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APPLICATIONS OF GAS–LIQUID CHROMATOGRAPHY IN PROTEIN CHEMISTRY

II. DETERMINATION OF AMIDE RESIDUES IN NANOMOLAR AMOUNTS OF PROTEINS

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SUMMARY

A method for the quantitative determination of amide residues in nanomolar amounts of proteins is described, based on dilute acid hydrolysis at 100°, followed by isothermal gas–liquid chromatography of the ammonia released by on-column neutralisation of the hydrolysate and quantitation by means of a conductometric detector. Amide contents are given for twenty well characterised proteins, as well as for asparagine and glutamine.

INTRODUCTION

Up to the present time three main methods have been used for the determination of amide residues in proteins. (1) Hydrolysis by dilute mineral acids for varying periods followed by neutralisation of the hydrolysate and estimation of the ammonia liberated. On the micro-scale this has usually been carried out by the Conway micro-diffusion method combined with colorimetric assay of the ammonia. (2) Determination of free ammonia during the ion-exchange determination of amino acids in protein hydrolysates. This suffers from the disadvantage that the conditions necessary for the complete hydrolysis of all amino acids (usually 6 *M* HCl for 24–72 h) are not optimal for ammonia, and additional ammonia will be set free by the decomposition of certain amino acids such as serine and threonine. Thus incorrect results may be obtained unless the results from several hydrolyses of different duration are extrapolated to zero time. (3) Assignment of glutamine and asparagine residues during sequencing of enzymatic hydrolysates. This is probably the most precise method, but obviously is not always available.

The need for a precise amide determination applicable to minimal amounts of valuable proteins in this laboratory drew our attention to alternatives to the usual methods, and in particular to the possibility of isothermal gas–liquid chromatography (GLC) of ammonia at relatively low temperatures. On-column liberation of ammonia

from acid hydrolysates would also minimise atmospheric contamination, especially as very few nitrogenous compounds could pass through the column under low-temperature operation. An important problem was, however, the selection of a suitable quantitative detection system, since many of the commonly used GLC detectors, notably the stable and reliable flame ionisation detector, are insensitive to ammonia[1]. Our final choice was the sensitive and relatively specific conductance detector (Coulson[2]), especially since Cochrane and Wilson[3] have shown that this detector is able to detect ammonia at the nanogram level. The Coulson conductance detector is commercially available, but in this paper we describe a simple and stable conductance detector assembled from laboratory equipment which may be useful to those laboratories which have only occasional use for this detector.

Another problem encountered in the GLC of ammonia is persistent zone tailing in many chromatographic systems. In agreement with the results of Lindsay Smith and Waddington[4] with aliphatic amines, we have found that using polystyrene beads (Porapak Q), zone tailing may be virtually eliminated by pre-coating with polyethyleneimine and potassium hydroxide. Isothermal GLC of ammonia in this system could be carried out at the low operating temperature of 68°. Combination of this chromatographic system with on-column neutralisation of dilute acid hydrolysates with barium hydroxide has provided a highly specific method for the determination of amide residues on nanomolar amounts of proteins.

MATERIALS AND METHODS

Chemicals

Porapak Q (bead form, 80–100 mesh) was supplied by Waters Assoc. (Milford, Mass., U.S.A.). AnalaR-grade barium hydroxide and concentrated hydrochloric acid, Dowex 1-X8 (20–50 mesh), Amberlite Monobed Resin MB-1, analytical grade (20–50 mesh), polyethyleneimine, L-glutamine and L-asparagine were supplied by BDH, (Poole, Great Britain). N-Acetyl-D-glucosamine was obtained from Hopkins & Williams (Chadwell Heath, Great Britain).

Proteins

α-Chymotrypsin (3.4.4.5) (prepared from four times recrystallized chymotrypsinogen A), bovine insulin, crystalline bovine trypsin (3.4.4.4), soya bean trypsin inhibitor (Kunitz) and twice recrystallized porcine pancreatic elastase (3.4.4.7) were obtained from BDH; crystalline sperm whale myoglobin, horse myoglobin, chicken lysozyme (twice crystallized) (3.2.1.17), bovine β-lactoglobulin (three times recrystallized), ovalbumin (five times recrystallized), crystalline bovine serum albumin, papain (ex *Papaya latex*, twice recrystallised) (3.4.4.10), and porcine pepsin (three times recrystallized) (3.4.4.1) from Koch-Light Labs. (Colnbrook, Great Britain); crystalline ribonuclease A (2.7.7.16) from Boehringer (Mannheim, G.F.R.); ribonuclease B, horse heart cytochrome *c* and chymotrypsinogen A (six times recrystallized) from Miles-Seravac (Maidenhead, Great Britain); horse myoglobin from Serva (Heidelberg, G.F.R.); and bovine serum albumin (crystalline) and porcine pepsin (3.4.4.1) from Armour Pharmaceutical Co. (Eastbourne, Great Britain).

