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Note

Amino acid analysis: a replacement solvent for flushing ninhydrin reagent

L. B. JAMES
John Curtin School of Medical Research, G.P.O. Box 339, Canberra, ACT 2601 (Australia)
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At the conclusion of an amino acid analysis it is essential to remove all trace of ninhydrin reagent from some of the flow lines of the instrument. If left to stand in the lines this reagent will form a precipitate that could be extremely difficult to remove and most certainly will cause otherwise unnecessary component replacement. The reagent is usually removed by prolonged pumping of water during the course of the analyzer shut-down procedure; which in current models of analyzers is an automatic programmed operation. This communication questions the suitability/wisdom of using water as a flushing solvent during the analyzer shut-down cycle, and demonstrates the disadvantages arising from its use in the Beckman Model-System 6300 amino acid analyser.

EXPERIMENTAL

All buffers and ninhydrin reagent were supplied by Beckman, and chromatograms along with results (peak integration and conversion to nanomoles of amino acid) were obtained with a Hewlett Packard 3390A reporting integrator. The suggested replacement solvent contained 600 ml of ChromAR™ dimethyl sulphoxide (supplied by Mallinckrodt, South Oakleigh, Australia), 300 ml of water from a Milli-Q Reagent Water System (Millipore, Bedford, MA, U.S.A.) and 1 ml of thiodiglycol concentrate (Pierce, Rockford, IL, U.S.A.). All analytical determinations were made with an amino acid calibration mixture supplied by Beckman (Palo Alto, CA, U.S.A.) (Cat No. 338088). The samples were automatically loaded into the ion-exchange column by the turntable loop-loader. The capacity of the loop was 50 μl, and 5 nmol of amino acids were contained in that volume. The composition of ninhydrin reagent usually consists of: (1) solid ninhydrin, (2) a solvent for the ninhydrin, *e.g.* methyl cellosolve or dimethyl sulphoxide, (3) an acetate buffer with either lithium, sodium or potassium as the cation, and (4) a reductant, *e.g.* stannous chloride, titanous chloride or the reduced form of ninhydrin, hydrindantin, may be added directly as a solid. For the ninhydrin reagent to react effectively with amino acids the presence of the reduced form hydrindantin is essential[1].

RESULTS AND DISCUSSION

Because of the toxicity of the solvent methyl cellosolve[2], daily exposure to its

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vapour or skin contact is undesirable,[3] and hence its use in ninhydrin reagent preparations is becoming restricted, and dimethyl sulphoxide becoming the predominantly used solvent. The practice of adding reducing agents to effect partial ninhydrin reduction[1,4,5] is also being replaced by the direct addition of hydrindantin to the mixture. And it is this hydrindantin, which is practically insoluble in water, that gives rise to the problem encountered when the ninhydrin pump and its flow-line is flushed out with water. Filling this microbore tubing with water prevents the analyzer from being immediately ready for use from the shut-down mode. Fig. 1 shows a section of the schematic flow lines of the Beckman 6300 analyzer. Reservoirs 1–5 contain buffers used for eluting the amino acids from the ion-exchange column, reservoir 6 contains alkali reagent (for column regeneration), reservoir 8 contains ninhydrin reagent and reservoir 7 is filled with water. The use of water in reservoir 7 is recommended in the operator's handbook for the analyzer. The flow line from Reservoir 7 continues via a Bubble trap to the reagent selector valve (A), from this valve to the

