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OPEN-FLASK ALKALINE HYDROLYSIS OF AMINO ACID PHENYLTHIOHYDANTOIN DERIVATIVES

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SUMMARY

It is suggested that the open-flask system for the acid hydrolysis of proteins described earlier can also be used to advantage for the alkaline hydrolysis of amino acid phenylthiohydantoin derivatives obtained in protein sequential analysis according to Edman. Results obtained under nitrogen and argon atmospheres are reported for the hydrolysis of 0.5 μmole of each phenylthiohydantoin derivative, which is the amount of protein that is usually employed in an automatic sequenator.

INTRODUCTION

The identification of the terminal amino acid in the sequential degradation (both manual and automatic) of proteins according to Edman and co-workers[1,2] is achieved by identifying the corresponding phenylthiohydantoin derivative that is obtained in each sequential step. These derivatives can be identified in many ways, such as paper[3], thin-layer[4], column[5] and gas–liquid chromatography[6], mass spectrometry[7] and thin-layer electrophoresis[8]. Van Orden and Carpenter[9] converted these derivatives into the corresponding amino acids by means of alkaline hydrolysis and then used ion-exchange chromatography of the free amino acids. This technique was subsequently improved by Africa and Carpenter[10]. They carried out the alkaline hydrolysis, by the sealed-vial method, of amounts of phenylthiohydantoin ranging from 1 to 1.5 μmole and observed that, by increasing the amount of the derivative in the hydrolysis vial, the recovery of the corresponding amino acid also increased. Moreover, they found that recoveries improved markedly when the alkaline hydrolysis was performed in the total absence of oxygen. They attributed the partial destruction of the phenylthiohydantoin derivatives during alkaline hydrolysis to oxygen residues trapped in the sealed vials. They consequently developed an elaborate method of completely eliminating oxygen before hydrolysis.

In our laboratories, the sealed-vial method for the acid hydrolysis of proteins has been routinely replaced with the open-flask method under an inert gas atmosphere[11] with complete success, and therefore the application of a similar technique was considered suitable for the alkaline hydrolysis of these derivatives.

This paper presents the results obtained by the hydrolysis of 0.4–0.5 μmole

of each phenylthiohydantoin derivative with 0.1 *N* sodium hydroxide solution in an open flask under argon and nitrogen atmospheres. It should be emphasized that the amounts of the derivative (0.4–0.5 μmole) employed are of the same order as those obtained in a sequential step of the automatic Edman sequenator.

EXPERIMENTAL

Materials

Phenylthiohydantoin derivatives. The amino acid phenylthiohydantoin derivatives were purchased from Fluka (Buchs, Switzerland) and kept under vacuum over phosphoric anhydride. The phenylthiohydantoin derivatives of aspartic acid, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine were dissolved in ethyl acetate (Merck, Darmstadt, G.F.R.). The phenylthiohydantoin derivatives of arginine and histidine were dissolved in a 1% aqueous solution of acetone. The phenylthiohydantoin derivatives of serine and threonine were dissolved in a 5% acetone solution of ethyl acetate.

Argon S. S-grade argon purchased from SIO (Turin, Italy) was employed.

Nitrogen. High-purity nitrogen containing not more than 5 ppm of oxygen was employed.

Pyrogallol solution. This solution, through which the inert gas was bubbled prior to entry into the hydrolysis flask, was obtained by dissolving 30 g of pyrogallol in 60 ml of hot water. To this solution, 160 ml of an aqueous potassium hydroxide solution (1:2 w/v) were added, and 1 ml of the final solution is able to absorb 10–12 ml of oxygen.

