Journal of Chromatography, 494 (1989) 87–99
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 4857

SIMPLE APPARATUS FOR CAPILLARY ZONE ELECTROPHORESIS AND ITS APPLICATION TO PROTEIN ANALYSIS

V ROHLÍČEK and Z DEYL*

Institute of Physiology, Czechoslovak Academy of Sciences, Vídeňská 1083, 142 20 Prague (Czechoslovakia)

(First received March 24th, 1989, revised manuscript received May 10th, 1989)

SUMMARY

The construction of a simple apparatus for capillary zone electrophoresis is described, consisting of an optical system allowing direct absorbance measurement in the capillary in UV light, an evaluating electronic module and a high-potential source An attempt was made to achieve maximum sensitivity with a simple construction In the electronics, care was taken to obtain a quiet baseline and to optimize the signal-to-noise ratio That part of the noise which is of the frequency band of the signal is filtered off Both suction and electrophoretic sample introduction are possible According to operator's choice, the apparatus can be run under constant voltage or current and is protected against overloading The high-potential electrode chamber contains separate buffer and sample compartments and its construction offers an easy interchange between the running and sampling positions The applicability of the system to the separation of amino acids as phenylthiocarbamyl derivatives, peptides and both artificial and naturally occurring protein mixtures is demonstrated

INTRODUCTION

Capillary zone electrophoresis was recently reviewed by Karger et al [1] and the diverse chemistries applicable to this separation procedure were summarized One of the main areas in which capillary zone electrophoresis seems promising is the separation of proteins and their aggregates and fragments. Here it is likely to develop into a complementary technique to high-performance liquid column separations and electromigration separations in gel media A number of studies of the separation of amino acids, peptides and proteins have been reported [2–5] However, whereas the separation of low-molecular-

0378-4347/89/$03 50

mass peptides is relatively simple and can achieve sub-attomole levels, the separation of high-molecular-mass species is difficult owing to unpredictable adsorption of the solutes on the capillary wall This can be overcome either by running the separation at extremely high [6] or low [7] pH values or by the addition of, e.g , a low-molecular-mass amine to the buffer to counteract the negatively charged silica capillary surface [8] Modification of the capillary wall is another possibility [7] The potential of the selectivity of capillary zone electrophoresis in the area of protein chemistry has been demonstrated in the separation of seven low-molecular-mass peptides differing only in a single amino acid [7]

Whereas the principle of the method is relatively simple, its materialization is subject to a number of practical problems Although commercial apparatus has been announced [9], all the recent reports involve the use of laboratory-assembled devices, with general diagrams [8,10] without construction details Therefore, we describe here a simple, versatile apparatus suited particularly for amino acid, peptide and protein separations, and demonstrate its practical applicability Standard protein mixtures, protein extracts from tissues, protein cleavage fragments and protein aggregates were successfully separated

EXPERIMENTAL

Materials

For testing the applicability of the system, both artificial and naturally occurring mixtures of amino acids, peptides and proteins were used Standard series of amino acids (Millipore-Waters, Milford, MA, U S A) were converted into phenylthiocarbamyl derivatives prior to analysis (Pico-Tag Amino Acid Analysis Manual) A series of peptide standards (trypsin inhibitor, insulin chains A and B, angiotensinogen and actinomycin) was purchased from Serva (Heidelberg, F.R.G) Standard proteins were taken from the molecular mass standard kit supplied by Serva

Samples of muscle protein extract were prepared from rat hind-leg muscle homogenate using 2 5 m*M* borate buffer (pH 9 6), the extract was spun off at 20 000 *g* for 10 min and the supernatant was used directly for analysis

Samples of elastin were prepared by the method of Lansing et al [11] from adult bovine aorta and rabbit auricular cartilage

Type I collagen was prepared in the laboratory by acid extraction of rat-tail tendons [12] and its quality was tested by polyacrylamide gel electrophoresis Collagen chain polymers were prepared by incubating 1% collagen solution in 0.5 *M* Tris–phosphate buffer (pH 7 0) with a 10-fold excess of galactose (Koch-Light, Colnbrook, U K) [13] at 37 °C for an indicated period of time Incubation buffer was removed by exhaustive dialysis and the samples were lyophilized and reconstituted in the running buffer

Dansylamide (Sigma, St Louis, MO, U S A.) was used as an uncharged solute for endosmotic flow determination

