CHROM. 5800

A new highly sensitive detection system for peptides and proteins in column effluents

An important problem in protein sequence studies is the separation of the peptides after enzymatic hydrolysis of the protein. Of the many methods which exist and can achieve this separation, column chromatography is an attractive one; however, the main difficulty of this method is the detection of the peptide material in the column effluent.

A highly sensitive automatic analyser has been designed for the detection of peptide material in column effluents and will be described in this paper. The underlying chemical principle is alkaline hydrolysis of the peptides, followed by staining with ninhydrin. The apparatus as described in this paper is outstanding in its sensitivity, reliability and economy. The very high sensitivity was obtained as a result of using Teflon tubing with a very small inner diameter and very slow pumps.

When column chromatography has to be applied repeatedly only a small amount of the effluent can be used for detection. Only 10 μl of each fraction were found to be necessary in this case. Even when fractions as small as 1 ml are taken, this means that only 1 % of the effluent is lost for the detection.

Operation of the apparatus

The operating principles of the apparatus can be divided into seven steps, *viz.* (1) sample application; (2) the mixing of the sample (a small amount of column effluent) with 10 *N* KOH to give a solution of sample in 5 *N* KOH; (3) incubation of the sample for 90 min in 5 *N* KOH at a temperature of 100°; (4) neutralisation of the solution with glacial acetic acid; (5) addition of ninhydrin solution; (6) incubation with ninhydrin solution for 5 min at a temperature of 100°; (7) reading of the optical density at 570 nm with a micro flow-through cuvette, and recording the extinction of the solution.

Resolution of the operating problems in steps 2–7. Sample application will be discussed separately. The main problems presented by steps 2–7 are the two incubation steps, 3 and 6. Step 3, which takes place over a very long period of time, is especially difficult. A solution has been found after studying the properties of liquid streams in Teflon tubes[1]. It was found that the diffusion and peak distortion decreases when the diameter of the tubing decreases. This means that when incubation for 90 min is performed in a "large" diameter tube, *e.g.* 2-mm cross-section, peak distortion is important, but when this incubation is performed in a "small" diameter tube, *e.g.* 0.3 mm, this peak distortion is far less important.

Establishment of these facts suggested the design of the apparatus, *viz.* long incubation times should be performed in long, small-diameter Teflon tubing. Air or nitrogen bubbles are not necessary in this case, because peak distortion is small. Mixing and addition of reagents (steps 2, 5 and 6) can be performed by means of low-velocity syringe pumps with micro T-connectors. Step 7 can be realized with a commercially available spectrophotometer–recorder combination.

Sample application. In conventional apparatus[2,3] a peristaltic pump has

been used and the sample, part of the effluent from a chromatographic column is sucked in. Here very slow syringe pumps are used, and suction is thus not possible

The problem of sample application has, however, been overcome, as is shown schematically in Fig. 1. Two very small sample loops (A and B) with a volume of 10 μl are coupled to a four-way microvalve. Column effluent is pumped from the column (III) via the sample loop B to the fraction collector (IV). Distilled water is pumped from the low-velocity syringe pump (I) via sample loop A to the detection system (II). When the fraction collector changes to the next tube the valve turns through 90° and sample loop A is positioned in the effluent line. The contents of loop B, 10 μl of column effluent, are pumped to the detection system. When the timing is right, and the volume of the tubing IV to the fraction collector is just half a fraction every 10-μl sample will be representative of the corresponding fraction. The retention time of the apparatus, approximately 2 h, is very reproducible, so it is possible to link every peak on the recorder diagram with its corresponding 10-μl sample.

