HYDROLYSIS OF THE PEPTIDE BOND AND AMINO ACID MODIFICATION WITH HYDRIODIC ACID

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Abstract

Reaction of hydriodic acid with peptides and proteins has been studied. At the boiling point, hydrolysis of the peptide bond, particularly stable bonds linking valine and isoleucine residues, is facile. Several amino acids react with constant-boiling hydriodic acid but the only reactions detrimental to the amino acid analysis are the reduction of serine with concomitant formation of alanine, and the destruction of tryptophan. Gentler conditions of hydrolysis with diluted hydriodic acid are required for analysis of serine. Good results for analysis of proteins for amino acids may be obtained after a 6-hr hydrolysis period.

I. Introduction

Hydrolysis of proteins in 6N HCl at 110°C is not only slow but generally requires prolonged treatments for different times if accurate results are to be obtained for some amino acids. One single hydrolysis for 22 hr followed by amino acid analysis gives a good indication of the amino acid composition when allowance is made for the partial destruction of threonine, serine, and cystine, and the possibly low values for methionine, valine, isoleucine, leucine, and tyrosine. Tryptophan is, of course, either partially or totally destroyed by this procedure.

Recently, Roach and Gehrke (1970) proposed the use of 6N HCl at 145°C for the rapid hydrolysis of proteins whilst Liu and Chang (1971) have suggested that 3N *p*-toluenesulphonic acid has advantages over hydrochloric acid, particularly for the determination of tryptophan.

This paper examines the use of hydriodic acid as a reagent to hydrolyse proteins. Hydriodic acid has been used extensively as an analytical reagent for the determination of alkoxyl groups (Inglis 1971) where it is considerably more effective than hydrochloric acid in cleaving ether bonds. Hydriodic acid was recommended by Baernstein (1936*b*) for the hydrolysis of proteins after a series of papers (Baernstein 1932, 1934, 1936*a*) in which he described its use for the determination of methionine and cysteine. He did not, however, have the sensitive chromatographic techniques of today to study the quantitative nature of the hydrolysis for all of the amino acids in proteins and surprisingly, the more recent literature contains no further references to the liberation of amino acids from proteins on hydrolysis with hydriodic acid.

II. Materials and Methods

(*a*) *Materials*

Lysozyme (salt-free), Worthington Biochemical Corporation; ribonuclease (in 0·1% phenol), Worthington Biochemical Corporation, was dialysed and lyophilized; crystalline beef insulin, Commonwealth Serum Laboratories; myoglobin (crystalline, salt-free), Pierce Chemical Co., was treated with acetone–hydrochloric acid (procedure of Theorell and Åkeson 1950) to remove the haeme. The peptides used were kindly supplied by Dr. F. H. C. Stewart of these laboratories.

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(b) *Hydriodic Acid*

Colourless hydriodic acid (M.A.R.), Hopkins & Williams. This reagent contains 0·03% hypophosphorous acid (H_3PO_2). For analyses in the presence of iodine either this reagent after exposure to the air or redistilled hydriodic acid (*pro analysi*), Merck, was used. The 2·5N reagent was prepared by mixing equal amounts of water and constant-boiling hydriodic acid.

(c) *Conditions of Hydrolysis*

Hydrolyses were carried out either in sealed evacuated tubes (Inglis and Liu 1970) or by refluxing in the presence of a stream of dry nitrogen ("oxygen-free" grade) (Nicholls and Gruen 1969; Mondino and Bongiovanni 1970). For the sealed-tube technique the glass tube was constricted before addition of the hydriodic acid (1 ml to 1–5 mg protein) which was sucked into the tube by immersing the tube in an alcohol–dry ice bath. This procedure prevents formation of iodine on the sides of the tube during the glass-working.

In one experiment in which extra hypophosphorous acid was added to reduce the iodine present in a hydriodic acid solution, there was an explosion on cracking the tube with a hot glass rod presumably because spontaneously combustible phosphine was formed. This does not occur with the usual reagent (0·03% H_3PO_2) or with the open-flask hydrolysis technique.