With proteins and peptides, samples containing 0.5 mg/ml of the analysed standards or 1 mg/ml of a protein mixture were applied. All samples were introduced electrophoretically by applying 10 kV for 6 s, with the exception of amino acids, where a period of 15 s was used to introduce the sample into the capillary

Apparatus

The apparatus for capillary zone electrophoresis is shown in Fig 1 In principle, it consists of an optical system with a UV light source, electrode chambers, photodetectors, evaluating electronics module and high-potential power supply

Optical system As the light source, a deuterium lamp (a) in a thermal shield (b) fed with an electronically stabilized current source combined with an automated starting circuit (A) was used. The light passes through a selected interference filter Up to five different filters are available by rotating a revolving filter holder (c) from the instrument panel One position of this holder cuts off the light to permit checking (or compensation) of the dark current of the photodetectors An auxiliary mirror (d) reflects the light to the concave mirror (e), which focuses the light beam into the slit in the capillary plug-in The light intensity at the output of the capillary is evaluated by a signal photodetector (g)

The reference photodetector (f) is placed beside the concave mirror (e) The

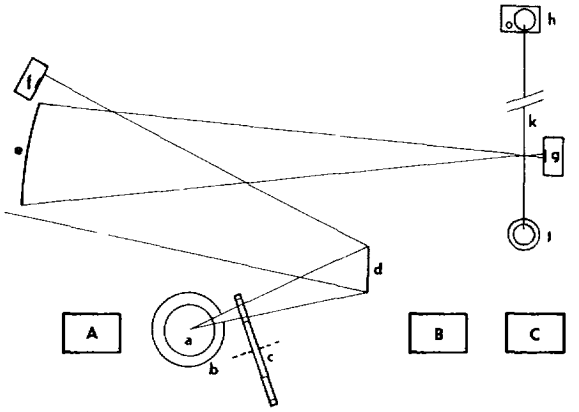

Fig 1 Schematic diagram of the apparatus a = Deuterium tube, b = thermal shield, c = revolving holder with interference filters, d = auxiliary mirror, e = concave mirror, f = reference photodetector, g = signal photodetector, h = high-potential electrode chamber, j = terminal electrode chamber, k = capillary A = Current source and starting circuit for the deuterium tube, B = evaluating electronics, C = high-potential source

capillary plug-in is inserted in a holder that allows precise adjustment of the plug-in in the path of the light beam (not shown).

Interference filters offering measurement at 220 and 280 nm were used

Electrode chambers Both electrode chambers are made of Perspex and equipped with carbon electrodes. The terminal electrode chamber (j) is air-tight to allow sample introduction by aspiration. The high-potential electrode chamber (h) contains two compartments, one filled with the running buffer and the other containing the sample. Both the sample and buffer compartments have their own electrodes connected to the high-potential power supply. This set-up allows electrophoretic sample introduction. As demonstrated in Fig 2, the high-potential electrode chamber is placed in a frame that permits its precise movement and interlocking between two positions, i e , between the running position and the sample introduction position. The capillary is fixed in a holder by a tightening screw and in either position penetrates through the hole in the wall of the appropriate compartment

Capillary An untreated fused-silica capillary (100 μm I.D., 300 μm O.D.) (Institute of Physics of the Electro-Physical Research Centre, Slovak Academy of Sciences, Bratislava, Czechoslovakia) protected with a silicone-rubber layer was used in our experiments