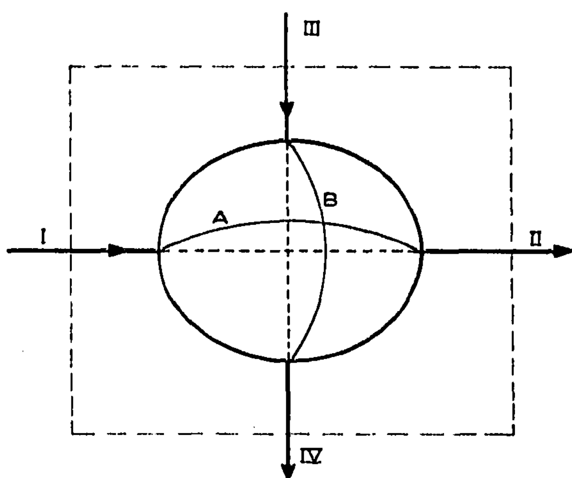

Fig. 1. Diagram of the sample applicator. A and B are two sample loops, 10 μl each. I = distilled water from pump with flow rate $\frac{1}{2}$ ml/h; II = connection with detection system; III = flow from column via B or A to fraction collector (IV).

Description of the apparatus

Fig. 2 gives a schematic diagram of the apparatus. Pump I delivers distilled water (1) and 10 N KOH (2). The sample, part of a column effluent (3), is introduced via the sample applicator IV, and is mixed with 10 N KOH in the manifold V to give a solution of peptides in 5 N KOH. The peptides are hydrolyzed in the Teflon reaction coil VI (length 21 m, I.D. 0.3 mm). Hydrolysis is performed at 100° for 1 h 30 min After hydrolysis the alkaline solution is neutralized with glacial acetic acid and mixed with ninhydrin reagent. The mixture is then heated at 100° for 5 min in the Teflon coil VIII (length 2.5 m, I.D. 0.3 mm). The ninhydrin reagent is the same as is used in routine amino acid analysis[4].

After reaction with ninhydrin reagent the optical density is measured at 570 nm in a spectrophotometer equipped with micro flow-through cells. Two flow cells with optical path lengths of 2 mm and 10 mm, respectively, were used. It is necessary to place a back-pressure system as shown in Fig. 3 after the flow cells, otherwise

the liquids in coils VI and VIII will tend to boil. A recorder is used in combination with the spectrophotometer to give a recording of the chromatographic run. The apparatus can be used continuously for approximately 24–28 h. After this period of time the pumps have to be refilled.

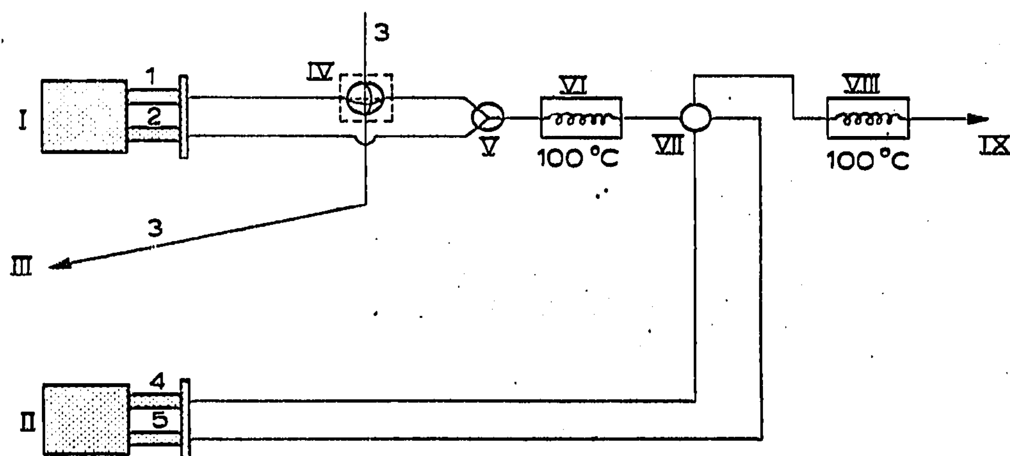

Fig. 2. Schematic diagram of the apparatus. I = pump: 1 = distilled water, 2 = 10 N KOH; II = pump: 4 = glacial acetic acid, 5 = ninhydrin reagent according to SPACKMAN *et al.*[4]; III = fraction collector; IV = sample applicator: 3 = flow from column, via sample applicator to fraction collector; V = three-way manifold; VI = reaction coil, length 21 m, I.D. 0.3 mm, held at 100° in a boiling water bath; VII = four-way manifold, or two three-way manifolds; VIII = reaction coil, length 2.5 m, I.D. 0.3 mm.; IX = to spectrophotometer.

