure is a headache. Since the solvent has only a slight odor, care is required in order to avoid harmful concentrations.

In the intervening years, new solvents have become available which could be tested as substitutes for methyl Cellosolve. A continued search has resulted in the finding that dimethyl sulfoxide has 1.5 times the solvent action of methyl Cellosolve for hydrindantin. In addition, dimethyl sulfoxide has a high boiling point (189°) and, among laboratory reagents, is one of the least toxic (9, 10), being more tolerable by ingestion or injection than glycerol or ethanol.

The first use of dimethyl sulfoxide in a ninhydrin reagent was by Kirschenbaum (11) who used a 60% methyl Cellosolve-40% dimethyl sulfoxide mixture; the solvent action of the combination served to minimize the tendency for precipitates to form in the capillary lines of an amino acid analyzer. The present experiments were undertaken to arrive at a formulation which required no methyl Cellosolve.

After experimentation with sodium and potassium acetate buffers, the use of lithium acetate was found to be necessary to obtain a fully soluble dimethyl sulfoxide-buffer mixture and to eliminate the possibility of precipitation of salts when the ninhydrin reagent mixes with the sodium citrate-containing effluent from an ion exchange column (4). In addition, the new reagent is more stable; this advantage results primarily from the absence of peroxide formation, which is a problem with methyl Cellosolve; with the dimethyl sulfoxide solution hydrindantin (2) rather than stannous chloride (1, 4) can be used as the reducing agent. This change avoids the formation of precipitates that may arise from the presence of tin salts in the Ninhydrin solution.

EXPERIMENTAL PROCEDURE

Materials—Industrial grade dimethyl sulfoxide, manufactured by Crown Zellerbach (Chemical Products Division, Camas, Washington 98607), was purchased from the regional distributor in 5-gallon containers with polyethylene closures,2 as packaged by the manufacturer. When the solvent has been repackaged in 1-gallon bottles, we found occasionally that plastic cap liners soluble in dimethyl sulfoxide had been inserted. If there is evidence of this, the solvent should not be used; the dissolved plastic may precipitate

the manufacturer. If any suspended material is present, the sulfoxide is drawn by suction into a glass filter flask through a sintered glass filter tube (Corning 39580-60C) with polyethylene tubing connections.

Ninhydrin and hydrindantin (anhydrous) were from Pierce Chemical Company (Rockford, Illinois) and lithium hydroxide-H$_2$O (reagent grade) was from Matheson Coleman and Bell.

**Lithium Acetate Buffer, /, pH 5.2**—The constituents for 10 liters of buffer are 1680 g of LiOH·H$_2$O and 2930 ml of glacial acetic acid (reagent grade). Weigh the LiOH in a hood (to avoid breathing the dust) and add it in the hood to about 4 liters of water in a Pyrex jar or bottle marked for 10 liters. Stir with a polyethylene paddle (S-7174, Scientific Glass Apparatus Company, Bloomfield, New Jersey). When the hydroxide is about half-dissolved, add the acetic acid. The solution gets warm enough to dissolve the hydroxide rapidly but not hot enough to boil; the vigorous bubbling is caused by carbonate which is present in the base. Make the solution nearly to volume and measure the pH at a 1:3 dilution. If the pH is not pH 5.20 ± 0.05, adjust the solution with acetic acid or LiOH; the addition of about 10 g of LiOH·H$_2$O or 10 ml of acetic acid per 10 liters is required per 0.01 pH unit. Cover the vessel (Saran wrap) and allow the solution to come to room temperature overnight. Make to final volume and filter with slight suction through a sintered glass filter tube (see above), Tygon lines can be used on the filter tube. Store at room temperature. The scale of preparation can be increased to 20 liters without difficulty.

**Reagent Solution for Amino Acid Analyzer**—For use with analyzers operated under the general conditions described by Spackman, Stein, and Moore (4), the solution is prepared under nitrogen by the same type of procedure given in that communication. Three liters of dimethyl sulfoxide (pour in hood) and 1 liter of the 4 M lithium acetate buffer are added to the 4-liter filling bottle. The magnetic stirrer (e.g. Thermolyne SL-7225, with a smooth wall 3-inch bar, Bel-Art F 3711) should be capable of giving vigorous stirring. The effluent nitrogen is bubbled through water in a 2-liter flask to trap traces of organic solvent. After nitrogen has been bubbled through the dimethyl sulfoxide-buffer mixture for 15 min, 80 g of ninhydrin are added and will dissolve readily if the crystals are dropped into a deep vortex produced by the stirrer operated at as high a speed as possible. If the stirring is too slow during the subsequent addition of hydrindantin (2.50 g/4 liters), the crystals may clump and the solution process will be slow. Since the mixture is very stable under nitrogen, no difficulty is encountered if it is necessary to stir the mixture for 20 to 60 min to obtain complete solution of the hydrindantin. The final solution is driven by nitrogen into the reservoir bottle of the analyzer (4).

When the new reagent is first used in an analyzer which may have some precipitate in the lines from the use of a stannous chloride-methyl Cellosolve reagent, the solvent power of dimethyl sulfoxide may loosen some solids from the walls of the tubing. The pumping of undiluted sulfoxide reagent through the reaction coil for 10 min is a useful initial cleaning step. If the operating pressure of the system rises, the lines should be checked for blockage, particularly at connection points, and the capillaries cleared with 26 gauge copper wire. After the lines are clear, we have not had the slightest evidence of precipitate formation with the dimethyl sulfoxide-containing reagent.