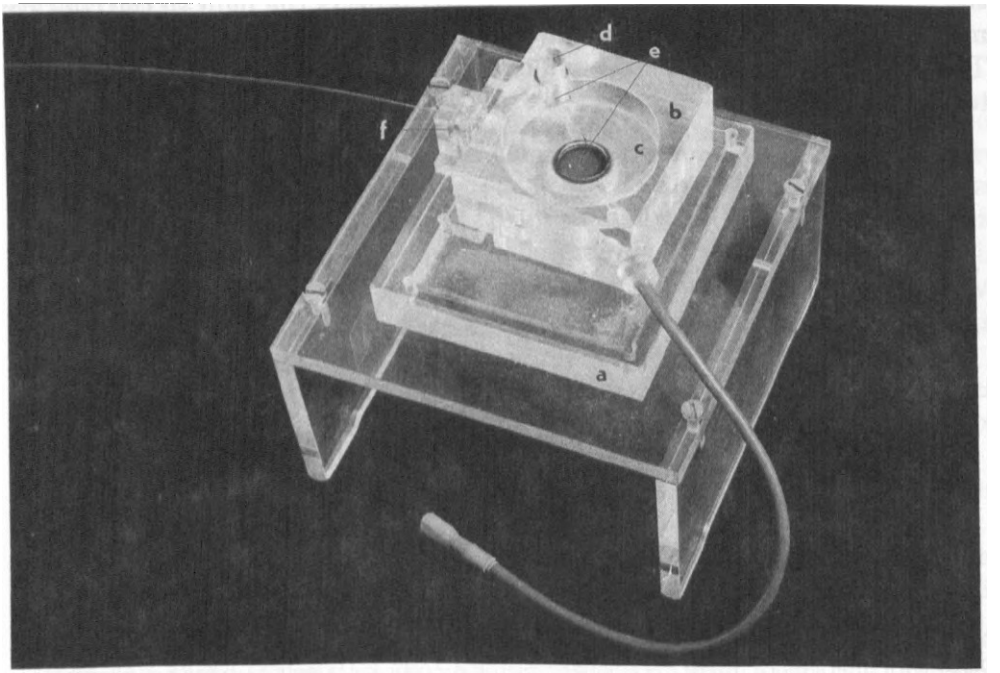

Fig 2 High-potential electrode chamber a = Frame, b = electrode chamber, c = buffer reservoir, d = sample compartment, e = carbon electrodes, f = capillary holder

Photodetectors A Hamamatsu R 1826 vacuum tube (Hamamatsu Photonic, Shimokanzo, Japan) was used for the detection of UV light This phototube, together with the preamplifier, is mounted in a metal box A current–voltage conversion circuit with an electrometric amplifier serves as a preamplifier Attention was paid to careful electrostatic shielding of the phototube by a system of diaphragms, to prevent capacitive transmission of electric interferences from the capillary The second point was proper construction of those parts of the circuit which require a high degree of insulation The band width of the UV photodetector is about 20 Hz The time response of the apparatus to the signal depends, however, on the low-pass filter

Both the signal and reference photodetector are of the same design

Evaluating electronic module A simplified diagram of this module is presented in Fig. 3 Both signals from the photodetectors are connected with logarithmic amplifiers (a) In the reference signal path an attenuator (b) is inserted for zero setting at the beginning of the experiment Next, both signals are subtracted in a difference amplifier stage (c)

Before outputting the signal to the line recorder, the signal passes through a tunable low-pass filter (d) (Bessel type, fourth order) which suppresses that part of the noise which has a higher frequency than the highest frequency expected during the analysis The cut-off frequency of the low-pass filter can be set between 0 1 and 2 Hz

High-potential power supply. The high-potential source is of the high-frequency type The simplified diagram is shown in Fig. 4 The potential from the

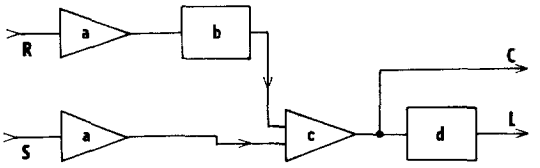

Fig 3 Evaluating electronics a = Logarithmic amplifiers, b = attenuator, c = difference amplifier, d = low-pass filter R = input from the reference photodetector, S = input from the signal photodetector, C = output to the computer, L = output to the line recorder

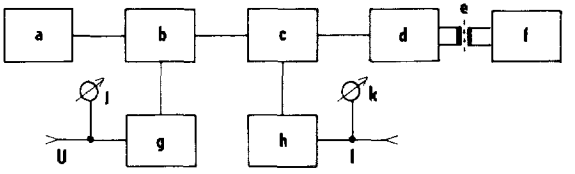

Fig 4 High-potential source a = Oscillator, b, c = attenuators, d = power stage, e = high-tension transformer, f = voltage multiplier, g = voltage comparator with preset, h = current comparator with preset, j = voltmeter, k = microammeter U = input from the divider connected to the high-potential output, I = input from the current sensor