Proteins supplied as crystalline or lyophilized powders were used without further purification. Elastase was supplied as a suspension in water. The suspension

was centrifuged, and the solid re-suspended in water and centrifuged. The process was repeated twice, and the protein was dried over P_2O_5. Papain was also supplied as a suspension, but as it is soluble in water, it was dissolved in water and precipitated with redistilled acetone. This process was repeated three times, and the protein finally dried over P_2O_5 *in vacuo*.

Instrumentation

A Pye Series 104 gas chromatograph was used as the basic chromatographic unit, purified nitrogen being employed as carrier gas. The output from the column was led into a gas–liquid mixer–separator unit (Figs. 1 and 2), which was supplied with a constant flow of de-ionized water. The detailed construction and dimensions of the mixer–separator unit which was fabricated from a 5 × 2 × 1 cm thick block of perspex are shown in Fig. 2. The liquid output from the separator was divided into two streams, one passing through the conductance cell (Radiometer Type CDC 314, cell constant 0.316, total volume 1 ml), and thence to the reservoir (Fig. 1). The bypass stream together with nitrogen gas from the column passed directly to the reservoir. Water from the reservoir was continuously recycled by a centrifugal pump through a jacketed 40 × 1.5 cm column, the lower portion of which was packed with 50 ml of Dowex 1-X8 anion-exchange resin (20–50 mesh) in the hydroxyl form, while the upper section was packed with 25 ml of Amberlite MB-1 mixed bed resin (20–50 mesh). Water emerging from this column at a flow-rate up to 15 ml/min had an electrolytic conductance of less than 0.1 μS. The conductance cell, mixer–separator and reservoir were placed in a water-bath maintained at 25 $\pm$ 0.5°, and water from the bath was also circulated through the jacket of the ion-exchange column. Winnett and Illingsworth[5] have demonstrated that control of the water temperature is essential for reproducible operation of the Coulson conductometric detector, there being a mark-

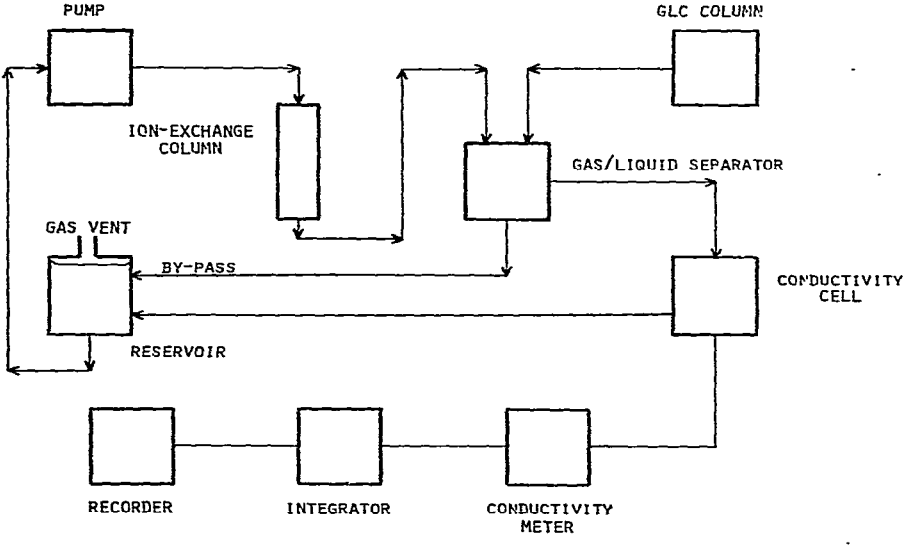

Fig. 1. Block diagram of the detector system.

