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RAPID IDENTIFICATION OF AMINO ACID DERIVATIVES FROM THE SEQUENATOR

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SUMMARY

A simple procedure is described for converting thiazolinones, from the protein sequenator, to the more stable phenylthiohydantoins without using the conventional HCl conversion procedure. The thiazolinones are applied to a silica gel plate and converted to phenylthiohydantoins by heating at 140° for 5–10 min, in the presence of heptafluorobutyric acid, prior to chromatography. After chromatography, measurements of the yields of the derivatives on the plate can be made with a variable-wavelength thin-layer chromatography scanner. This is shown to be a useful adjunct for identifications.

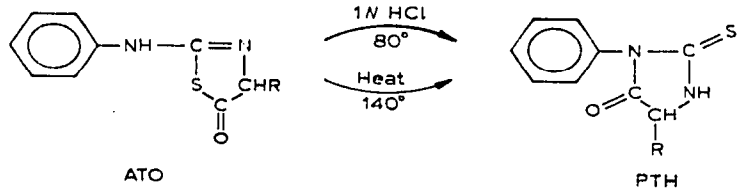
INTRODUCTION

The automation of the final step of the Edman degradation procedure was not considered to be necessary when Edman and Begg[1] designed their original sequenator. Instead, they collected the 2-anilino-5-thiazolinones (ATOs) in a refrigerated fraction collector and manually converted them to the 3-phenyl-2-thiohydantoins (PTHs). However, this conversion step is tedious and time-consuming, involving an initial drying, treatment at 80° for 10 min with 1 *N* HCl, multiple extractions with ethyl acetate and finally evaporation of the organic solvent to concentrate the PTH solution. The reaction conditions represent a compromise since some residues (*e.g.*, ATO-glycine) are slow to convert whilst others such as ATO-serine are rapidly decomposed. Furthermore, ethyl acetate extraction only partially removes the PTHs of arginine, histidine and cysteic acid from the aqueous acid layer, hence these derivatives must be identified after concentration of the aqueous phase.

Wittmann-Liebold[2] has streamlined protein sequencing by incorporating in a modified Beckman sequencer an automatic device which carries out the conversion step using 20% trifluoroacetic acid containing dithiothreitol. After conversion the volatile acid is removed and the products are extracted and transferred to the fraction collector with a mixture of 1,2-dichloroethane and methanol. This overcomes the need to search for very polar PTHs in an aqueous phase.

We describe here a much simpler procedure especially suitable for thin-layer

chromatography (TLC) of the PTHs. It is based on an early observation of Edman[3] that the conversion of ATOs to PTHs can be accomplished by heat alone, that is



ATO PTH

R = side chain of the animo acid residue

EXPERIMENTAL

All the usual amino acid residues that occur in proteins were studied by sequencing apo-myoglobin (purified according to Hapner *et al.*[4]), glucagon (Sigma, St. Louis, Mo., U.S.A.), lysozyme (Worthington, Freehold, N.J., U.S.A.) and a helical fragment from wool[5] with an Edman–Begg sequenator[1] built with their collaboration. Reagents were purified as recommended by Edman and Begg.

The solutions of ATOs from the sequenator were dried in a stream of nitrogen, 100-μl aliquots of ethyl acetate were added, and 10- to 50-μl portions of these concentrates were loaded onto an aluminium-backed TLC plate containing the fluorescent indicator Kieselgel 60 F_{254} (Merck) followed by 1 μl heptafluorobutyric acid. A multispotter (Unimetrics, Joliet, Ill., U.S.A.) was used to apply accurate volumes onto the plate but the heating strip of the instrument was kept below 40°. After loading, the plate was placed in an oven for 10 min at 140°; it was then quickly cooled to room temperature and the reference PTH standard mixtures were loaded onto the plate immediately prior to TLC[6]. When PTH-arginine or PTH-histidine was expected, the plate was re-run in the solvent system of Inagami[7] (xylene–95% ethanol–acetic acid, 50:50:0.5). The plates were scanned at selected wavelengths using a Shimadzu dual wavelength Model CS-900 TLC scanner (Shimadzu Seisakusho, Kyoto, Japan) operated as a single-beam instrument in a reflectance mode.