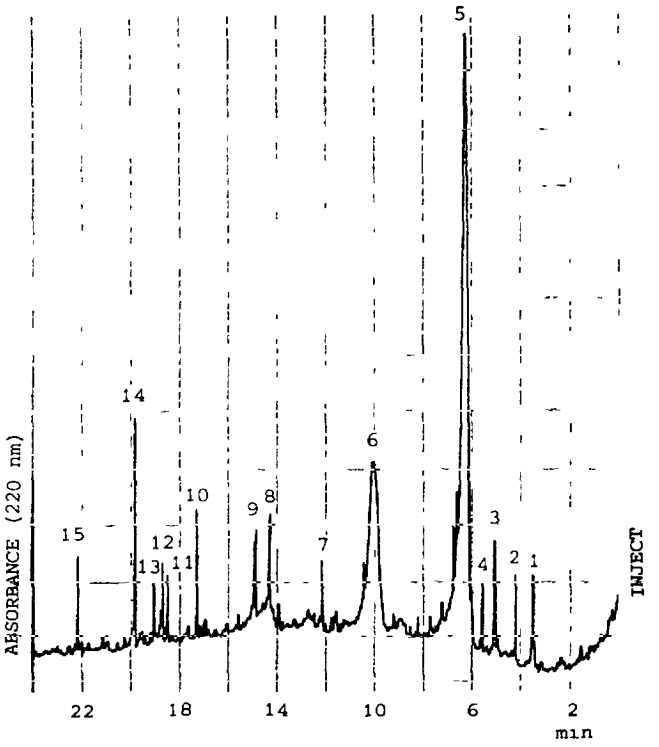

Fig 5 Electrophoretic separation of a standard amino acid mixture as phenylthiocarbamyl derivatives Conditions 2 5 pmol of each amino acid, separation from the right to the left, 5 m*M* borate buffer (pH 9 6), untreated silica capillary, 50 cm long to the detector, 100 μm I D , 20 kV per capillary, 25 μA Peaks 1 = arginine, 2 = lysine, 3 = leucine, 4 = isoleucine, 5,6 = reagent peaks, 7 = combined peak of phenylalanine and histidine, 8 = combined peak of valine and proline, 9 = threonine, 10 = serine, 11 = alanine, 12 = glycine, 13 = tyrosine, 14 = glutamic acid, 15 = aspartic acid Retention time of uncharged solute (dansylamide) was 43 min

oscillator (a) passes through two attenuators (b, c) to the power amplifier (d) The high potential is obtained from a cascade voltage multiplier (f) connected to a high-tension transformer (e) The multiplier is a separate plug-in unit that can be reversed to change the polarity The electronic stabilization is provided by comparing the preset voltage/current with the actual value of these magnitudes The output potential from these comparators (g, h) determines the degree of attenuation of the signal from the oscillator In this way that value (voltage or current) which reaches the preset value is electronically stabilized In this manner the device behaves as either a constant-voltage or constant-current source. In the constant voltage mode, the preset value of the current is a current limit, so protecting the system from overloading In its present version the maximum voltage obtainable is 20 kV