Specifications for the different parts of the apparatus

Valve for sample application. Labotron Model 002002 automatic two-way motor valve.

Syringe pumps. Two Labotron Model LDP 11 A syringe pumps, fitted with a Model 001952 motor and Model 001958 pump system. The delivery rate is 2 × 0.5 ml/h for each pump.

Manifolds. One three-way manifold, Labotron Model 001864, and one four-way manifold, Labotron Model 001863, were used. It is also possible to use three three-way manifolds.

Reaction coils. Habia thin-walled Teflon tubing with an I.D. of 0.3 mm and an O.D. of 0.9 mm. The Teflon coils are refluxed in boiling water when the apparatus is in operation.

Connections. Teflon tubing of various diameters can be used for connections before the sample applicator and after the spectrophotometer. Teflon tubing with a very small inside diameter (0.3 mm) should be used in the sample path. An exception to this is the connection between the first and the second flow cell of the spectrophotometer. A Teflon tube, length 15 cm and I.D. 0.4 mm, has been used.

Back-pressure system with manometer. The individual parts of the back-pressure system are described in the legend to Fig. 3. The manometer is an Econosto manometer, 0–25 atm, with a stainless-steel Bourdon gauge. A special fitting was made to connect the manometer to the system. The manometer is positioned after the spectrophotometer and before the back-pressure device. The working pressure is approximately 3 atm.