(d) *Amino Acid Analyses*

The amino acid analyses were carried out on a Beckman–Spinco model 120B analyser with the dimethylsulphoxide–ninhydrin reagent of Moore (1968). Sodium citrate buffers of the customary formulation were used; the long column was run with first buffer at pH 3·26 (57°C) to enable cystine to be eluted between alanine and α-amino-n-butyric acid. For determination of small amounts of the amino acid in the homoserine position it was necessary to use buffer of pH 3·05 to improve the resolution from glutamic acid.

III. Results

Hydrolyses of lysozyme were carried out at the boiling point of hydriodic acid in a stream of pure nitrogen for periods of from 1 to 20 hr. The results are shown in Table 1.

After only 1 hr most of the protein (at least 90% on a weight basis) was hydrolysed to free amino acids. These results indicate that accurate analyses would be possible for all the remaining amino acids except alanine, serine, and tryptophan provided suitable times of hydrolysis and operation of the amino acid analyser were used. For the amino acids lysine, histidine, glutamic acid, glycine, leucine, tyrosine, and phenylalanine, hydrolysis for only 1 hr is sufficient; however, for the amino acids arginine, asparatic acid, methionine, threonine, valine, and isoleucine, hydrolysis for at least 2 and preferably 4 hr is desirable. Under the normal operating conditions of the amino acid analyser cysteine and proline are eluted together (Moore and Stein 1963) and were not determined separately, although proline analyses for myoglobin (see Table 3), which does not contain cystine, gave quantitative results. Because of the difficulty of cystine analysis after acid hydrolysis, the quantitation of cystine and the separation of cysteine from proline will be the subject of another paper (see Inglis, Nicholls, and Roxburgh 1971). The amino acids methionine and threonine are converted to homocysteine thiolactone (Baernstein 1934) and α-amino-n-butyric acid respectively.

These reactions do not present any problems of determination since homocysteine thiolactone (which elutes after arginine) is recovered quantitatively from methionine and the small amounts of α-amino-n-butyric acid (which elutes before valine) can be added to the value for threonine.

Serine, however, is the most difficult amino acid to quantitate under reflux conditions with hydriodic acid. It is extensively destroyed during the hydrolysis with a concomitant increase in the alanine value. Moreover, two other peaks attributable to the serine breakdown can be found in variable amounts in the hydrolysate. One peak, which elutes in the position of cysteic acid, was greatly decreased upon prolonged

TABLE 1

AMINO ACID ANALYSES OF LYSOZYME AFTER HYDROLYSIS IN REFLUXING 5N HI

Amino Acid	No. of Residues	No. of Residues Found after:				
		1 hr	2 hr	4 hr	8 hr	20 hr
Lys	6	5·9	6·2	6·0	6·1	6·4
His	1	1·0	1·0	1·0	1·1	1·2
Glu	5	5·1	5·0	5·0	5·0	5·0
Gly	12	11·9	11·9	11·9	12·3	12·3
Val	6	5·0	5·7	5·9	6·1	5·8
Ile	6	4·9	5·6	5·7	5·9	5·6
Leu	8	8·0	8·0	8·0	8·0	8·0
Tyr	3	2·8	3·0	2·9	3·1	3·1
Phe	3	2·8	3·0	2·9	3·0	3·1
Arg	11	9·0	11·1	10·5	10·8	11·0
Asp	21	18·2	19·6	19·9	20·3	20·1
Met }	2	0·8	0·0	0·0	0·0	0·0
Homocysteine thiolactone }	0	1·4	1·8	1·7	1·8	1·9
Thr }	7	5·4	5·4	5·8	5·8	4·0
α-Amino-n-butyric }	0	0·0	0·3	0·5	0·9	3·0
Pro+CysH	2	6·2	6·9	6·0	5·7	5·3
½Cys	8	2·1	0·8	0·8	2·3	3·2
Ala	12	13·5	14·0	15·3	17·5	21·3
Ser	10	5·6	3·7	3·9	2·9	<1
Peak before Glu	0	1·2	1·1	1·0	0·8	0·0
Peak at cysteic acid	0	2·6	3·4	1·5	0·7	0·0
Trp	6	1·0	1·4	1·4	1·7	0·9