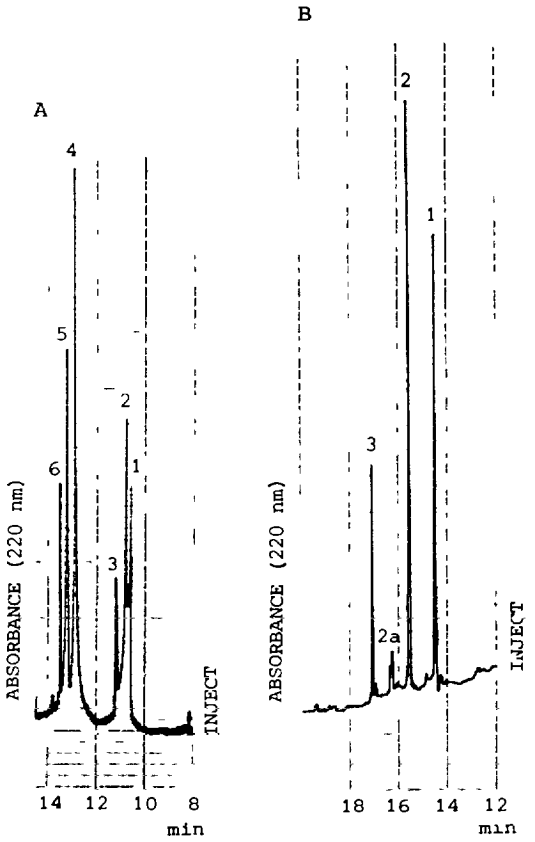

Fig 6 Separation of a standard peptide mixture analysis of a solution containing 0 5 mg/ml of each peptide Separation from the right to the left Other conditions as in Fig 5, except for the buffer concentration (2 5 m*M*) (A) No additive to the buffer, (B) the buffer and the sample solution were made 10 m*M* with respect to cadaverine Note the improved separation of the first three standards Peaks 1 = trypsin inhibitor, 2 = insulin, chain A, 2a = unknown, 3 = insulin, chain B, 4 = angiotensinogen, 5 = actinomycin, 6 = unknown

RESULTS

The apparatus developed for capillary zone electrophoresis offers satisfactory results with different solutes by the simplest means. The practical applicability of the device is shown in Figs. 5–8 Clearly, it is possible to separate all the main types of chemical entities that are involved in protein chemistry, i e , amino acids, peptides, constituting polypeptide chains and whole protein molecules Fig. 5 demonstrates the separation of a standard amino acid mixture in the form of phenylthiocarbamates The amount separated is 2 5 pmol of each amino acid

