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Loss of C-terminal amino acids by hydrazidation during hydrazinolysis

Since the introduction of the method in 1952[1], hydrazinolysis has been very useful for the determination of the C-terminal groups of proteins and peptides. It has, however, always required correction factors, some of them very large, owing to unexplained low recoveries of many of the freed amino acids (see *e.g.* ref. 2). The origin of some of these losses has been examined here in connection with the study of the C-terminal groups of the calf thymus histones[3].

Experimental and results

The C-terminal amino acids of these proteins are alanine, glycine and lysine, and one estimate of the correction factors for these amino acids in the hydrazinolytic procedure has been made by heating the free amino acids in hydrazine at 100°. Samples were run directly in the amino acid analyser. Other samples were completely hydrolysed in 6 *N* HCl and were found to give the full yield of the parent amino acids taken. Further hydrazinolysed samples were chromatographed on Whatman No. 1 paper in duplicate in two solvents. The spots were detected by the ninhydrin reagent of LEWIS[4], which gives useful colour differences with different amino acids and derivatives, and the duplicate chromatograms were dipped in the reagent of ANDREAE[5], which gives a brilliant blue reaction with hydrazides. The ferric chloride stock solution for this was made up in 0.1 *N* acetic acid to avoid precipitation of hydroxide. The results are collected in Table I.

TABLE I

FORMATION OF AMINO ACID HYDRAZIDES FROM FREE AMINO ACIDS DURING HYDRAZINOLYSIS

In all cases the slower ninhydrin-reacting spots correspond in position to the free amino acids. Distances are measured to the centres of the spots.

Amino acid	*Hydrazinolysis (h at 100°)*	*% hydrazidated*	*Paper chromatograms*			
			Solvent A[a] *(cm moved)*		*Solvent B*[b] *(R_F × 100)*	
			NH[c]	*Hydrazide*[d]	*NH*[c]	*Hydrazide*[d]
Alanine	15	66	15.7; 25	24	54; 61	59
Glycine	22	80	11.2; 21.7	21.7	45; 50	50
Lysine	15	41	5.1; 6.8	7.0	36	36

[a] Butan-1-ol–acetic acid–water (3:1:1, by vol.).
[b] Propan-2-ol–0.1 *N* NH_4OH (4.25:1, by vol.).
[c] Ninhydrin reagent[4].
[d] Detected by the ANDREAE reagent[5].

Discussion

It can be seen that an extra ninhydrin-reactive spot is formed in all three cases on hydrazinolysis, and these spots are also strong reducing agents from which the parent amino acids can be regenerated by acid hydrolysis. These extra substances are indistinguishable chromatographically from the hydrazides of these amino acids

prepared by short hydrazinolysis of their esters. The same products also appeared when peptides of these amino acids (Gly–Ala, Leu–Gly and Gly–Lys) were hydrazinolysed for 10 h at 100°, in addition to the expected free C-terminal amino acids.

It is concluded that the major losses of C-terminal amino acids of proteins determined by the hydrazinolytic procedure are due to the formation of the amino acid hydrazides, $NH_2 \cdot CHR \cdot CONH \cdot NH_2$, although with arginine, cysteine, cystine and glutamic acid (which forms pyrrolidone carboxylic acid hydrazide[6]) other considerations also apply. The reaction is not unexpected, since acyl hydrazides such as acetohydrazide can be readily made by heating a mixture of hydrazine and acetic acid without any need to use the ester. It should be mentioned here that a recent excellent paper[7] using catalytic hydrazinolysis at 80° has shown greatly reduced losses of C-terminal amino acids in this method.

Chester Beatty Research Institute,
Institute of Cancer Research, Royal Cancer Hospital,
Fulham Road, London, S.W. 3 (Great Britain)

D. M. P. PHILLIPS

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