hydrolysis and was considered to be due to phosphoserine formed from the interaction of serine with oxidation products of hypophosphorous acid. In fact, when H_3PO_2 was added in similar amounts to a hydrochloric acid hydrolysis (22 hr, 110°C, sealed tube), approximately 12% serine was converted to this derivative and no other deleterious effects were noted. The other peak, which elutes in the position of homoserine just preceding glutamic acid, remained relatively constant with time and only decreased when almost all the serine had been converted to alanine. This suggests that the compound is possibly iodoalanine or some other intermediate in the conversion of serine into alanine, but attempts to isolate this material by paper chromatography were unsuccessful.

Analyses of model peptides (see Table 2) confirmed the observations made for the analyses of lysozyme. Thus, valylvaline and isoleucylvalylglycine were both

TABLE 2

HYDROLYSIS OF MODEL SUBSTANCES WITH REFLUXING 5N HI

Substance	Hydrolysis Time (hr)	Percentage Recovery					
		Val	Ile	Gly	Ser	Peak before Glu	Ala
Val-Val	4	95					
Ile-Val-Gly	4	95	96	101			
AcGly-SerNH$_2$	2			100	63	10	16
Ser	2				73	9	10
Ser	4				56	8	25
Ser	23				8	2	81

TABLE 3

NUMBER OF RESIDUES OF AMINO ACIDS PER MOLE FOUND FOR PROTEINS AFTER REFLUXING IN 5N HI

Amino Acid	Ribonuclease: 6-hr hydrolysis		Insulin: 6-hr hydrolysis		Myoglobin: 2-hr hydrolysis	
	Expected	Found	Expected	Found	Expected	Found
Lys	10	10·0	1	1·1	19	19·4
His	4	4·0	2	2·0	12	11·9
Arg	4	4·0	1	1·1	4	3·9
Glu	12	12·2	7	7·5	19	19·3
Gly	3	3·0	4	4·3	11	11·2
Val	9	8·8	5	4·9	8	7·5
Ile	3	2·8	1	0·9	9	8·8
Leu	2	2·1	6	6·2	18	18·0
Tyr	6	5·7	4	3·7	3	3·0
Phe	3	2·9	3	2·8	6	6·1
Asp	15	15·2	3	3·1	8	8·2
Met }	4	0·0	0	0·0	2	0·0
Homocysteine thiolactone }	0	4·0	0	0·0	0	1·8
Thr }	10	9·0	1	0·7	5	4·3
α-Amino-n-butyric }	0	0·6	0	0·2	0	trace
Pro	4	4·2*	1	1·1	4	3·9
½Cys	8	7·4	6	0·6†	0	0·0
Ala }	12	18·1	3	5·8	17	18·9
Ser }	15	7·0	3	0·5	6	3·4
Peak before Glu	0	1·8	0	0·0	0	0·0
Peak at cysteic acid	0	0·2	0	0·0	0	0·5
Trp	0	0·0	0	0·0	2	0·6

* Hydriodic acid containing iodine used. † 5·2 as cysteine.

completely hydrolysed in 4 hr in refluxing hydriodic acid. Quantitative fission of Val-Val and Ile-Val bonds in proteins usually requires at least 72 hr with hydrochloric

acid at 110°C. After a 2-hr hydrolysis using hydriodic acid containing no H_3PO_2 both serine and acetyl glycylseryl amide gave only one unknown peak (that which eluted in the homoserine position). Moreover, longer hydrolysis times produced greater destruction of serine and higher recoveries of alanine.

Ribonuclease, myoglobin, and insulin show similar behaviour to lysozyme on hydrolysis (see Table 3). These proteins all yielded excellent valine and isoleucine recoveries and indeed gave similar results for most of the amino acids, although the analyses were marred by the large conversion of serine to alanine.

