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AN EXPERIMENTAL STUDY OF AMINO ACID DEGRADATION UNDER OPEN FLASK HYDROLYTIC CONDITIONS

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SUMMARY

The authors suggest that the open flask system for acid hydrolysis of proteins could also be used to advantage when it is necessary to submit small quantities of pure and isolated proteins to acid hydrolysis. This method is simpler and offers a higher repeatibility, regarding the removal of dissolved oxygen, as against the sealed vial system. An experimental study of the behaviour of an artificial protein amino acid mixture, under standard open flask hydrolytic conditions, in constant boiling hydrochloric acid and in an oxygen-free atmosphere, is reported. The interaction of tryptophan and cystine under these conditions with formation of cysteine was investigated. It was found that this amino acid can severely interfere with the chromatographic separation of proline, when sodium buffers are employed in the elution of resin columns. The separation of these two amino acids has been achieved by employing a method which uses lithium buffers and which has been previously described by one of the authors for the separation of amino acids and related compounds in physiological fluids.

INTRODUCTION

Protein cleavage by acid hydrolysis in order to obtain amino acid solutions suitable for application to chromatographic analytical columns, is now usually accomplished, when isolated proteins are under investigation, according to the well known sealed vial system. On the other hand, when the amino acid analysis of the protein content of foods and feeds is required, owing to the necessity of working with larger samples, the hydrolysis of these proteinaceous materials is preferably carried out according to the open flask method, under a nitrogen atmosphere[1].

Since the latter method, from an operational point of view, is much easier and, as far as the dissolved oxygen removal is concerned, offers a better degree of repeatibility as against the sealed tube, it is suggested that, even for the hydrolysis of isolated proteins, whenever the sample quantity is not extremely low, the open flask method would be preferable.

Acid hydrolysis of proteins, even under controlled conditions, results in the loss, sometimes complete, of certain amino acids as HIRS *et al.*[2], TRISTRAM AND SMITH[3] and SMITH AND STOCKELL[4] have reported when employing the sealed tube method.

The experimental study reported here was therefore carried out in order to check the amino acid degradation as well as the reciprocal interference of some amino acids under open flask hydrolytic conditions. In particular, the behaviour of tryptophan and cystine by themselves as well as in the presence of each other was investigated.

The stability of tryptophan, under acidic conditions, when not in the presence of cystine and in the absence of oxygen, was confirmed[5–8].

The formation of cysteine as a product of the oxidation–reduction reaction between cystine and tryptophan was also confimed[6,9] and it was found that the formation of this compound from cystine can severely interfere with the determination of proline when using our method of protein amino acid analysis[10] employing Amberlite IR-120 crushed resin with the buffer elution system according to SPACKMAN *et al.*[11]. On the other hand, WAINER[12] has reported that, by employing the method of PIEZ AND MORRIS[13] for the protein amino acid analysis with Chromobead resin Type A, the cysteine peak merges with proline. Consequently, a study has been carried out in order to find a means of ridding the proline peak of cysteine. This was achieved by using the method for the determination of amino acids and related compounds in physiological fluids described in a previous paper by MONDINO[14], which employs an Amberlite IR 120 crushed resin column which is eluted with a lithium buffer system.

MATERIALS AND APPARATUS

Hydrochloric acid

36% analytical grade hydrochloric acid (Merck) diluted with water 1:1 (v/v) was twice distilled in a quartz Heraeus bidistiller; in this way constant boiling hydrochloric acid solution, about 6 *N*, was obtained.

Nitrogen

High purity grade nitrogen, containing less than 5 p.p.m. of oxygen was employed.

Pyrogallol solution

In order to obtain nitrogen absolutely free of oxygen, the high purity nitrogen was bubbled through a pyrogallol solution, which was obtained by dissolving 30 g of pyrogallol in 60 ml of warm water. To this solution, 160 ml of KOH in water (1:2, w/v) were added. 1 ml of the final solution is capable of absorbing between 10 to 12 ml of oxygen.