Apparatus

An automatic amino acid analyzer developed in our laboratories, described in earlier papers[12–14] and now manufactured by Optica (Milan, Italy) under the name Aminolyzer, was employed for the automatic ion-exchange chromatography of the amino acids freed from the phenylthiohydantoin derivatives after alkaline hydrolysis. The separation of the neutral amino acids was achieved using an Amberlite CG-120 resin column eluted with sodium buffers as described previously[12]. Fig. 1 shows a chromatogram of acid and neutral amino acids obtained by this procedure. The separation of the basic amino acids was performed on an Aminex A-5 resin column eluted with a lithium buffer. This separation method was previously described by Mondino *et al.*[15]. The preparation of the buffer solutions, of the ninhydrin colour reagent and of the calibrating amino acid solution and the analytical parameters were reported in the earlier papers.

Method of hydrolysis

One millilitre of each of the phenylthiohydantoin derivative solutions (containing 0.5 μmole) was placed in a ground-glass three-necked 25-ml conical flask. After stoppering the two lateral necks, the solution was evaporated to dryness in a Büchi rotary evaporator at 40°. The dry residue was re-dissolved in 5 ml of 0.1 *N* sodium hydroxide solution into which argon or nitrogen (de-oxygenated by passage through the pyrogallol solution) had previously been bubbled for 3 h.

The flask was immediately placed on a heating mantle and a five-bubble con-

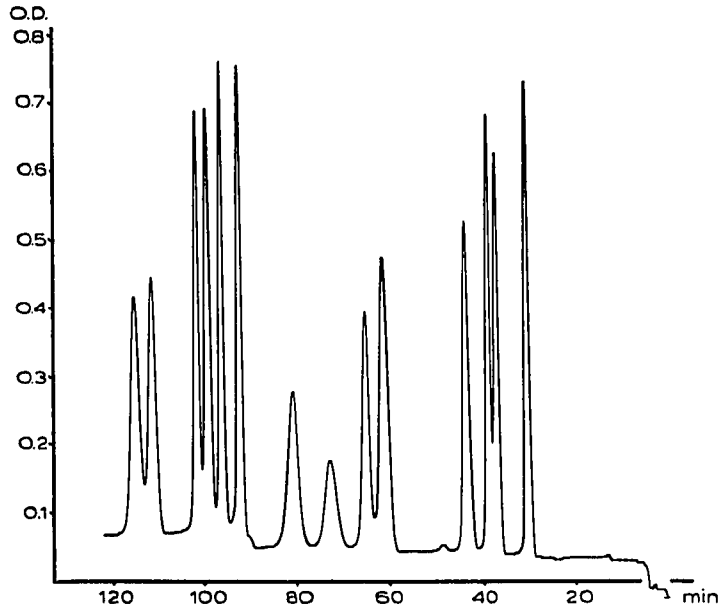

Fig. 1. Example of a chromatogram obtained with a calibration solution of acid and neutral amino acids (100 nmoles of each), read on the 570-nm channel. Peaks, from right to left: aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cystine (50 nmoles), valine, methionine, alloisoleucine, isoleucine, leucine, tyrosine and phenylalanine.

denser was inserted over the flask. Inert gas was immediately bubbled through the solution at the flow-rate of 0.5 ml/min. A Brooks (Stockport, Great Britain) 8744 pressure reducer was used to reduce the pressure of the argon or nitrogen from the high-pressure bottle and the flow-rate was measured with a Brooks 2-1355V rotameter. After bubbling for 1 h, the heat was switched on and the solution was boiled gently for 16 h. The alkaline solution was then cooled and neutralized with about 2.5 ml of 0.2 *N* hydrochloric acid, the solution was evaporated to dryness in a Büchi rotary evaporator at 40° and the dry residue was re-dissolved in exactly 2 ml of 0.2 *N* hydrochloric acid. A 0.4-ml volume of this solution was loaded on to each chromatographic column. If the analysis could not be performed immediately, the solution was kept in a freezer at about −20°.