Separation of a standard peptide mixture is shown in Fig 6 The first three

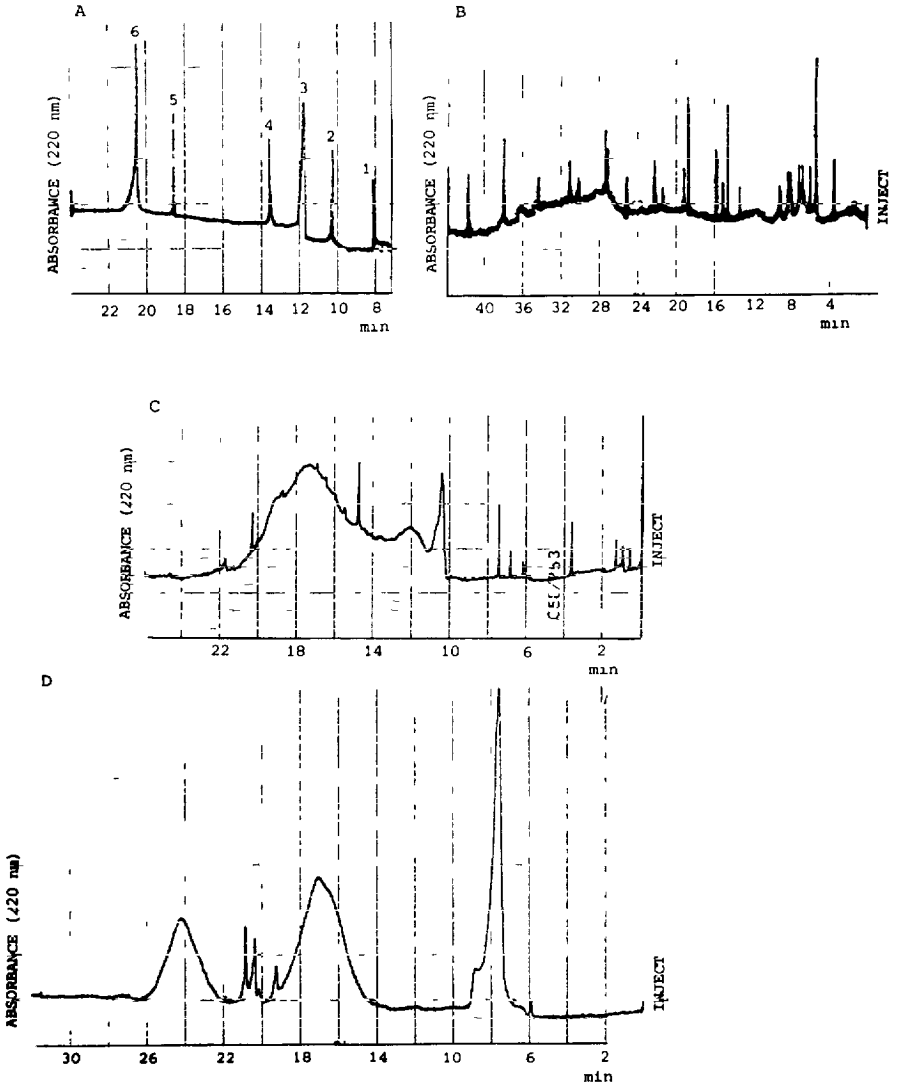

Fig 7 Separation of model protein mixtures Untreated silica capillary (60 cm × 100 nm I D), 2 5 m*M* borate buffer (pH 9 8), 5 m*M* with respect to cadaverine (A) Standard protein mixture Peaks 1 = cytochrome c from bovine heart, 2 = ribonuclease from bovine pancreas, 3 = chymotrypsinogen, 4 = aldolase from rabbit muscle, 5 = bovine serum albumin, 6 = ferritin (B) Separation of crude muscle protein extract (C, D) Separation of elastins solubilized by the method of Lansing et al [11] (C) preparation obtained from bovine aorta and (D) preparation obtained from rabbit auricular cartilage

most rapidly emerging members of the series are incompletely resolved under the conditions specified in Fig 6A. The addition of cadaverine, aimed at preventing wall adsorption of positively charged peptides, gave a considerably im-

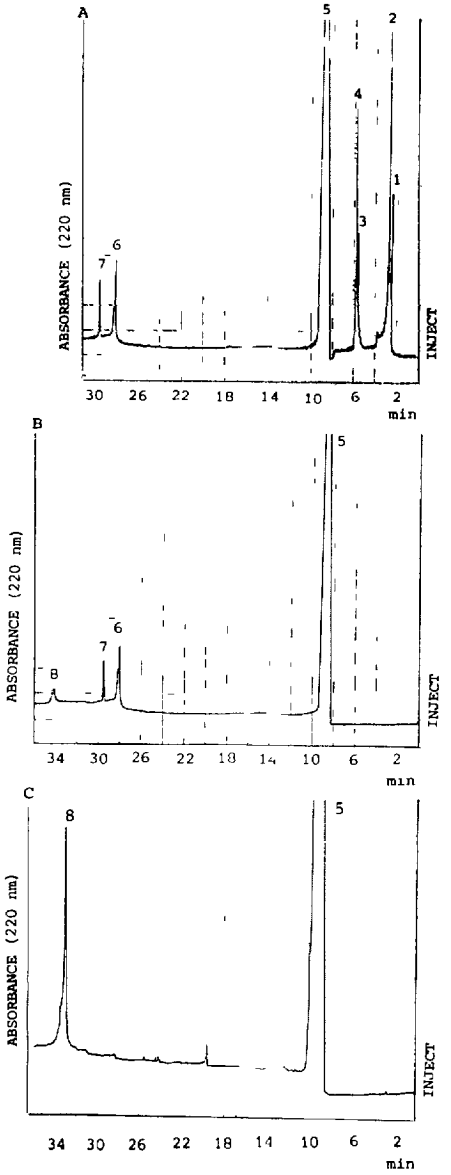

Fig 8 Separation of collagen type I α-chains and their polymers Conditions as in Fig 7 Peaks 1 = α-2, 2 = α-1, 3 = β-1,2, 4 = β-1,1, 5 = chain trimers (predominantly), other peaks represent higher-chain polymers Polymerization of collagen α-chains through incubation with galactose (A) after 24 h at 37 °C, (B) after 6 days, (C) after 29 days

proved separation (Fig 6B), although at the expense of an increase in retention time and a decrease in electroosmotic flow

Fig 7 summarizes examples of the separation of diverse protein mixtures

The separation of a standard protein mixture consisting of bovine heart cytochrome c, bovine pancreatic ribonuclease, chymotrypsinogen, rabbit muscle aldolase, bovine serum albumin and horse spleen ferritin is shown in Fig 7A There is some residual tailing, particularly with horse spleen ferritin, which persists even after the addition of the organic cation (cadaverine) to the running and sample buffers The other parts of Fig 7 show the results obtained with naturally occurring mixtures Fig 7B represents a profile of rat muscle proteins, the occurrence of peaks at high retention times probably reflects the presence of proteins with low p*I* values in this preparation Fig 7C and D represent profiles of proteinous fragments occurring in samples of elastin solubilized by the method of Lansing et al [11] The difference between preparations obtained from bovine aorta and rabbit ear cartilage emerges from comparison of the two profiles