RESULTS

The most convenient method we have found for following the conversion of the ATOs is to scan the plate with a TLC plate scanner at 270 nm (the absorption maximum for PTHs). Table I compares the results from the usual HCl conversion and heat conversions carried out in the presence and in the absence of organic acids at 140° for 10 min. Lower temperatures and longer heating times can be used but these conditions represent the quickest conversion rate with minimal extraneous thermal degradations. The results for the leucyl, seryl, valyl, and glutamyl residues of apo-myoglobin indicated that heat conversions are best effected in the presence of heptafluorobutyric acid and in fact the yields were marginally superior to those after the conventional HCl procedure.

Table II shows some results of the study on the heat conversion reaction (in the presence of heptafluorobutyric acid) of ATOs from the sequenator. Yields (as

TABLE I

EFFECT OF ACID CATALYSTS ON HEAT CONVERSION OF AMINO ACID THIAZOLINONES

Amino acid	*Relative yields**			
	HCl conversion	*Heat conversion*		
		—	*HFBA***	*$-SO_3H$****
Leucine	87	70	100	71
Serine§	43	73	100	44
Valine	82	100	100	44
Glutamic acid	80	79	100	29

* Based on peak areas of TLC scan of residues obtained from the sequenator; HFBA value set at 100.

** HFBA = Heptafluorobutyric acid (1 μl).

*** 3 *M* *p*-toluenesulfonic acid (1 μl).

§ Only the peak of the dehydro derivative was observed at 270 nm.

calculated from peak areas on the TLC scanner) of phenylalanine were similar for times of from 5 to 30 min at 140° but the amount of background gradually decreased as heating continued. Yields of PTH-proline and PTH-glycine after heat conversion for 10 min in the presence of heptafluorobutyric acid were marginally better than those after HCl conversion but the yield of PTH-glycine decreased significantly with longer conversion times. On the other hand, the threonyl residue formed a dehydro product (X) with a maximum yield after 5–10 min; thereafter it proceeded to break down to another product (Y), and both products appear in the PTH-phenylalanine region of the plate (Fig. 1 is a photograph of the plates). PTH-S-carboxymethylcysteine is also partially converted by HCl, and completely converted by heat to what appears to be a dehydro product which has an identical R_F value to PTH-alanine. The R_F values, using the conditions of Inglis and Nicholls[6], for the products from the more labile amino acid thiazolinones after heat conversion (140°, 10 min) are given in Table III.

Photographs under short-wavelength UV light of the TLC plates after chro-

TABLE II

EFFECT OF THE TIME OF HEAT CONVERSION ON THE YIELD OF AMINO ACID PHENYLTHIOHYDANTOINS

Amino acid	*Relative yields* of derivatives after various heating times** (min)*			
	5	*10*	*15*	*30*
Phenylalanine	106	100	105	96
Threonine (derivative X)	99	100	23	6
S-Carboxymethylcysteine (derivative)	115	100	73	56
Glycine	75	100	74	53
Proline	140	100	92	105

* Based on peak areas of TLC scan of residues from the sequenator; area for 10-min heat conversion set at 100.