The undiluted reagent will bleach from a reddish color to light yellow in the presence of oxygen; this color change provides a test of the impermeability of the plastic tubing from the ninhydrin reservoir bottle to the ninhydrin pump and from the pump to the valve manifold. If that tubing is Teflon, which is permeable to oxygen, the reagent can be seen to bleach as it stands in the lines. We have replaced all the ninhydrin lines, up to the valve manifold, with 1-inch outer diameter Saran tubing (Pyramid Plastics, Palatine, Illinois 60067). The filling line to the ninhydrin reservoir bottle is 3/16-inch Saran; any Teflon or Teflon tubing in the nitrogen-filled protecting system should be similarly replaced.

The reagent can be kept in the dark at room temperature (25°C) for about 2 weeks without developing sufficient background color to affect significantly the adjustment of the base-line of the 440 nm channel of the analyzer (4). An advantage of the dimethyl sulfoxide mixture is that, if desired, the reagent bottle can be stored in a refrigerator at 7-10°C without any risk of precipitation. When kept in the cold, the reagent is usable even after 1 or 2 months of storage. A 1- or 2-cu ft refrigerator can be placed beside the analyzer and the Saran leads extended to the pump and the nitrogen reservoir.

The dimethyl sulfoxide may cause swelling of a polystyrene bead filter on the ninhydrin pump line and result in undesirable back pressure. A glass wool filter in this position of the system is preferable.

The only disadvantage that we have observed with this reagent is that dimethyl sulfoxide does yield a noticeable odor if the solvent is allowed to accumulate in drains that are infrequently used. An analyzer can be operated with the reagent without any detectable odor if the effluent is collected in a covered bucket and the contents periodically emptied into a sink which is subsequently well rinsed with water.

Although dimethyl sulfoxide is reported to be capable of carrying some organic chemicals through rubber gloves (12), rubber, vinyl, or polyethylene gloves protect the user fully from the blue stains from ninhydrin in the normal handling of this reagent. The use of gloves is recommended; if hands are stained by direct contact with the solution, we find that the blue coloration caused by this reagent is more readily scrubbed off than is the case with methyl Cellosolve-containing reagent solutions.

**Reagent for Manual Ninhydrin Method**—A similar formulation, with 3 g of hydrindantin per liter, can be used for the manual ninhydrin reagent (2). In this case, the ninhydrin and the hydrindantin are dissolved in the pure dimethyl sulfoxide first, and the buffer subsequently added. The reagent, stored under nitrogen in a refrigerator at 7-10°C, is stable for months. This mixture has replaced the earlier case for all of the manual ninhydrin analyses in our laboratory. When alkaline hydrolysis is employed prior to ninhydrin analysis, the 30% acetic acid recommended by Fruchter and Crestfield (13) for neutralizing the alkali should be replaced by 15% acetic acid for optimum color yields with the dimethyl sulfoxide-lithium acetate reagent solution.

**Results**

**Color Yields**—The yields of color in the ninhydrin reaction with amino acids are almost identical with the dimethyl sulfoxide and methyl Cellosolve reagent solutions. The improved protection against oxidation afforded by the substitution of Saran for Teflon...
lines in an analyzer usually results in a 2 to 3% increase in color yields.8

Stability—Part of the instability of ninhydrin-hydrindantin solutions in methyl Cellosolve is undoubtedly a result of the tendency of the solvent, as an ether, to form peroxides. It was essential to obtain supplies of methyl Cellosolve which gave a negative peroxide test (less than 3 ppm). When dimethyl sulfoxide is used as the solvent, there is not this initial problem and the principal difference to be noted in the results is the much slower rate of oxidation (as evidenced by bleaching of the color) of hydrindantin or diketohydrindylidene-diketohydrindamine when solutions are allowed to stand exposed to air and light. For example, if tubes containing the blue color (absorbance about 0.40 at 570 nm) obtained in the usual manual ninhydrin analysis with the methyl Cellosolve system are allowed to stand overnight, they retain about 50% of their absorbance. Under the same conditions, samples obtained with the dimethyl sulfoxide-containing mixture retain 98% of their initial absorbance after 16 hours.

On the automatic analyzer, we have obtained less than a 1% decrease in color yields after storage of the reagent for 1 month. If there is a drop in color yield, and flushing of the nitrogen reservoir system with prepurified nitrogen does not prevent the loss on the next filling, the system should be examined for leaks which may be allowing access of air to the stored solution.

DISCUSSION

The earlier papers on the uses of this type of ninhydrin reagent (1–4, 13) define the sensitivity of the method and the sizes and pH

3 Standard solutions of amino acids which have been stored for several months may be low in glutamic acid because of the formation of pyrrolidone carboxylic acid. If the color yields for glutamic acid and alanine are not nearly the same, a fresh standard of glutamic acid should be tested or the alanine color yield used as a practical approximation.

value of aqueous samples that can be handled by the buffered ninhydrin-hydrindantin solutions; the same specifications all apply to the dimethyl sulfoxide-containing reagent. The present reagent has been tested on a number of amino acid analyzers for 2 years with fully satisfactory results. It should be possible to substitute dimethyl sulfoxide for methyl Cellosolve in most quantitative ninhydrin methods, including those that use cyanide as a catalyst for hydrindantin formation (Rosen, Berard, and Levenson (14)). The change to a lithium buffer in the reagent may be a necessary part of such adaptations.

REFERENCES

Amino Acid Analysis: Aqueous Dimethyl Sulfoxide As Solvent for the Ninhydrin Reaction
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