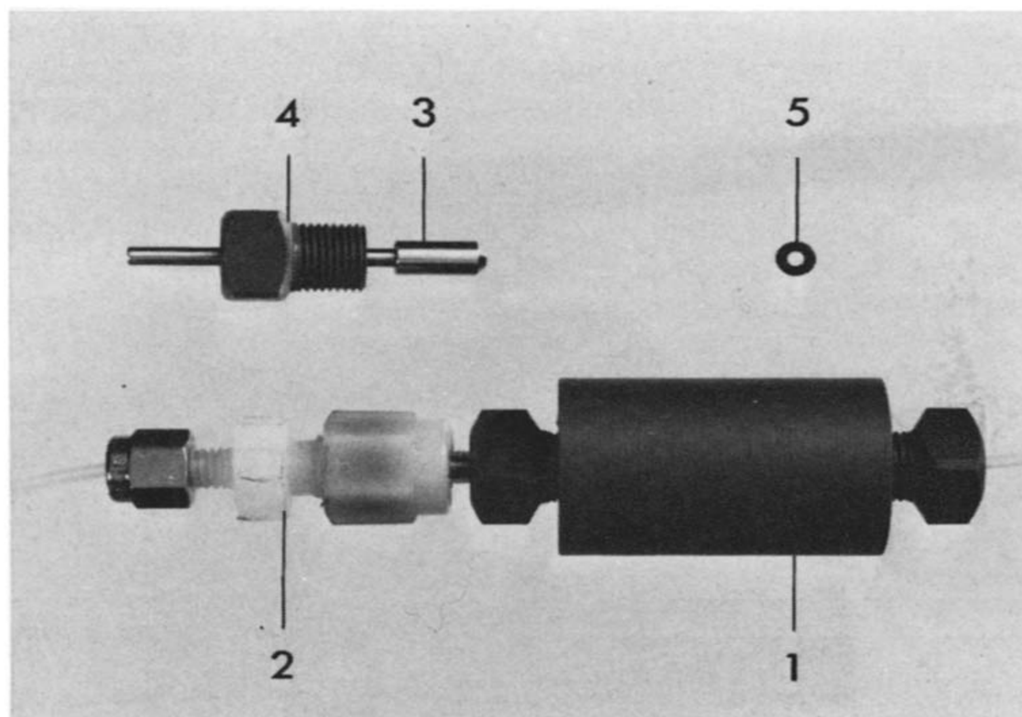

Fig. 3. Back-pressure system. 1 = Labotron Model 001537 connector with two O-fittings; 2 Crawford Model 200-6-1 reducing union, Zytel or stainless steel; Zytel or Teflon ferrules are recor mended; 3 = stainless-steel insert; 4 = screw of an O-fitting, slightly bored out; 5 = O-ri of the O-fitting. A small stainless-steel filter with a diameter of 4 mm has been placed und the insert (3) and O-ring (5) in the connector. This filter was cut from a Beckman Model 9385 stainless-steel filter. A small amount of fine ion-exchange resin was placed in the insert (3) to gi a back-pressure of approximately 3 atm to the system.

Filling valves for the pumps. In order to fill the pumps with the very corrosi reagents it was found advisable to use filling valves. One Labotron Model 0018(double two-way valve for each pump was found to be satisfactory.

Spectrophotometer–recorder combination. A Labotron UDC 1 with a Hartma and Braun Polycomp 2 recorder was used. Two channels are necessary for the app ratus, as described in this paper.

Cuvettes. Two microcuvettes were used, one with a 2-mm light path and o with a 10-mm light path. The cuvette with the smaller light path must be plac first, otherwise baseline troubles occur.

Connectors. Fig. 4 shows a standard Labotron connector, adapted for the ve small inside diameter tubing used.

Fraction collector. The fraction collector used was an LKB Model 7000. Th fraction collector has a connection for a Uvicord. This connection gives a conta pulse when the fraction collector makes a step.

Contact mechanism for the sample applicator. The contact mechanism w designed with three contacts, 1, 2 and 3. At the beginning of a sequence, contac 1 and 2 are closed and the contact mechanism is coupled with the Uvicord conta of the fraction collector. When the fraction collector makes a step a contact pul is given, and contacts 1 and 3 are closed. At the next step of the fraction collect

contacts 1 and 2 close again, etc. The sample applicator is coupled to the contact mechanism. When contacts 1 and 2 are closed loop A is in the sample line, when 1 and 3 are closed loop B is in the sample line.

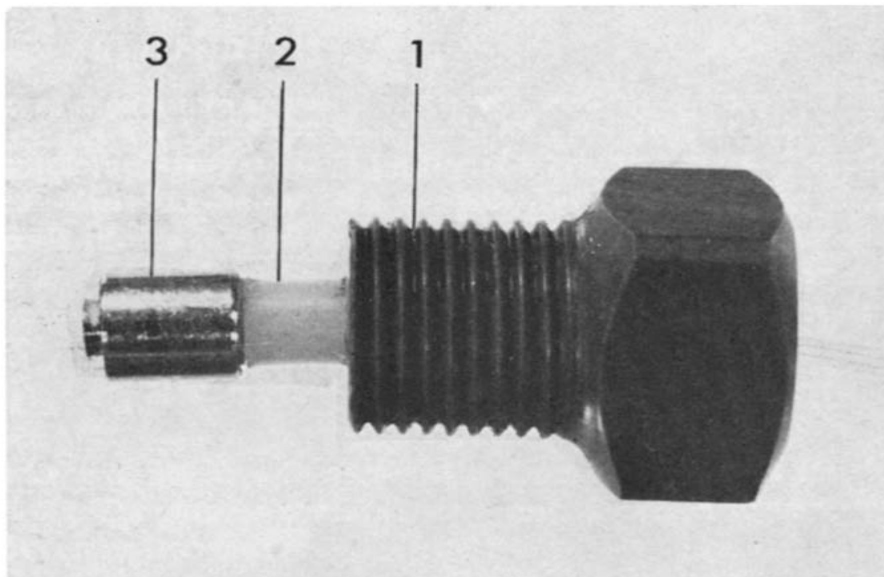

Fig. 4. O-fitting adapted to small-diameter Teflon tubing. 1 = Screw; 2 = small Delrin cylinder, with a hole adapted to the outside diameter of the Teflon tubing; 3 = insert of the O-fitting.