Fig 8 shows the separation of collagen type I constituting α-chains and their polymers, which arise after in vitro reaction with galactose This figure is shown as an example because the chain polymers constituting peaks 5–8 in this profile are so large that they cannot be analysed by polyacrylamide gel electrophoresis as they do not enter even dilute polyacrylamide gels The separation of the first four peaks resembles conventional gel separations, offering distinct peaks of α-2, α-1, β-1,2 and β-1,1 collagen type I polypeptide chains

DISCUSSION

A monochromator is commonly used to obtain the desired wavelength, but in this work interference filters were employed to simplify the construction of the device The larger band width of these filters compared with the monochromator does not represent a limitation because of broad-band absorption of most proteins On the other hand, the intensity of light is higher. The only disadvantage of this type of filter, namely the presence of higher-order maxima, is unimportant as the phototubes selected are of the solar blind type Although less sensitive, they have the advantage of lower thermal noise, so that the final signal-to-noise ratio is better

From the point of view of the record, the noise component puts serious limitations on the sensitivity of the apparatus; there are three frequency bands that can be recognized in the signal· (a) a frequency band over the maximum frequency originating from the absorption change in the capillary due to the separated components of the sample; (b) a frequency band containing the information about the separated solutes, and (c) a frequency band from zero up to the lowest frequency b This band involves such changes as baseline drift (e g d c instability of the electronic circuitry).

It is relatively easy to minimize component (a) by capacitive feedback in the preamplifier and by filtering it off using a suitable low-pass filter Because it is difficult to estimate the maximum frequency of interest, it is advantageous

to construct this filter as tunable, whereas the feedback limitation in the preamplifier has to be chosen with sufficient reserve.

For obvious reasons, the noise in the frequency band (b) cannot be additionally suppressed and therefore it must be limited in its sources (i e., the light source, photodetectors, preamplifiers and capacitive transmission of electric disturbances from the capillary)

Perfect electronic stabilization of the current feeding the deuterium tube is essential. However, even if the current is constant weak fluctuations of the light flux in the discharge occur For this reason, a reference signal is picked up and the degree of compensation of the light source fluctuations depends on the parallelism of both logarithmic amplifiers and the degree of the common-mode rejection ratio in the difference amplifier

The most effective means of diminishing the noise originating in the photodetectors and preamplifiers is to have a high intensity of detected light Therefore, the optical system has to be designed with the highest light efficiency. Although there is no problem in obtaining sufficient illumination in the reference path (with the reference being placed next to the concave mirror, Fig. 1), the main restriction in the signal path is the slit, the dimensions of which must be carefully estimated. The maximum absorption of the light takes place in the middle part of the fluid column in the capillary, whereas the side parts reduce the degree of light modulation. Our experience has shown that the width of the slit should not exceed 40% of the inside diameter of the capillary The length of the slit is a compromise between the light beam limitation and the resolution capability. It appears that 0 1% of the capillary length is acceptable.

The noise from the phototube is given by the type selected and can be slightly reduced by lowering the anode potential to its minimum for linear operation (saturated current) at the maximum expected illumination A suitable electrometric amplifier, particularly with a low $1/f$ noise component, should be used. Also, the choice of the feedback resistor in the current-to-voltage convertor circuit is of considerable importance Only a resistor of the highest quality should be used It is essential to prevent any leakage current by suitable construction of the circuitry

Because the photodetector from the optical point of view (dispersion of the light beam by the capillary wall) should be as close to the capillary as possible, there is a risk of capacitive transmission of electrical disturbances from the capillary to the phototube These disturbances originate from the switching on and off of the high potential, from the alterations due to the inhomogeneous conductivity of the fluid column with passing solutes and if the high potential is increased during the run [7]. To minimize the capacitive transmission, it is possible either to use a system of diaphragms or a light guide. Because of the light losses with a light guide, the former alternative was our choice

It is difficult to estimate what the lowest frequency of interest is (point c)

A baseline shift caused by partial adherence of the solute to the capillary wall at the detector can also be indicated in this way. Therefore, the use of a high-pass filter (similar to case a) cannot be recommended and the zero shift must be minimized in the device itself