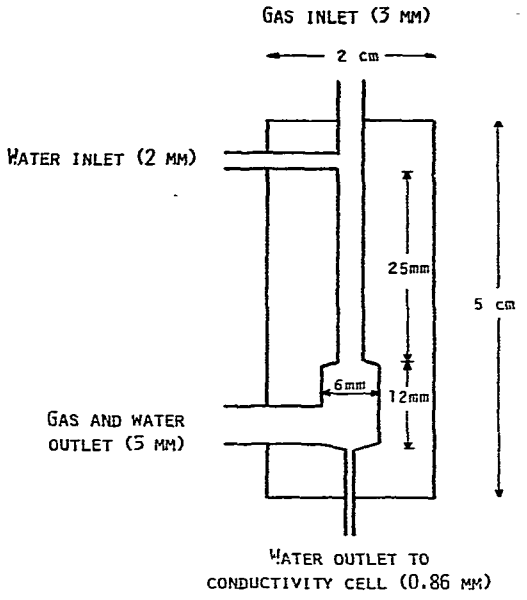

Fig. 2. Dimensions of the gas–water mixer and separator.

ed reduction of sensitivity at higher temperatures. This effect was minimal in the temperature range 22–26°.

The electrolytic conductance between the electrodes of the Radiometer CDC 314 cell was measured by a Radiometer CDM 3 conductance meter, operating at an a.c. frequency of 2 kHz on the 50 μS/full-scale deflection range. This corresponds to an output of 1.0 V, which was applied to an Autolab Model 6300 digital integrator with a printed output and peak areas were expressed in counts/sec. The output from the integrator could be varied and was usually operated at a quarter of the maximum output, which gave reasonable peak sizes when applied to a Pye Unicam Series AR 25 potentiometric recorder operating on the 10-mV input range.

Methods

Water content of the protein samples. Protein samples were not dried before hydrolysis, the water content of each protein being determined on 10–20 mg samples by drying *in vacuo* (better than 0.1 Torr) over P_2O_5. The weight of protein taken for analysis was then corrected for water content. This procedure obviated weighing problems with proteins which became very hygroscopic on intensive drying, but may be impractical when only very small samples of the protein are available.

Ammonia content of the protein samples. Chibnall *et al.*[6] showed that certain protein samples contained free ammonia (presumably as the ammonium salts of the dicarboxylic amino acids), which vitiated the results of amide analyses, and suggested a method for its estimation. The protein (10 mg) was dissolved or suspended in methanolic HCl (1.0 ml, 0.033 *M*), and at once precipitated with diethyl ether (2.0 ml). The mixture was centrifuged, the supernatant liquid removed, and the process repeated. A known amount of methylamine hydrochloride was added to the combined

supernatant liquids as internal standard, and the solvent removed. The residue was dissolved in 1 *M* HCl (25 μl), and 1-μl samples of this solution were analysed by the GLC method described below.

Protein hydrolysis for amide determinations. Although the recommended method for the determination of the amide content of proteins involves hydrolysis with 2 *M* HCl for various peroids of time (2–8 h) and extrapolation to zero time (Wilcox[7]), others, *e.g.*, Chibnall *et al.*[6], Marks *et al.*[8], and Spiro and Spiro[9] have used a single 3-h hydrolysis period.

We have investigated the rate of release of ammonia from β-lactoglobulin by 2 *M* HCl at 100°, using the GLC analytical method described in this paper. The results are given in Table I, and show that the release of ammonia is substantially complete (98%) in 2 h, and remains constant until at least 4 h. Increased amounts are obtained after 24-h hydrolyses, presumably due to degradation of serine and threonine. In agreement with the workers cited above, we have therefore adopted a standard hydrolysis time of 3 h, with 2 *M* HCl at 100°.

TABLE I

RATE OF RELEASE OF AMMONIA FROM β-LACTOGLOBULIN BY 2 *M* HCl AT 100°

Time (min)	*Ammonia released (moles)*
5	1.20
15	16.79
30	25.42
60	26.99
90	26.93
120	27.63
180	27.61
240	27.69
420	27.93
1440	31.92

The protein (0.2–1.3 mg, sufficient to give 4–14 μg of ammonia after hydrolysis) was dissolved or suspended in 2 *M* HCl (0.4 ml) containing a known weight of internal standard (methylamine hydrochloride) and hydrolysed for 3 h in a sealed glass tube. The hydrolysate was centrifuged, and the supernatant liquid evaporated to dryness using a rotary evaporator at reduced (water pump) pressure. In agreement with Wilcox[7], we have found that this concentration procedure involves no loss of amine hydrochlorides. The residue was dissolved in 1 *M* HCl (25–40 μl) and 1-μl aliquots injected directly into the column.