Amino acids

The following amino acids were employed: aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, lysine, histidine and arginine; all were obtained from Fluka.

Amino acid solutions

The amino acids were dissolved in constant boiling hydrochloric acid at the amount of 750 nmoles of each amino acid per ml of solution. This concentration was chosen so that we obtained, in the case of the solution of the complete mixture,

the same order of magnitude of the proportion protein–acid as that usually employed in the sealed vial system (1/500).

Reaction flask

A 500 ml three neck reaction flask manufactured by Quickfit (catalog No. FR 500/4S/44A) was employed. The reflux condenser was inserted in the central neck and the nitrogen bubbler, catalog No. MF 5-ST 53/24, was introduced into one of the lateral necks. Through the other lateral neck, which was kept stoppered throughout the reaction time, the samples could be withdrawn. The flask was heated on an electric thermoregulated heating mantle, regulated in such a way that uniform and gentle boiling of the solution took place.

Rotating evaporator

A rotating film evaporator manufactured by Büchi, provided with a 50 ml evaporating flask, and heated by means of a water steam bath, was employed.

Amino acid analyser

An amino acid analyser "Aminolyzer", manufactured by the Optica Co. of Milan, was employed for the amino acid automatic analysis.

METHOD

The amino acid solutions, containing, as previously said, 750 nmoles of each amino acid per ml of solution, were prepared in volumes of 250 ml and then introduced into the hydrolysis flask. Before warming, the oxygen was removed from the solution and the apparatus, by bubbling the solution with the oxygen-free nitrogen at a flow rate of 2 ml per sec for 20 min. The heating was then started and the solution was brought to boiling point. Throughout the reaction time the nitrogen was kept bubbling at 1 ml per sec, the gas flow rate being measured by means of a rotameter.

Samples were withdrawn at zero time and at 24, 48 and 120 h by means of a 10 ml pipette, to which a "pro-pipette" was connected. Before the sample withdrawal, heating was discontinued in order to stop the boiling and to get a temperature drop in the solution down to 104–105°. The nitrogen bubbling during sampling was maintained and the top of the reflux condenser was stoppered in order to avoid a flow of air taking place through the open sampling neck of the reaction flask with a consequent contamination of the solution by oxygen.

The samples were kept in a freezer at —30°. Before processing for the amino acid analysis, they were allowed to stand some time at room temperature and then 1 ml, exactly measured, was introduced into the rotating film evaporator and evaporated to dryness. This operation was repeated 3 times, adding each time about 1 ml of distilled water. Finally, the residue was dissolved in the evaporator flask by addition of 1 ml, exactly measured, of 0.1 *N* HCl.

0.1 ml of this solution were then employed for the analysis of the amino acids on each column according to the method described by MONDINO[10] in a previous paper. In order to improve the precision and degree of accuracy in the evaluation of the proline peak, the sensitivity of the second colorimeter, reading at 440 nm, has been expanded to 0.5 O.D. units for a full scale deflection as against 2 O.D. sensitivity main-

tained on the first colorimeter, reading at a 570 nm wavelength. The cuvette light path was 20 mm long on both colorimeters.

In order to separate the peaks of proline and cysteine, which emerge together when sodium buffers are employed for the column elution, and which both have an absorbance maximum at 440 nm, the method for amino acid and related compound determination described by MONDINO[14] was employed. This method, applied to columns of Amberlite IR-120 crushed resin, employs lithium salt buffers as eluting agents. With this system the cysteine peak emerges well separated before the proline peak. The sensitivity on the colorimeter reading at 440 nm was set at 0.5 O.D. units for a full scale deflection and a cuvette having a 10 mm light path was employed.

Since in our analytical system, as previously reported[10,14,15], peak evaluation is possible by simply measuring the height of the peaks over the base line in mm, the peak heights of the chromatograms of the samples at 24, 38 and 120 h have been compared with the peak heights of the chromatograms run for the samples taken at zero time, which were considered equal to 100.