Experimental and results

Every 10-μl sample gives a peak, the height of which represents the quantity of ninhydrin-positive material present. The width of the peak at half height proved to be constant, independent of the height of the peak, as is demonstrated in Table I. The two cuvettes, 2-mm and 10-mm light path lengths respectively, gave rise to different peak widths, but the area under the peak, calculated according to the $H \times W$ method, proved to be proportional to the length of the light path, as would be expected.

Tabel II gives the results of the linearity tests on the apparatus. Because of the linear relationship between peak area and light path, results for only one cuvette

TABLE I

PEAK WIDTH AT DIFFERENT PEAK HEIGHTS

Glycine (mg/ml)	*Height × width, H × W (min)*						*Quotient* $\frac{H \times W\ 10\ mm}{H \times W\ 2\ mm}$
	Peaks obtained from 2-mm cuvette			*Peaks obtained from 10-mm cuvette*			
1 × 0.265	1.098	3.96	4.348	+	—	—	
1/2 × 0.265	0.511	3.92	2.003	1.572	6.16	9.684	4.84
1/4 × 0.265	0.256	4.00	1.024	0.792	6.24	4.942	4.83
1/8 × 0.265	0.111	3.92	0.435	0.342	6.32	2.161	4.97
1/16 × 0.265	0.053	4.08	0.216	0.174	6.46	1.124	5.20
1/32 × 0.265	0.025	4.00	0.100	0.077	6.20	0.477	4.77
1/64 × 0.265	0.013	—	—	0.047	6.12	0.288	
Mean ± S.D.	—	3.98 ± 0.06	—	—	6.26 ± 0.12	—	4.92 ± 0.17

TABLE II

TEST OF THE LINEARITY OF THE DETECTION

Glycine (mg/ml)	*Peak height*[a]		*Quotient*[b]		*Triglycine (mg/ml)*	*Peak height*[a]	*Quotient*[b]	*Haemoglobin (mg/ml)*	*Peak height*[a]	*Quotient*[b]
1 × 0.265	1.098	1.320			1 × 0.250	1.260		1 × 0.153	0.280	
			2.14	2.30			2.42			2.12
1/2 × 0.265	0.511	0.575			1/2 × 0.250	0.520		1/2 × 0.153	0.132	
			1.99	2.38			2.16			2.24
1/4 × 0.265	0.256	0.242			1/4 × 0.250	0.240		1/4 × 0.153	0.059	
			2.30	2.20			2.35			2.10
1/8 × 0.265	0.111	0.110			1/8 × 0.250	0.102		1/8 × 0.153	0.028	
			2.09	2.20			2.31			2.54
1/16 × 0.265	0.053	0.050			1/16 × 0.250	0.048		1/16 × 0.153	0.011	
			2.12	2.17			1.85			2.20
1/32 × 0.265	0.025	0.023			1/32 × 0.250	0.026		1/32 × 0.153	0.005	
			1.92	2.09			2.16			
1/64 × 0.265	0.013	0.011			1/64 × 0.250	0.012				

[a] Peak height as obtained with the 2-mm cuvette for glycine and triglycine and with the 10-mm cuvette for haemoglobin.
[b] Quotient of pairs of consecutive peak heights.

are given. Tests were carried out with the amino acid glycine, the peptide triglycine and the protein haemoglobin. A duplicate experiment is shown for glycine. When looking at the results, as given in Table II, we observe that the height of the peak is not precisely proportional to the concentration of the ninhydrin-positive material in the sample. An explanation of this fact cannot readily found. The deviation from linearity is not very large, however, and the quotient "Q" is reasonably reproducible: when the concentration is 2 times lower, the peak height is approximately 2.2 times lower.