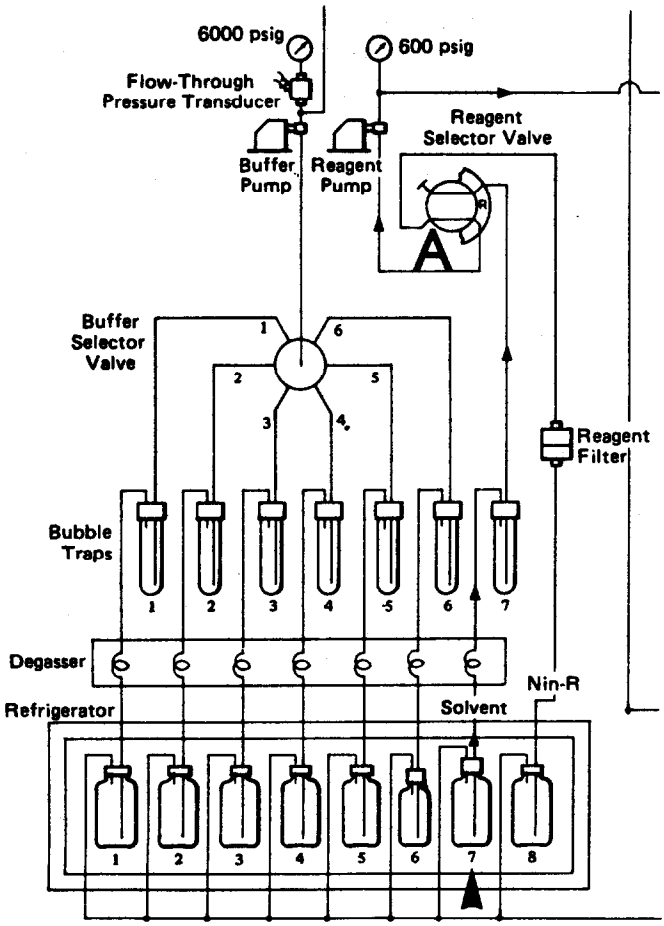

Fig. 1. Schematic diagram of the liquid flow lines of the amino acid analyzer.

pump and on to the mixing chamber (where ion-exchange column eluent and ninhydrin come into contact) and finally to the reactor cartridge, colorimeter bubble injector valve, flow-meter, drop detector and waste collection vessel, in that order respectively.

It is time consuming and wasteful to have to pump ninhydrin and buffer for asppproximately 90 min, so as to purge the flow line; even a trace of water appears to affect the potency of the ninhydrin reagent. Colour development is impaired possibly through the presence of residual oxygen from the replaced water converting the hydrindantin to ninhydrin and thus establishing an imbalance of these two substances in the ninhydrin reagent. The necessity of both forms being present has been mentioned before[1]. Preliminary line purging takes the form of "dummy" analytical determinations, whereby sample dilution buffer is subjected to analysis. This results in no peaks on the chromatogram other than that showing airborne ammonia contamination of the buffer. Further, the chromatogram baseline is not steady, as seen in Fig. 2A. The analysis is complete after 45 min, and the second commenced. The chromatogram shown in Fig. 2B is typical of a "start-of-the-day" second analysis,