RESULTS AND DISCUSSION

In Table I the results of 5 experiments on the hydrolytic treatment of a mixture of the 18 protein amino acids are reported as single amino acid percentages of the initial quantity at 24, 48 and 120 h of treatment. Mean values, standard deviations and coefficients of variation are also reported.

Examining these results, it can be seen that aspartic acid, glutamic acid, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine and arginine are stable under these hydrolytic conditions. Threonine and

TABLE I

RESULTS OF 5 EXPERIMENTS IN WHICH A MIXTURE OF THE 18 PROTEIN AMINO ACIDS HAS BEEN HYDROLYSED BY THE OPEN FLASK METHOD

M = mean; S.D. = standard deviation; c.v. = coefficient of variation.

	24h							
	1	*2*	*3*	*4*	*5*	*M*	*S.D.*	*c.v.*
Aspartic acid	100.3	101.0	99.1	102.2	100.4	100.6	1.06	1.05
Threonine	98.1	98.8	96.8	97.6	97.8	97.8	0.73	0.74
Serine	94.3	95.0	94.0	95.3	95.8	94.8	0.73	0.77
Glutamic acid	99	99.4	97.7	100.6	98.5	99.0	1.08	1.09
Proline	116.8	120.8	113.4	115.6	114.0	116.1	2.94	2.53
Glycine	100.4	100.3	99	100.7	99.2	99.9	0.77	0.77
Alanine	101.0	100.4	99.2	100.6	99	100.0	0.89	0.89
Cystine	62.9	66.1	68.4	63.3	63.2	64.8	2.40	3.71
Valine	100.6	100.9	99.4	100.0	99.2	100.0	0.74	0.74
Methionine	101.5	99.9	101.4	98.6	99.3	100.1	1.28	1.28
Isoleucine	99.5	101.2	102.0	102.0	99.1	100.8	1.38	1.38
Leucine	99.8	100.2	101.8	100.9	98.8	100.3	1.13	1.13
Tyrosine	100.8	100.0	102.1	101.6	99.1	100.7	1.21	1.20
Phenylalanine	101.8	99.6	99.8	100.6	98.7	100.1	1.17	1.17
Tryptophan	55	55.7	54.8	54.8	55.9	55.2	0.52	0.94
Lysine	98.2	97.6	99.1	99.7	102.2	99.4	1.78	1.79
Histidine	99.1	99.6	99.5	99.5	102.1	100.0	1.21	1.21
Arginine	99.1	100.6	98.3	99.3	102	99.9	1.45	1.45

