# A NON-VOLATILE BUFFER WITH IMPROVED PERFORMANCE IN AUTOMATED PROTEIN SEQUENCING

G. S. BEGG and F. J. MORGAN
*St. Vincent's School of Medical Research, Melbourne, Victoria 3065, Australia*

Received 5 April 1976

## 1. Introduction

The design of the protein sequenator allows the reactions of the phenylisothiocyanate degradation to be performed with high efficiencies, making it possible to determine extended amino acid sequences in proteins. A repetitive yield of 98% was originally reported permitting the assignment in a single degradation of the $NH_2$-terminal sixty residues of humpback whale apomyoglobin [1]. Such high yields required meticulous attention to the purity of reagents and solvents, especially with respect to aldehyde contamination. However *N,N,N',N'*-tetrakis(2-hydroxypropyl)ethylenediamine (Quadrol) could not be obtained completely free from aldehydes although its properties made it suitable for use in the sequenator. In practice most published automated sequence determinations using chromatographic methods to quantitate amino acid derivatives have reported repetitive yields of 92–96%, e.g. [2,3]. However, since a high repetitive yield is a critical factor in permitting long amino acid sequence determinations it is extremely important to be able readily to maximize repetitive yields. We wish to report a simple modification of the original reaction conditions whereby repetitive yields of 98% or more, as estimated from gas chromatographic quantitation, are routinely obtained by the substitution of *N,N,N',N'*-tetrakis(2-hydroxyethyl)-ethylenediamine (THEED), which is readily obtained free from aldehydes, for Quadrol in the coupling buffer.

Presented in part at the Australian Biochemical Society Protein Structure and Function Summer Workshop, March 1-3, 1976.

Abbreviations: Quadrol (Registered trade mark, Wyandotte Chemicals Corporation), *N,N,N',N'*-tetrakis(2-hydroxypropyl) ethylenediamine; THEED, *N,N,N',N'*-tetrakis(2-hydroxyethyl) ethylenediamine; PTH, 3-phenyl-2-thiohydantoin.

## 2. Materials and methods

*N,N,N',N'*-tetrakis(2-hydroxyethyl)ethylenediamine (THEED) was synthesised from ethylene oxide and ethylene diamine [4]. THEED (Lot 73864) was also obtained commercially from ICN-K&K Laboratories, Inc. (Plainview, N.Y.), and used without further purification. THEED from both sources was free from aldehyde as determined by the Tollens' reaction [1]. All other reagents and solvents were purified according to published methods [1,5]. Some degradations were performed with Quadrol buffer (Batch A1119) from Beckman Instruments, Palo Alto, Calif. Sperm-whale myoglobin (Pierce Chemicals, Rockford, I11.) was repurified as described for humpback whale myoglobin [1] and converted to apomyoglobin.

Automated sequence determinations were performed as previously described [1] with the following modifications:

Reagent 2 (coupling buffer): 1 M THEED in 1-propanol–water, 3:4 (v/v), pH 9.0, with trifluoroacetic acid (glass electrode, 20°C).

Solvent 2: Ethyl acetate, containing 0.1% acetic acid (v/v) and 15% 1-propanol (v/v).

Quantitation of PTH-amino acids was performed by gas-liquid chromatography, essentially according to the method of Pisano and Bronzert [6], using a

Packard Becker Gas Chromatograph Model 421, fitted with a computing integrator (Autolab Minigrator, Spectra Physics, Santa Clara, Calif.). Calculations were made on the average of five injections at each step. Gas chromatographic quantitation provides a measurement of individual PTH-amino acids released at each step. Measurement by ultraviolet absorption of the PTH-amino acid derived from automated degradations will include contributions from overlap and non-specific background. This leads to an overestimation of the absolute yield of PTH-amino acids at any step as degradation proceeds and in our hands results in a calculation of repetitive yields several percent higher than those calculated from gas chromatography.

### 3. Results and discussion

Automated degradation of sperm-whale apomyoglobin (250 nmol) using the Quadrol buffer system gave average repetitive yields of up to 94% as measured by gas chromatography. Repetitive yields were calculated from the yields of PTH-valine, from step 1 to step 10. Fig.1A illustrates a degradation using Quadrol buffer (Batch A1119, Beckman Instruments) giving a repetitive yield of 94.0%. This result is in the range of efficiencies reported from other laboratories. When the modifications to the reagents described above were introduced, a significant improvement in yield was observed. The repetitive yield in fig.1B was calculated to be 99.6%. In this example, the only change in the reaction conditions was the substitution of THEED buffer for the Quadrol buffer together with the addition of 15% 1-propanol to the ethyl acetate (Solvent 2). The improvement in absolute yields at steps 10 and 21, resulting from improvement in repetitive yields, is obvious when compared to fig.1A. We have consistently observed similar improved yields with THEED buffer. The repetitive yield averaged over a period of five successive degradations of sperm-whale apomyoglobin using the THEED buffer system was 98%. There are inherent difficulties in accurately calculating the efficiency of sequenator operations, especially when small variations are encountered, although there is a published report of statistical treatment of sequenator yields [2]. However, the improvement in absolute yields in this case clearly demonstrates a real improvement in repetitive yield.

THEED is similar to Quadrol in many respects being a liquid buffering in the same regions but is less volatile and less viscous. Its synthesis is achieved at lower temperatures than that of Quadrol, which is possibly a major reason for the absence of aldehydes in THEED preparations. Molecular distillation of commercial THEED (145°C at $10^{-3}$ mm Hg) under conditions similar to those described for Quadrol (120°C at $10^{-3}$ mm Hg) [1] results in conversion of the originally Tollens negative THEED to Tollens positive material.