The sensitivity per nmole of ninhydrin-positive material after hydrolysis has also been calculated. Table III presents the values obtained. Triglycine was found to have exactly the same sensitivity as glycine and it was concluded that triglycine is completely hydrolyzed after 90 min of incubation in 5 *N* KOH. The colour yield per nmole of haemoglobin is much lower (the amount in µmoles of ninhydrin-positive material per mg of protein was calculated by amino acid analysis). This observation leads to the conclusion that haemoglobin is only partially hydrolyzed.

This proved also to be the case when checking this with bovine serum albumin. The sensitivity proved to have values between 0.070 and 0.100 per nmole of ninhydrin-positive material in the 10-mm cuvette.

TABLE III

SENSITIVITY OF DETECTION AT DIFFERENT CONCENTRATIONS OF GLYCINE, TRIGLYCINE AND HAEMOGLOBIN

Glycine			*Triglycine*			*Haemoglobin*		
nmoles	*Sens./nmole*		*nmoles*	*Sens./nmole*		*nmoles*	*Sens./nmole*	
	2 mm	*10 mm*		*2 mm*	*10 mm*		*2 mm*	*10 mm*
35.5	0.035	—	39.7	0.032	—	—		
17.6	0.031	0.096	19.9	0.026	0.081	—		
8.8	0.028	0.088	9.9	0.024	0.079	12.4	0.0061	0.023
4.4	0.025	0.078	5.0	0.020	0.067	6.2	0.0057	0.021
2.2	0.023	0.077	2.5	0.019	0.063	3.1	0.0055	0.019
1.1	0.022	0.071	1.25	0.021	0.068	1.6	0.005	0.018
0.55	0.022	0.082	0.63	0.019	0.067	0.8	0.006	0.014

Conclusions

Although the chemical principles used in the apparatus described have already been applied in conventional designs of peptide analysers[2,3], a large increase in sensitivity was achieved after a study of the properties of liquids flowing in Teflon tubes with a very small inside diameter. It was found that when the inside diameter of the tubing used is very small and very slow pumping syringe pumps were used, no segmentation is necessary.

These pumps also proved to be very well suited for pumping the corrosive liquids 10 *N* KOH, glacial acetic acid, and the ninhydrin reagent used in the system. The system could be run for at least 24 h before refilling the pumps with a very small amount of reagent was necessary.

Only a 10-µl sample of every fraction is taken for hydrolysis and detection.

The sensitivity for glycine and triglycine was 0.070–0.100 per nmole of ninhydrin positive material and amounts even smaller than 1 nmole could be detected, the practical limit being approximately 0.2–0.6 nmole.

Carrying out the incubation in very small-diameter Teflon tubing and the use of very slow syringe pumps could provide a solution to many other complicated chromatographic detection problems.

The authors wish to express their thanks to Prof. Dr. H. BLOEMENDAL for his continuous interest in this work, and to Drs. C. H. DICKHOUT for giving the first author the time and opportunity to complete this work.

Department of Biochemistry,
University of Nijmegen,
Geert Grooteplein Noord 21,
Nijmegen (The Netherlands)

J. J. T. GERDING
H. J. M. KEMPE
B. J. M. LAMER
M. H. GERDIN

1 J. J. T. GERDING, B. J. M. LAMERS, TH. ZELDERS AND L. J. BEENTJES, in preparation.
2 G. N. CATRAVAS, *Anal. Chem.*, 36 (1964) 1146.
3 R. L. HILL AND R. DELANEY, *Methods Enzymol.*, 11 (1967) 348.
4 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
5 *Labotron Data Sheet* 683 L.D.P.

Received October 25th, 1971

* Present address: Johannes or Juliana Hospital, Zaandam, The Netherlands.