	48 h							
	1	*2*	*3*	*4*	*5*	*M*	*S.D.*	*c.v.*
Aspartic acid	100.6	101.2	99.7	101	98.8	100.3	1	1
Threonine	95.3	93.7	95.4	96.6	96.8	95.6	1.24	1.30
Serine	87.5	88.4	89.1	90.2	90.6	89.2	1.27	1.43
Glutamic acid	99.5	98.8	96.9	99.6	101.4	99.2	1.62	1.63
Proline	128.7	128.8	131	134.3	127.7	130.1	2.64	2.03
Glycine	100.6	100.8	98.5	101.6	99.3	100.2	1.24	1.24
Alanine	100.6	100.2	98.7	101.7	99.4	100.1	1.15	1.15
Cystine	52.3	49.9	49	48.8	53.2	50.6	1.99	3.9
Valine	100.5	100.4	99.2	99.9	98.8	99.8	0.74	0.75
Methionine	98.6	101.6	99.1	101.3	100.2	100.2	1.32	1.31
Isoleucine	98.4	98.1	98	100	101.4	99.2	1.48	1.49
Leucine	99.2	100	99	100.5	101.5	100.1	1.02	1.02
Tyrosine	100.4	100.9	99.3	100.5	98.6	99.9	0.96	0.96
Phenylalanine	102	101.5	100	100.4	100.8	100.9	0.81	0.80
Tryptophan	38.8	41	39.9	40	40.1	40.0	0.78	2.00
Lysine	98.2	100	97.6	99.7	98.8	98.9	1	1.01
Histidine	98.4	101.8	99.1	101.1	98.6	99.8	1.54	1.55
Arginine	100.3	100	99.8	101.4	92.8	99.9	1.15	1.15
	120 h							
	1	*2*	*3*	*4*	*5*	*M*	*S.D.*	*c.v.*
Aspartic acid	101.1	101.2	102.2	100.1	98.3	100.6	1.48	1.47
Threonine	80.0	89.3	90.3	87.7	86.8	88.6	1.37	1.55
Serine	72.1	70.9	70	73.4	72.8	71.8	1.39	1.93
Glutamic acid	101.9	101	99.3	99.1	98.2	99.9	1.51	1.51
Proline	147.1	143.0	150.0	148.1	151.2	147.9	3.16	2.14
Glycine	101.8	101.3	101.6	101	98.1	100.8	1.52	1.51
Alanine	102.5	101	101.8	100.3	98.7	100.9	1.46	1.45
Cystine	38.9	36.9	37.2	36.5	36.6	37.2	0.98	2.63
Valine	101.2	100.8	100.9	100.1	98.7	100.3	1.00	1.00
Methionine	100.2	101.9	99.6	101	99.3	100.3	1.12	1.12
Isoleucine	99.9	101.4	100.3	98.7	98	99.7	1.34	1.34
Leucine	101.6	101.9	101.5	99.9	99.1	100.8	1.23	1.22
Tyrosine	102.1	102.7	100	99.5	99.2	100.7	1.59	1.58
Phenylalanine	102.3	101.1	100.9	100.5	98.5	100.7	1.38	1.37
Tryptophan	11.1	11.6	11.5	11.8	11.4	11.5	0.27	2.25
Lysine	100.5	101.5	101.8	99.5	99.4	100.5	1.11	1.10
Histidine	99.7	100.8	102.8	99.3	98.8	100.5	1.41	1.05
Arginine	98.6	98.9	99	101.2	99.1	99.4	1.04	1.05

serine are slightly degraded, while cystine and tryptophan are severely destroyed. The values of proline are shown to be increasing, due to an artifact which occurs when amino acid analysis is carried out under conditions which do not allow the separation of cysteine from this amino acid.

Considering the trend of the degradation curves of threonine and serine reported in Fig. 1, it can be assumed that the degradation of these two amino acids is free from any interference, at this concentration level, by other amino acids. This has been confirmed by treating threonine and serine alone under the same hydrolytic conditions and examining samples taken after 24, 48 and 120 h of boiling in an open flask. The results obtained in this way are in perfect agreement with those obtained when

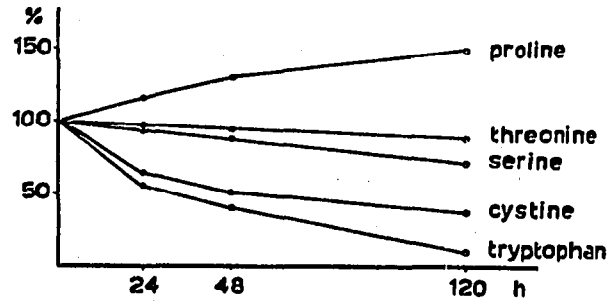

Fig. 1. Degradation curves (mean values of 5 results) of threonine, serine, tryptophan and cystine in the presence of each other and in the presence of all the other 18 protein amino acids. The artifacted curve of proline is also shown.

threonine and serine are treated in the presence of all the other protein amino acids.

The degradation curves of cystine and tryptophan, in the presence of all the other amino acids, are also illustrated in Fig. 1. The behaviour of these amino acids when treated separately, and alone, in the open flask under the conditions and at the concentration levels previously described, is completely different; both tryptophan and cystine are perfectly stable if boiled alone in hydrochloric acid in the absolute absence of oxygen. This result obtained in our open flask apparatus is in agreement with literature data[6] for the sealed tube system and can be taken as a test of the complete absence of oxidative conditions.