GLC of ammonia. (a) Preparation of the GLC column. Porapak Q (80–100 mesh, 7.44 g) was placed in a flask, just covered with methanol and a solution of polyethyleneimine (0.4 g) and KOH (0.16 g) in methanol was added. After thorough mixing the excess methanol was removed using a rotary evaporator, and the coated material dried at 110° for 18 h. The amounts given correspond to a coating of 5% polyethyleneimine and 2% KOH. The coated material was rapidly screened and the 80–100 mesh fraction packed into a 1.5 m × 4 mm all-glass helical column. The top

5 cm of the column were left unpacked. The column was conditioned by passing nitrogen at a flow-rate of 100 ml/min through the column at 150° for 24 h.

(b) Transfer and neutralisation of the acid hydrolysates. Neutralisation of the hydrolysates was achieved by injecting an aliquot into a plug of barium hydroxide formed at the top of the column (Ayres[10]). Commercial AnalaR grade barium hydroxide octahydrate was recrystallized from water and dried at 90° overnight. It was then ground sufficiently finely to pass through a 150-mesh screen, and a 2.5-cm plug of the powdered material was placed on the top of the coated Porapak Q in the column. The column was then replaced in the chromatograph oven, operating conditions established and a 1.0-μl aliquot of the hydrolysate injected directly through the septum. Barium hydroxide on-column neutralisation has many practical advantages, and if operated below 80° no pyrolysis products are released into the column. The system has, however, certain disadvantages. As previously observed by Ayres[10], no more than 10–15 μl of water could be injected into the barium hydroxide plug, either in aliquots or as a single injection, without deterioration in column performance. This necessitated changing the plug after at most fifteen 1-μl sample injections. Also H_2SO_4 could not be used for the hydrolysis of proteins, as successive injections of this acid also resulted in a progressive decrease in the response to ammonia. No such effect was observed with HCl. Neutralisation of the hydrolysate prior to injection gave low ammonia values, possibly due to loss into the partial vacuum produced during manipulation of the injection syringe.

(c) GLC operating conditions. The operating parameters (water flow-rate, cell/bypass flow ratio, carrier gas flow-rate and column and injection port temperatures were adjusted to give: (i) A good detector response without excessive peak tailing. As previously observed by Coulson[2], a decreased water flow-rate increased the sensitivity, but led to peak tailing and distortion. (ii) Rapid elution of ammonia and methylamine to give sharp peaks and a short analysis time.

The optimum conditions were found to be: nitrogen carrier gas flow-rate, 70 ml/min; oven temperature, 68°, isothermal; injection port temperature, 68°; water flow through conductance cell, 4.0 ml/min; water flow through bypass, 7.0 ml/min; detector sensitivity, 50 μS f.s.d.; paper chart speed, 0.2 cm/min.

Under these operating conditions, methylamine, dimethylamine, ethylamine, and trimethylamine could be eluted in addition to ammonia. Their retention times are given in Table II. Methylamine was chosen as internal standard as the zone pro-

TABLE II

RETENTION TIMES OF AMMONIA AND SOME ALIPHATIC AMINES UNDER EXPERIMENTAL CONDITIONS AS DESCRIBED IN THE TEXT

Amine	*Retention time (sec)*
Ammonia	66
Methylamine	222
Dimethylamine	552
Ethylamine	625
Trimethylamine	952

file and molar response were very similar to that of ammonia, but it was well separated from the latter.

A typical experimental protocol is given in Table III. The coefficient of variation both of the molar ratio ammonia/methylamine and of the amide analyses was of the order of 2%, suggesting that the precision of the determination of the molar ratio is the predominant factor in determining the overall precision of the analysis.

TABLE III

HYDROLYSIS OF LYSOZYME (DRY WEIGHT 0.216 mg) FOR 3 h AT 100° IN 2 *M* HCl (0.4 ml, CONTAINING 49.15 μg OF METHYLAMINE)

A = Zone areas of ammonia standards; B = zone areas of methylamine standards; C = relative molar responses of ammonia to methylamine; D = zone areas of ammonia from the hydrolysate; E = zone areas of methylamine internal standards; F = micrograms of ammonia released from 1 μl of the hydrolysate.