** In the presence of heptafluorobutyric acid (1 μl).

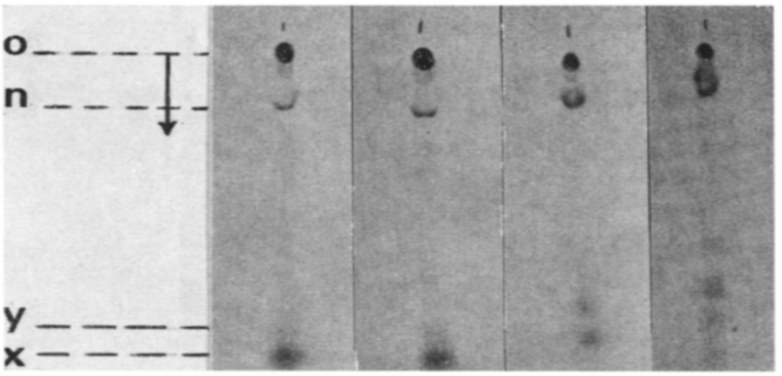


Fig. 1. UV photographs of products of heat conversion of ATO-threonine with 1 μl of heptafluorobutyric acid after 5, 10, 15 and 30 min, respectively, at 140°. The fastest moving spot (X) gradually fades and a second, slightly slower spot (Y) increases in intensity. *n* is an artifact of the heat conversion procedure but it does not interfere in the identification of the PTH-amino acids. O = Origin.

TABLE III

RESIDUES SUBSTANTIALLY DECOMPOSED BY HEAT CONVERSION OF AMINO ACID THIAZOLINONES AT 140° FOR 10 min

Amino acid	R_F *of products formed**			*Comments*
	1	*2*	*3*	
Serine	0.38	0.40	—	No PTH-Ser. Product 1 is major spot under UV light. Absorbs at 320 nm. Ninhydrin positive. Product 2 is yellow.
S-Carboxymethylcysteine	0.40	—	—	No PTH-Cys (S-carboxymethyl derivative). Absorbs at 320 nm. Ninhydrin positive.
Threonine	0.43	0.48	—	No PTH-Thr. Product 2 is major spot under UV light. Both products absorb at 320 nm. Weak ninhydrin response.
Tryptophan	0.41	0.43	—	PTH-Trp yield lower than with HCl conversion. Product 2 is yellow.
Lysine	0.04	0.37	0.50	Low yields of each product. Higher loading than normal required for identification. All ninhydrin positive.

* Chromatography in ethylene chloride–acetic acid (60:7)[6]. Relative R_F values for PTH-Lys (phenylthiocarbamyl derivative), PTH-Ala, PTH-Trp and PTH-Phe were 0.37, 0.40, 0.41 and 0.45, respectively.

matography of the first ten residues of apomyoglobin are shown in Fig. 2. The residues in the two photographs were loaded in equivalent amounts but those in the upper photograph were converted to PTHs by the usual 1 *N* HCl procedure whereas in the lower plate the concentrated ATO solutions were loaded directly onto the plate and heat converted in the presence of heptafluorobutyric acid. Both plates show that significant overlap occurred after step 5 (the glycine residue) in this sequenator run. The lower photograph does not show additional yellow products obtained with the seryl and tryptophyl residues on heat conversion as these are not visible under UV light. However, the heat conversion results compare favourably with those after HCl conversion in that the lanes are slightly cleaner, yields are at least as good, except for PTH-