The behaviour of tryptophan and cystine in the presence of each other when

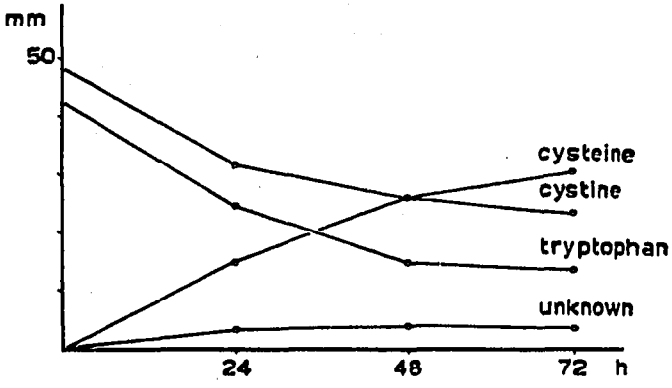

Fig. 2. Degradation curves of cystine and tryptophan treated in the presence of each other and in the absence of the other amino acids. The formation curves of cysteine and of another unknown reaction product are also illustrated.

treated for 72 h in the open flask, but in the absence of all the other amino acids has also been investigated. The results of this experiment are reported in Table II and in Fig. 2. The cysteine values were read on the second colorimeter at the 440 nm wavelength, as the absence of proline permitted the evaluation of cysteine.

Literature reports[6,9] state that, when tryptophan and cystine are both present in an acidic hydrolysis medium, in the absence of oxygen, sugars and metals, an oxidation–reduction equilibrium takes place, with the formation of 2 molecules of cysteine for every molecule of cystine and with consequent tryptophan oxidation. The oxidation–reduction reaction between tryptophan and cystine under the con-

TABLE II

RESULTS OBTAINED BY TREATING TRYPTOPHAN AND CYSTINE IN THE PRESENCE OF EACH OTHER AND IN THE ABSENCE OF THE OTHER AMINO ACIDS

The amino acid values are expressed as peak heights in mm over the base line of the chromatograms.

Time (h)	*Cystine*	*Tryptophan*	*Cysteine*	*Unknown*
0	48	42.5	0	0
24	32	24.5	15	3.5
48	26	15	26	4
72	23.5	13.5	30.5	3.5

ditions of our experiment gives rise, in addition to cysteine, to an unknown substance which, in the short column amino acid chromatogram eluted with sodium buffers, emerges just before tryptophan, as can be seen in Fig. 3.

With regard to cysteine, if the long column amino acid analysis is carried out by elution with sodium buffers, this amino acid cannot be separated, in that it has the same retention volume and the same absorbance maximum, when reacted with ninhydrin color reagent, as proline. The apparent increase in the proline values which can be seen in Table I and in Fig. 1, is satisfactorily explained in this way.

All our efforts, such as varying the column operational parameters, *e.g.* resin volume, temperature and pH, in our protein amino acid analysis method[10], in order to find suitable conditions for the separation of these two amino acids, were unsuccessful.

Since WAINER[12], who employed a different resin and the gradient buffer system of PIEZ AND MORRIS[13] for column elution, also did not succeed in obtaining this separation, it can be assumed that cysteine cannot be resolved from proline when sodium buffers are used as eluting media.

The resolution of this doublet became possible by employing the method for amino acids and related compounds in physiological fluids described by MONDINO[14] which uses lithium buffers for eluting Amberlite IR-120 crushed resin columns. Both

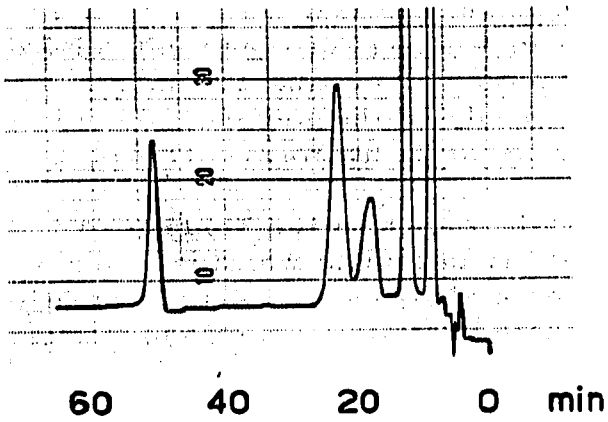

Fig. 3. Short column chromatogram of the reaction products which are formed when tryptophan and cystine alone are boiled for 48 h under hydrolytic conditions. The first two peaks from the left are ammonia and tryptophan. Just after tryptophan the peak of an unknown substance can be seen.