Hydriodic acid, suitably diluted with water, can give results similar to those produced by constant-boiling hydrochloric acid. The results obtained after hydrolysis of proteins using 2·5N HI revealed virtually no evidence for the demethylation of methionine or other side reactions; methionine, threonine, and serine showed greater than 95% recoveries. Hydrolysis with 2·5N HI, in sealed tubes, at 120°C for 6 hr produced very similar results to reaction at 110°C for 22 hr and these results were equivalent to those obtained after hydrolysis with 6N HCl (110°C, 22 hr). Hence it would be possible to determine serine and alanine using the diluted hydriodic acid reagent at 120°C and retain the advantage of a more rapid hydrolysis step. However, to obtain good valine and isoleucine recoveries, at least 2 hr hydrolysis with the constant-boiling reagent is necessary.

IV. Discussion

The experiments with hydriodic acid as a hydrolytic reagent for peptides and proteins have shown that it can be used profitably as an alternative to hydrochloric acid. Like hydrochloric acid, it is readily available and can be purified by distillation. Moreover, the hydrolysis conditions are not as exacting—the reducing nature of the acid is apparently safeguard enough against oxidations which may occur during hydrolyses with hydrochloric acid if traces of oxidants or metals are left in the reagent tube. Iodine (admittedly not a strong oxidizing agent) present in hydriodic acid is not of any consequence apart from affecting the amount of cystine reduced to cysteine during the hydrolysis. This is an important observation for if oxidizing agents were present, reaction with hydriodic acid would produce iodine. The excellent analyses for tyrosine, even in the presence of iodine, are significant in this respect since this amino acid can easily be modified during acid hydrolysis and additives such as phenol and thioglycollic acid have been recommended for preventing tyrosine destruction (Sanger and Thompson 1963).

Tryptophan was usually recovered in small and variable amounts after either short reaction times with boiling 5N HI or longer periods with diluted acid. Separate experiments using pure tryptophan did not clarify the reaction with hydriodic acid although no other amino acids were apparently formed from the tryptophan. The conversions of serine, threonine, and methionine to related amino acids raises the possibility that tryptophan might also be transformed by hydriodic acid to another compound which is not eluted from the column of the analyser.

Roach and Gehrke (1970) found that the hydrolysis of proteins in 6N HCl at 145°C for 4 hr gave similar results to 6N HCl at 110°C for 26 hr; however, yields of isoleucine, serine, methionine, and tyrosine were low for ribonuclease and to obtain

accurate values for many amino acids the results had to be extrapolated to "zero time" hydrolysis. On the other hand, a single short hydrolysis (2–6 hr) with constant-boiling hydriodic acid will give quantitative results for all amino acids, except serine and alanine, without the need to extrapolate values. Our work has shown that 2·5N HI either at 120°C for 6 hr or at 110°C for 22 hr gives comparable results to 6N HCl hydrolyses (110°C, 22 hr). The higher boiling point and the higher pK_a of hydriodic over hydrochloric acid (−11 versus −7, Albert and Serjeant 1962) are two properties which would help to explain the stronger hydrolytic nature of hydriodic acid.

The most useful features of hydriodic acid as a hydrolytic reagent for proteins are the ability to carry out rapid hydrolyses under simple reflux conditions and to quantitatively liberate amino acids (such as valine and isoleucine) which are difficult to hydrolyse with hydrochloric acid. For instance, it is very difficult to obtain more than 2 of the 3 Ile residues from ribonuclease after hydrolysis with hydrochloric acid for 22 hr at 110°C. From Table 3 it can be seen that 2·8 residues of Ile were recovered after only 6 hr hydrolysis with refluxing 5N HI. This is a general observation for all the proteins examined and indicates that this reagent has a useful place in peptide and protein analysis. Work in progress on the determination of cystine and the hydrolysis of stable amino acid derivatives supports this view.

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