Run No.	*Standards*			*Hydrolysate*		
	A	*B*	*C*	*D*	*E*	*F*
1	148 450	345 248	1.04			
	141 180	315 005	1.11	76 572	414 706	4.57
	155 447	349 953	1.10	82 047	461 545	4.40
	174 361	405 893	1.06	75 037	434 455	4.31
	185 640	437 600	1.05	68 547	382 876	4.47
	173 425	400 805	1.07	64 035	361 030	4.43
	162 852	365 893	1.10			
2 (repacked $Ba(OH)_2$)	161 093	401 563	0.99			
	183 288	434 643	1.04	75 917	444 815	4.46
	135 578	309 055	1.08	60 504	336 942	4.61
	155 892	368 192	1.05	70 099	404 693	4.45
	165 989	388 693	1.05	64 371	372 700	4.43
	175 400	411 303	1.05	72 768	416 495	4.48
	169 970	404 421	1.04			
Mean ± 1 S.D.			1.06 ± 0.022			4.46 ± 0.08
Coefficient of variation, %			2.07			1.78

RESULTS

Table IV gives the moles ammonia liberated per mole protein under our conditions of acid hydrolysis compared with the "theoretical" ammonia yields calculated from the corresponding sequence studies tabulated by Croft[11]. In the majority of cases the precision of analysis was good enough to assign the number of amide residues to ±1 residue in 20–30. Elastase was an exception and the low result must reflect the quality of our preparation, as it was not improved by further replicate analyses. The amide analyses of asparagine and glutamine were carried out on 100-μg samples, and serve as checks on the overall precision of the method. N-Acetyl-D-glucosamine was included because of its occurrence in glycoproteins. It will be seen that its contribution to amide nitrogen would be negligeable. There has been disagreement in the literature regarding the amide content of myoglobins[12–14]. Our results support the lower estimates in both whale and horse myoglobin. Only our prep-

TABLE IV

AMIDE AND FREE AMMONIA CONTENTS OF SOME PROTEINS AND OF RELATED COMPOUNDS

	μg free ammonia/mg of protein	*Moles NH_3 found/mole protein*	*Moles NH_3/mole protein calculated from sequence studies*
N-Acetyl-D-glucosamine	—	0.02	0
L-Asparagine	—	1.002	1
L-Glutamine	—	0.981	1
Bovine insulin	Negligeable	6.23	6
Sperm whale myoglobin	0.135	6.19	6 or 7
Seravac horse myoglobin	Negligeable	8.17	8 or 9
Koch-Light horse myoglobin	0.125	7.54	8 or 9
Horse heart cytochrome *c*	0.295	8.11	8
Soya bean trypsin inhibitor	0.798	13.71	14
Lysozyme	0.429	16.99	16
Ribonuclease A	Negligeable	16.76	17
Ribonuclease B	Negligeable	18.25	17
α-Chymotrypsin	3.112	22.87	23
Chymotrypsinogen A	0.077	23.87	24
Armour porcine pepsin	0.140	26.16	25
Koch-Light porcine pepsin	0.180	26.10	25
Papain	Negligeable	24.51	26
β-Lactoglobulin	0.1052	28.01	28
Trypsin	Negligeable	27.17	29
Ovalbumin	Negligeable	33.70	30–32
Elastase	Negligeable	26.92	33
Armour bovine serum albumin	Negligeable	33.72	34–37
Light bovine serum albumin	Negligeable	32.04	34–37

aration of α-chymotrypsin gave a significant free ammonia content by the method of Chibnall *et al.*[6].

DISCUSSION

It must be emphasized that although only 2–20 μg of the protein hydrolysate are actually used for a single analysis, the method as described does not approach the limits of sensitivity possible, but is rather chosen to avoid the difficulties of weighing low microgram amounts of possibly hygroscopic proteins. A tenfold higher sensitivity is available in the Radiometer CDM 3 conductance meter, although its use would involve modification to the zero setting arrangements of the actual recorder used. The volume of the conductance cell could also be reduced with advantage. Hall[15] has described a number of improvements to the original Coulson[2] detector, claimed to increase its sensitivity 20–50 times, the most important being a smaller conductance cell constructed of PTFE, and the replacement of water by 50% aqueous propan-2-ol as circulating liquid. These claims were only partially confirmed by Wilson and Cochrane[3]. We have not investigated them further, but it would appear that a picogram-scale ammonia determination with a precision ($\pm 2\sigma$) of $\pm 5\%$ or better is quite feasible.

The specificity of the method described here is very high, as not only must any contaminant pass through the GLC column with a retention time similar to that of ammonia, but it must also dissolve in water to give a conducting solution. An important advantage of the present method (in contrast to the micro-Conway diffusion method) is that it does not require rigorous cleaning and careful storage of glassware, as the only glassware used are the syringe and the sealed ampoules which are made from disposable Pasteur pipettes and used once only.

Although described here for amide determinations in proteins the method is obviously applicable for many similar ammonia estimations, *e.g.*, asparaginase or glutaminase determinations. The rapidity of the GLC technique makes it very suitable for multiple analyses, as in the investigation of enzyme kinetics.

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