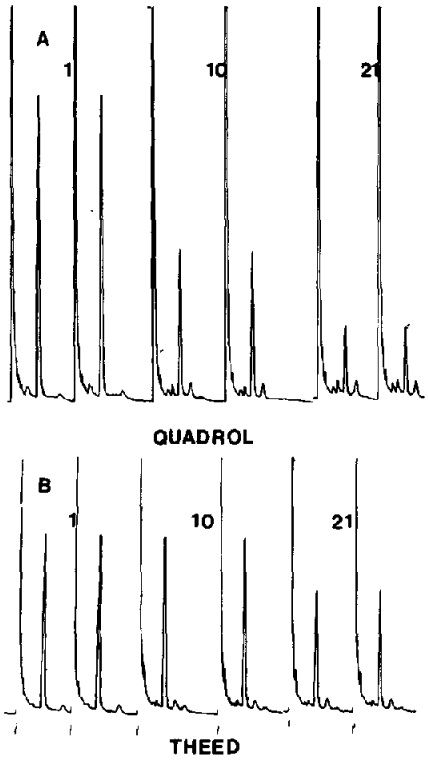


Fig.1. Gas chromatograms of PTH-amino acids derived from steps 1, 10 and 21 of automated Edman Degradations of sperm-whale apomyoglobin (approx. 250 nmol). Duplicate injections (4% of the sample) are shown at each step. Column: 4 ft × 2 mm i.d. 10% SP-400 on Chromosorb W at 210°C. Flame ionization detector. (A) Edman degradation with Quadrol buffer (Lot A1119, Beckman Instruments, Palo Alto). $16 \times 10^{-10}$ A, full scale. (B) Edman degradation with THEED buffer. $32 \times 10^{-10}$ A, full scale. Reagent batches were unchanged between the two experiments, except for the substitution of THEED for Quadrol, and the addition of 1-propanol to Solvent 2 to increase the solubility of THEED.

THEED behaves in a similar fashion to Quadrol in the protein sequenator and no special precautions need to be taken; however, being less viscous, THEED buffer flows more readily through the delivery lines. During the coupling reaction proteins appear to be less soluble in THEED than in Quadrol buffer, but this does not appear to affect the reaction adversely. THEED, unlike Quadrol, is only sparingly soluble in ethyl acetate; 1-propanol is added to the ethyl acetate to obtain adequate extraction of THEED by Solvent 2.

The use of THEED is very suitable for methods where identification of PTH-amino acids is made by gas chromatography [6] or by acid hydrolysis to the free amino acid [2,7]. However, commercially available THEED appears to contain contaminants which, although they do not affect the efficiency of the reaction, result in products visible by ultraviolet quenching of fluorescence in thin layer chromatographic systems used for PTH-amino acid identification. These contaminants chromatograph close to the origin in solvent system H and in the regions of leucine and proline in solvent system D [5]. They may therefore confuse the identification of amino acids in cases where thin layer chromatography alone is used as the method of identification.

Several explanations have been advanced for the side reaction responsible for the loss in $NH_2$-terminal yield at each cycle, including oxidative desulphuration [8] and elimination of aniline from the phenylthiocarbamyl protein [9]. However the increase in yield accompanying the use of aldehyde-free THEED seems to support the idea that blocking of the $NH_2$-terminus by aldehyde impurities may be the major source of the drop in $NH_2$-terminal yields [1]. High repetitive yields will allow the possibility of extended degradations in suitable cases, although other factors may operate to reduce the theoretical extent of the degradation in practice. However in cases where limited amounts of material are available such as biosynthetically-labelled prohormones [10], or trace proteins [11,12], the high yields obtainable with THEED together with the use of the recently introduced micro methods for automated sequence determination [13] should enable an improvement to be made in the amount of information obtainable from valueable biological materials.

**Acknowledgement**

Work in this laboratory is supported in part by grants from the National Health and Medical Research Council of Australia.

**References**

[1] Edman, P. and Begg, G. (1967) Eur. J. Biochem. 1, 80–91.
[2] Smithies, O., Gibson, D., Fanning, E. M., Goodfliesch, R. M., Gilman, J. G. and Ballantyne, D. L. (1971) Biochemistry 10, 4912–4921.
[3] Hermodson, M. A., Ericsson, L. H., Titani, K. and Neurath, H. (1972) Biochemistry 11, 4493–4502.
[4] Sherlin, S. M., Berlin, A. Y. and Rabinovich, F. E. (1941) J. Gen. Chem. (USSR) 11, 305–308.
[5] Edman, P. and Henschen, A. (1975) in: Protein Sequence Determination (Needleman, S. B., ed.), 2nd Edn., Springer-Verlag, Berlin, New York.
[6] Pisano, J. J. and Bronzert, T. J. (1969) J. Biol. Chem. 244, 5597–5607.
[7] Inglis, A. S., Nicholls, P. W. and Roxburgh, C. M. (1971) Aust. J. Biol. Sci. 24, 1247–1250.
[8] Ilse, D. and Edman, P. (1963) Aust. J. Chem. 16, 411–416.
[9] Laursen, R. A. (1975) in: Solid Phase Methods in Protein Sequence Analysis (Laursen, R. A., ed.), pp. 3–9, Pierce Chemical Co., Rockford, I11.
[10] Jacobs, J. W., Kemper, B., Niall, H. D., Habener, J. F. and Potts, J. T. (1974) Nature 249, 155–157.
[11] Schechter, I., McKean, D. J., Guyer, R. and Terry, W. (1975) Science 188, 160–162.
[12] Silver, J. and Hood, L. (1975) Nature 256, 63–64.
[13] Jacobs, J. W. and Niall, H. D. (1975) J. Biol. Chem. 250, 3629–3636.