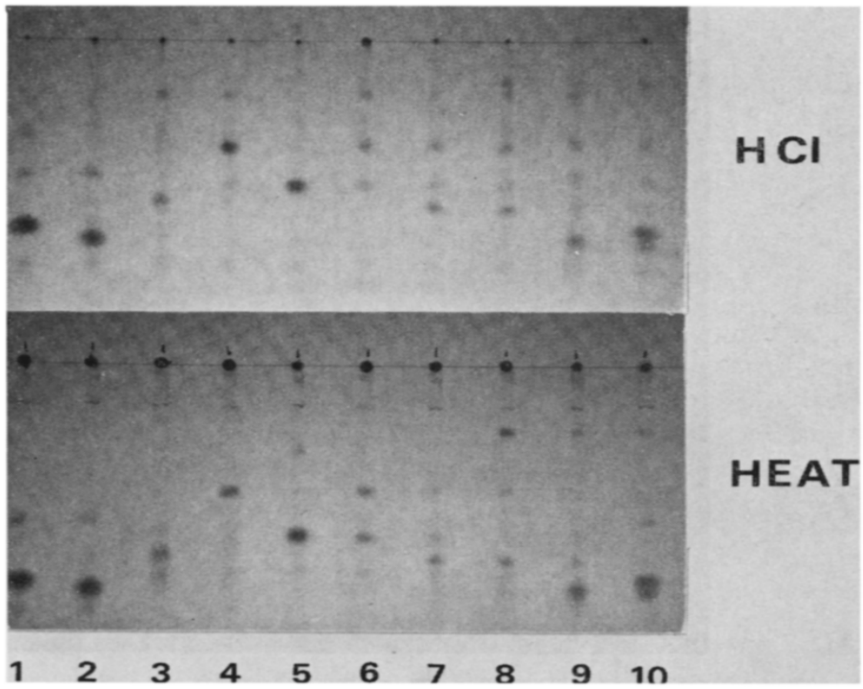


Fig. 2. UV photographs of TLC plates after chromatography of HCl-converted and heat-converted residues from a sequenator run on apomyoglobin. The amino acid sequence is: (1) valine, (2) leucine, (3) serine, (4) glutamic acid, (5) glycine, (6) glutamic acid, (7) tryptophan, (8) glutamine, (9) leucine, and (10) valine. In visible light, on the lower plate, there were two yellow spots just ahead of the seryl and tryptophyl derivatives, respectively.

tryptophan at step 7, and PTH-glutamine is not partially deaminated to give PTH-glutamic acid.

The change in absorption spectra of the various derivatives on heating can be a useful means of identification and in this work we were able to demonstrate this by scanning the TLC plates at selected wavelengths. Fig. 3 illustrates the results obtained for the ATO-threonine residue from the sequenator. The two derivatives X and Y are readily distinguished as dehydro compounds[8] by their strong absorption at 320 nm. Similarly the seryl and S-carboxymethyl derivatives exhibit greatly enhanced absorption at 320 nm. PTH-glutamic acid also yields a small amount of a product which is barely visible at 270 nm but easily detectable at 320 nm in the PTH-phenylalanine region. Reheating (at 140° for 1 h) the plate after chromatography helps to differentiate between PTH-asparagine and PTH-glutamine by producing characteristic thermal degradation products. After such treatment PTH-asparagine shows strong absorption at 350 nm whereas PTH-glutamine shows maximum absorbance at 320 nm. The PTHs of serine, threonine, glutamic acid, leucine and isoleucine behave similarly to PTH-glutamine with extra heating, giving maximum response at 320 nm, but only PTH-tyrosine and PTH-phenylalanine behave analogously to PTH-asparagine, giving maximum response at 350 nm. The remaining PTH amino acids all show broad absorption bands from 270–350 nm after this severe heat treatment. The initial scan-

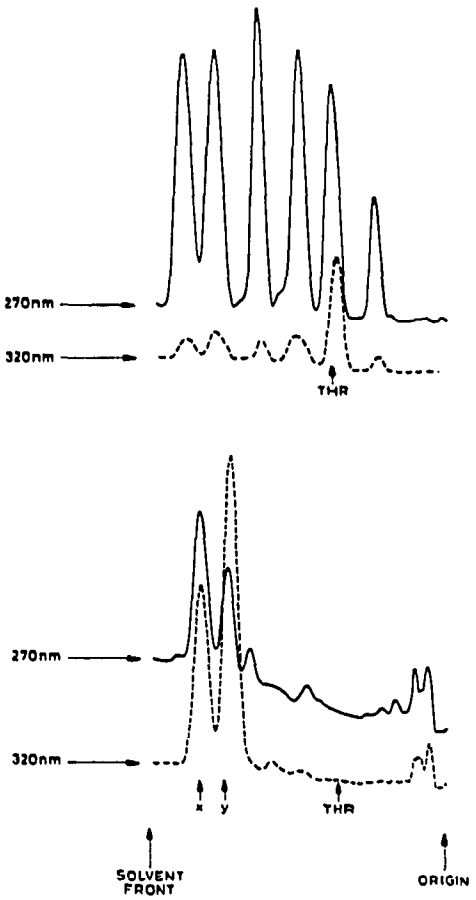


Fig. 3. Chromatograms obtained from the Shimadzu TLC scanner showing a reference mixture of six PTH-amino acids (above) and of a threonyl residue from the sequenator after heat conversion on the TLC plate (below).

ning run at 270 nm after the 10-min heat conversion is also helpful in identifying the prolyl derivative. PTH-proline only weakly quenches the fluorophore on the TLC plate and is relatively insensitive to other detection methods but gives almost equivalent response at 270 nm in the TLC scanner as the other PTH amino acids. Moreover, the measurement of peak areas at 270 nm from the scanner can be used to give a semi-quantitative estimation of the repetitive yields and degree of overlap between steps.