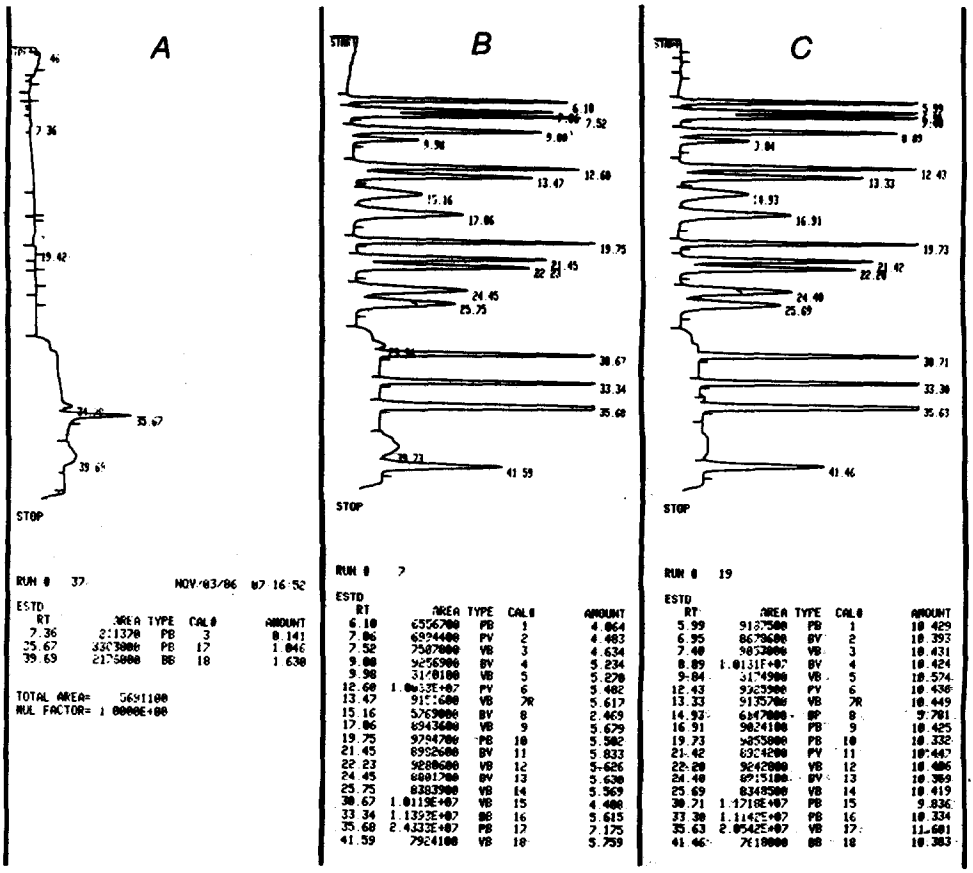

Fig. 2. Amino acid analysis chromatograms showing the "artifact" and its subsequent elimination.

and demonstrates that the ninhydrin reagent has not yet reached "full potency" because the peaks which represent aspartic acid, threonine and serine; with retention times of 6.10, 7.06 and 7.52 min respectively, at the start of the chromatogram, can be seen to be gradually increasing in magnitude towards the optimum, 5 nmol amount, being recorded as 4.064, 4.483 and 4.634 nmol respectively. The recording of the peak representing glutamic acid at 9.00 min in the second analysis shows that the ninhydrin reagent is now obtaining maximum colour development in the reactor cartridge. Thus, it will be that with the third "analysis" of the day that samples containing unknown amounts of the individual amino acids can be attempted with certainty.

In Fig. 2C is shown a chromatogram obtained after the water in reservoir 7 had been replaced with the new solvent mixture described above. The analyzer was placed directly into the analysis mode from the overnight shut-down mode and both ninhydrin reagent and buffer pumps were started whilst the samples were being loaded into the turntable (multiple sample loader) sample coils. Sample loading can be complete in a time interval of 9 min and the analysis commenced immediately. As can be seen there is no delay in the response of the ninhydrin reagent; optimum colour development is achieved instantaneously after the ninhydrin reagent displaces the new solvent mixture in the flow line. The aspartic acid peak (5.99 min) is registering the expected value of 10 nmol. The "10 nmol" amount recovery in this determination was obtained by switching the recorder to a higher sensitivity range. It also demonstrated the ability of the Beckman System 6300 to maintain a high standard of excellence in recording the outcome of the ninhydrin–amino acid reaction even though range changes are being introduced. Attention is drawn to the diminution of an artifact, that appears on chromatograms A and B in Fig. 2 with a retention time of approximately 39–40 min [between the ammonia (35.68 min) and arginine peaks (41.59 min)] after water is replaced with the new flushing solvent. In chromatogram C, this sample contaminant has almost disappeared.

Finally, the new flushing solvent makes use of the reduction properties of thiodiglycol, a substance that is used as a buffer additive to prevent oxidation of methionine during analysis; possibly its presence in the new solvent mixture exhibits this capacity to establish a reducing environment through which hydrindantin passes without change and in solution. The desirability of having a reducing potential in the new solvent can be readily appreciated when it is remembered that reagents used in analysis come into contact with metal pump parts and fittings; stainless steel. The use of dimethyl sulphoxide in the new solvent also presents no problems as regard the escape of its vapour into the laboratory atmosphere; the Beckman analyzer has a waste collection vessel with a charcoal vent filter installed. However for analyzer shutdown time intervals longer than 72 h, water should be placed in reservoir 7, and for extended shutdown, it is recommended that the ninhydrin reagent (reservoir 8) should also be filled with water.

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