Both the dark current of the phototube and the d.c. shift in the offset voltage of the preamplifier are temperature-dependent. Therefore, all the electronic circuits (with the exception of the preamplifier) were located in separate cases The main heat source which cannot be avoided is the deuterium tube (ca 25 W) To limit the transfer of this heat to the system, the deuterium tube is closed in a reflective thermal shield This shield, together with the tube holder, is thermally insulated by PTFE rings Cooling is further enhanced by circulation of air through a 25-cm-long PTFE-insulated "chimney". As a result of these provisions, a negligible temperature increase (less than 1 °C) in the region of the photodetector was observed

Compared with a phototube, a semiconductor photodetector has an order of magnitude higher absolute sensitivity in the UV region. In spite of this advantage, we did not use it The first reason is a much higher temperature dependence of the dark current than with vacuum tubes (the dark current doubles with every 5 °C increase in temperature), which will cause a zero shift of the baseline The second reason is its sensitivity to visible light, which is about ten times higher than that for UV light This feature makes it impossible to use interference filters because of their higher-order transmission maxima. An additional high-pass light filter for the region up to 200 nm is not commercially available. The very high sensitivity for visible light makes it necessary to build the optical system to be very light-tight, which involves difficulties mainly at the entrance of the capillary On the other hand, the use of solar-blind phototubes allows operation with minimum light shielding A different and slightly more complicated solution was described by Prusík et al [14].

The utility of the capillary zone apparatus described can be demonstrated by some representative separations; it can be used for amino acids, peptides and proteins With peptides and proteins, addition of a positively charged organic ion to the running buffer and sample solution and high pH resulted in a sufficient reduction in the adherence of these solutes to the capillary wall The effects of different polymethylenediamines, such as cadaverine or putrescine, in preventing protein adherence were almost identical Better separations at longer running times were obtained in the presence of these cations (Fig 6B) Under these conditions, even very complex mixtures such as crude muscle extracts can be satisfactorily resolved (Fig 7A)

The limit of detection depends on the solute The best results were obtained with collagen polymers (Fig 8), where even on application of about 10 fmol the signal can be distinguished from the baseline noise

Basically two mechanisms of separation appear to be involved· as demonstrated with a standard protein mixture, the proteins are eluted in order of

decreasing p*I* values On the other hand, proteins with nearly identical p*I* values can also be separated, as demonstrated in Fig 8

ACKNOWLEDGEMENTS

The authors thank Dr J Lochman (Astronomical Institute, Czechoslovak Academy of Sciences, Turnov, Czechoslovakia) for his help with the optical system and A Nordheim and W Hampel (Max-Planck-Institut fur Biophysik, Frankfurt am Main, F R G) for their help with realization of the mechanical system Thanks are also due to Dr M. Horákova and Dr I Syrový of our Institute for the preparation of muscle and structural protein samples and to Dr I Mikšík (of the same Institute) for preparing the samples of collagen chain polymers

REFERENCES

1 B L Karger, A S Cohen and A Guttman, J Chromatogr , 492 (1989) 585
2 Y -F Cheng and N J Dovichi, Science, 242 (1988) 562
3 P D Grossmann, K J Wilson, G Petrie and H H Lauer, Anal Biochem , 173 (1988) 265
4 F S Stover, B L Haymore and R J McBeath, J Chromatogr , 470 (1989) 241
5 H Ludi, E Gassman, H Grossenbacher and W Marki, Anal Chim Acta, 213 (1988) 215
6 H H Lauer and D McMangill, Anal Chem , 58 (1986) 166
7 R M McCormick, Anal Chem , 60 (1988) 2322
8 J W Jorgenson and K De Arman-Lukacs, Science, 222 (1983) 266
9 Leaflets Microphoretic Systems, Sunnyvale, CA, Applied Biosystems, Foster City, CA, HPE 100, Bio-Rad Labs , Richmond, CA, Beckman Instruments, Palo Alto, CA, Dionex, Sunnyvale, CA
10 M J Gordon, X -H Huang, S L Pentoney, Jr , and R N Zare, Science, 242 (1988) 224
11 A J Lansing, T B Rosenthal, M Alex and E W Dempsey, Anat Rec , 114 (1952) 555
12 G Chandrakasan, D A Torchia and K A Piez, J Biol Chem , 251 (1976) 6062
13 Z Deyl and V Rohlíček, J Liq Chromatogr , in press
14 Z Prusík, V Kašička, S Staněk, G Kuncová, M Hayer and J Vrkoč, J Chromatogr , 390 (1987) 87