DISCUSSION

For most amino acid thiazolinones heating on silica gel plates with 1 μl of heptafluorobutyric acid produces a compound that chromatographs identically to the compound (PTH-amino acid) obtained in a similar yield by 1 *N* HCl treatment at 80° for 10 min. Exceptions in this regard are the ATOs of threonine, serine, S-car-

boxymethylcysteine (all of which are also found to be partly degraded after HCl conversion), tryptophan, N^{ε}-phenylthiocarbamyllysine and, to a minor extent, glutamic acid.

The ninhydrin spray of Roseau and Pantel[8], together with the assistance of a copper nitrate spray[6], is very useful in facilitating identification of the conversion products. Colour development is now routinely carried out at 140° instead of 107°, for 3.5 min after the ninhydrin spray and for 1 min after the copper nitrate spray. The products X and Y from threonyl residues give only weak reactions with these sprays, thus permitting differentiation from the chromatographically similar PTHs of phenylalanine and valine. After heat conversion ATO-serine gives rise to two main spots: one has a visible yellow colour and is a most useful indicator for the presence of a seryl residue. This spot has the same R_F value as PTH-alanine but does not cause quenching of the fluorophore in the plate. The other spot runs slightly slower than PTH-alanine (and is the one calculated in Table I), and gives only a faint pink colour with ninhydrin, which changes to a brownish purple with copper nitrate, in contrast to PTH-alanine, which gives a bright pink colour with ninhydrin changing to a brown with copper nitrate. Similarly, the PTH-S-carboxymethylcysteine derivative may be distinguished from PTH-alanine by its lower yield and the weak orange-brown colour it gives with the ninhydrin spray. The ATO derivative from N^{ε}-phenylthiocarbamyllysine is partially heat-converted to two other products, one appearing near the origin and giving a strong pink colour with ninhydrin, the other near PTH-isoleucine but giving a weak blue ninhydrin colour. However, the formation of three compounds from the lysine residue appears to be the major weakness of the heat conversion procedure. Addition of dithiothreitol to the spot at the origin after loading resulted in slightly improved yields of all products. The behaviour of ATO-tryptophan is also noteworthy as heat conversion to PTH-tryptophan is not as effective as HCl conversion; on the other hand, ATO-tryptophan gives rise to a visible yellow species that strongly absorbs at 430 nm although it does not quench the background fluorescence in short-wavelength UV light.

We have observed that the substrate for the heat conversion is important. Preliminary work attempting to bring about heat conversions on paper chromatograms was completely unsuccessful; also we have obtained inferior results when using TLC plates which do not contain the zinc silicate fluorophore. Inorganic ions must play an important part in the mechanism of the heat conversion reaction possibly by increasing the stability of the labile ATOs. The heating step also removes some of the possible contaminating materials such as phenylisothiocyanate and diphenylthiourea but not phenylthiourea. The removal of diphenylthiourea is particularly advantageous as its presence interferes with the TLC identification of PTHs in the PTH-leucine region of the plate. Quadrol can be tolerated in small amounts only.

By retaining all the residues in one phase, problems associated with the identification of the basic residues of histidine and arginine are decreased, as has already been noted[2], although we find that these residues are best identified after TLC using long-wavelength UV light and a palladium-calcein spray[10]. Also, this heat conversion of ATOs to PTHs curtails the usual procedure by eliminating both the acid treatment and the solvent extraction steps. In fact, since confirmation of TLC identifications can be made by either hydrolysis to the free amino acid[11] or mass spectrometry[12] of the thiazolinone the conventional production of PTHs may be unnecessary.

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