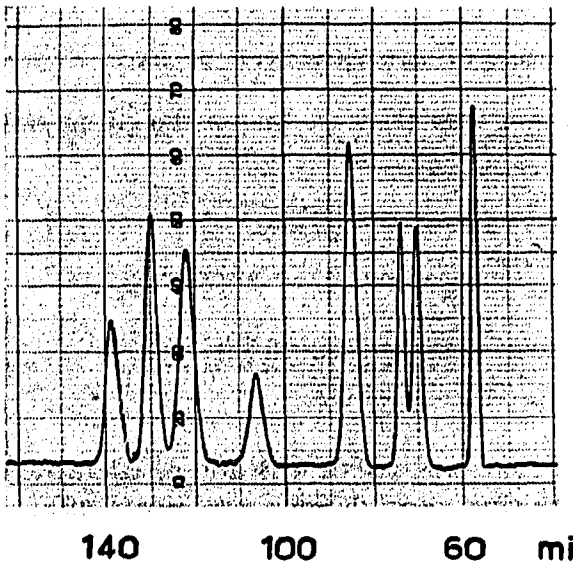

Fig. 4. Example of a separation with lithium buffers on Amberlite IR-120 crushed resin of proline from cysteine, originating from tryptophan. A sample of an 18 protein amino acid mixture after 48 h of hydrolytic treatment, has been chromatographed. The amino acids reported (200 nmoles load) are, from the left: alanine, glycine, proline, cysteine, glutamic acid, serine, threonine and aspartic acid, read on the second colorimeter at 440 nm, provided with a 10 mm light path cuvette and set at 0.5 O.D. for a full scale deflection.

peaks are read and recorded on the second channel when this is reading at 440 nm. In this system cysteine is eluted before proline as can be seen in Fig. 4, where an example of the separation of proline from cysteine with lithium buffers is shown.

It has to be stressed that, if a reducing sugar such as glucose, is added to the cystine–tryptophan mixture before acidic treatment, the formation of cysteine can be stopped.

Consequently, if a pure and isolated protein, containing in its molecule cystine and tryptophan residues, is submitted to acid hydrolysis by the open flask method in the total absence of sugars, metals and oxygen, the problem of the accurate determination of the proline residues, if present, can only be solved by employing lithium buffers to elute the resin columns of the automatic amino acid analyser.

Moreover, it has to mentioned that tryptophan, if present in a pure and isolated protein which does not contain in its chain any residue of cystine, can be directly determined by analysing the acid hydrolysate (provided that the previously described hydrolytic conditions are respected), without carrying out the hydrolysis of the protein in a basic medium.

Finally, some conclusions can be drawn with respect to the volumes of the solution submitted to hydrolysis and to the quantitity of protein which is available.

The volume of 250 ml which was employed in the above examples can be greatly reduced. Particularly, when sampling at intermediate hydrolysis times from a single flask is not required, the open flask method can be easily employed with volumes not exceeding 10 ml, provided that micro-chemistry glassware, easily available (Quickfit), is employed.

As far as the protein–acid ratio is concerned, it has to be stressed that any reduction of the concentration, which was chosen for the experiments reported here,

can be made, provided that the sensitivity limits of the analytical system available for the amino acid analysis are taken into consideration. As a matter of fact, a diminution of the protein–acid ratio will always improve the hydrolytic conditions, consequently minimising any interaction.

From this it can be deduced that, even in cases where only a few mg of protein are available for the investigation of the amino acid composition, the open flask method of acid hydrolysis can be adopted.

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