RESULTS AND DISCUSSION

Table I reports the percentage recoveries of the amino acid relative to the theoretical amount estimated to be obtained from the corresponding phenylthiohydantoin derivative subject to hydrolysis. The second and third columns give the percentage recoveries obtained from the basic hydrolysis under a nitrogen atmosphere, while the fourth and fifth columns give those obtained under an argon atmosphere. It can be seen that the results obtained when bubbling argon during hydrolysis are all higher than those obtained when bubbling nitrogen. Hence it can be assumed that, if oxygen is responsible for the partial destruction of the phenylthiohydantoin

TABLE I

RECOVERIES OF THE THEORETICAL AMOUNTS ESTIMATED TO BE OBTAINED FROM THE CORRESPONDING DERIVATIVES

Substance	*Recovery (%)**			
	N_2 atmosphere		*Argon atmosphere*	
	Exptl.	*Theoretical*	*Exptl.*	*Theoretical*
Asparagine	54	50.2	74	75
Glutamine	44	42	61.3	63
Threonine as glycine	nd	nd	45.4	43
Serine	nd	nd	Traces	Traces
Proline	80.5	81	88.6	91
Glycine	55	50.5	70	73
Alanine	41.2	38	54.6	56
Valine	60	62	70	70
Cystine	0	0	0	0
Methionine	45	43.3	57	54.5
Isoleucine		20.2	30.6	31
as isoleucine	22			
as alloisoleucine	31	30	43.5	42
Leucine	41	42	52	52.5
Tyrosine	46.2	44	63.4	65.8
Phenylalanine	47	47	64.6	63
Tryptophan	38	35.5	53.8	51.5
Lysine	nd	nd	nd	nd
Histidine	26	22	50.3	50
Arginine		6	2	2
as arginine	9			
as ornithine	12	10	37.1	38

* nd = not determined.

derivatives during hydrolysis, as reported by Carpenter and co-workers[9,10], argon is more capable, owing to its higher density, of removing oxygen from the solution.

It should be stressed that, when using this open-flask technique, there are fewer technical problems than in sealed-vial methods, especially as regards the total removal of oxygen. The recovery results given here are lower than those reported by Carpenter and co-workers[9,10]. It should be pointed out, however, that the amounts of phenylthiohydantoin derivatives that were subjected to hydrolysis were much smaller than those used by Carpenter and co-workers. In our laboratories, it has also been observed that, by hydrolyzing larger amounts of derivatives, the recoveries increased. It was nevertheless considered preferable to work with smaller amounts of phenylthiohydantoin derivatives, of the same order as those recovered from the automatic protein sequenator described by Edman and Begg[2]. In fact, with the sequenator, the amount of protein undergoing sequential analysis is usually about 0.5 μmole. Our results are therefore satisfactory for the qualitative analysis of the phenylthiohydantoin derivatives obtained from the sequenator.

Taking into account that the mean recovery is higher than 50%, the 0.4-ml volumes of the final solution that are loaded on to each column contain more than 50 nmoles of amino acid and consequently no particular problem of sensitivity arises

when using our system of automatic amino acid chromatography. More than one analysis can be performed for each sample.

As far as the quantitation of the samples of phenylthiohydantoin derivatives obtained from a sequential step is concerned, it would be advisable to perform a suitable number of hydrolyses and analyses in order to find a statistically significant factor by which to multiply the results obtained.

From the operational viewpoint, in order to speed up the process, it would be preferable first to chromatograph the sample for basic amino acids on a short column on which analysis is very rapid. Thereafter, if this chromatogram is blank, the search for acidic and neutral amino acids is carried out.

In general, the conversion of the phenylthiohydantoin derivatives into the corresponding amino acids involves some complications. Serine and cystine are almost totally degraded, while threonine is converted into glycine; consequently, for these three derivatives, an alternative method such as thin-layer chromatography must be employed. Isoleucine is partly converted into alloisoleucine. This does not actually constitute a problem inasmuch as alloisoleucine can easily be separated in the chromatogram from isoleucine and can then be identified and quantified. Arginine is partly converted into ornithine, which is why the basic amino acids are eluted with a lithium buffer capable of eventually separating ornithine from lysine and ammonia.

In conclusion, the chromatographic identification of amino acids after alkaline hydrolysis of the phenylthiohydantoin derivatives, despite the above-mentioned drawbacks, can be recommended for the sequential investigation of proteins.

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