CHROM. 4950

THE APPLICATION OF THIN-LAYER ELECTROPHORESIS AND CHROMATOELECTROPHORESIS ON SEPHADEX G-25 TO THE ANALYSIS OF AMINO ACID AND PEPTIDE COMPOSITION OF BIOLOGICAL FLUIDS

A. KLEIN, J. CHUDZIK AND M. SARNECKA-KELLER

Laboratory of Animal Biochemistry, Institute of Molecular Biology, Jagiellonian University, Kraków (Poland)

(Received July 27th, 1970)

SUMMARY

Some examples of the application of thin-layer electrophoresis on Sephadex G-25 and of the use of this technique with paper chromatography to the examination of amino acid and peptide composition of urine and blood plasma are given. Thin-layer electrophoresis appears to be a very convenient method for a preliminary rapid analysis, especially of urine. In most cases the deproteinization and desalting of samples are not required, and the procedure can be completed with photodensitometric analysis. The chromatoelectrophoresis is a more tedious and time-consuming method, but it enables the demonstration of variations in the level of individual amino acids and other ninhydrin-positive substances.

INTRODUCTION

In 1968 we reported the successful separation of artificial mixtures of amino acids and low-molecular-weight peptides by means of thin-layer electrophoresis and chromatoelectrophoresis on Sephadex G-25 (ref. 1). In this paper we describe the results of the application of the same methods to the analysis of amino acids and peptides present in urine and blood plasma of human or mammalian origin.

EXPERIMENTAL

Materials

The analyses were carried out on citrated blood plasma or 24-h samples of urine. The volume of the samples applied depended on the level of total nonprotein α-amino nitrogen present in the biological fluid under study. This was estimated in deproteinized urine or blood plasma with ninhydrin according to ROSEN[2]. In all experiments Sephadex G-25 fine (Pharmacia, Uppsala, Sweden) was used, the same portion being repeatedly regenerated by the procedure given in the previous paper[1].

Methods

Thin-layer electrophoresis. The technique of thin-layer electrophoresis was the same as previously described[1] with only slight modifications. The size of glass plates was 6 × 36 cm and the time of electrophoresis 4 h. Samples of normal and pathological human urine were mostly used for analysis without any initial treatment. Mouse urine, especially from males, was deproteinized with 96% ethanol, the ratio of urine and alcohol being 1:4. The same method was also applied to the deproteinization of plasma.

Chromatoelectrophoresis. The use of descending paper chromatography with thin-layer electrophoresis on Sephadex G-25 was carried out in the same way as described for standard mixtures[1]. The solvent system *n*-butanol–acetic acid–water (144:13:43) used in the chromatographic procedure was the only modification applied in these experiments. Glass plates, 24 × 36 in size, were used in most cases. In a few experiments the distance of development in paper chromatography was twice as long as usual. In these cases, after the development of the chromatograms, the paper strips were cut in two, and for each half a separate glass plate of the same size as before was employed. The samples of urine and blood plasma were deproteinized and desalted with Dowex 50 X8. The elution of the amphoteric substances from the ion exchanger was carried out with aqueous 2 *N* NH_3 which was then removed by evaporation under reduced pressure at room temperature. In some experiments a different deproteinization method was employed for blood plasma. The sample was evaporated to dryness under reduced pressure at *ca.* 25°, treated 3 times with 5 volumes of acetone containing 5% 6 *N* HCl, and the acetonic extracts were centrifuged and HCl removed by evaporation to dryness several times with water under the former conditions. In each comparative series of analysis, the same procedure of deproteinization and desalting was always used.

Staining and quantitation of the spots. After drying the plates in air at room temperature, the spots were dyed by carefully spraying the gel surface with a 2% acetone solution of ninhydrin. The color was developed at room temperature or at 60°. In some cases replicas were made on Whatman No. 1 paper strips and dyed with ninhydrin. A visual evaluation of the color intensity of the ninhydrin-positive spots obtained in the course of electrophoretic separation was checked by photo-densitometric analysis. For this purpose photo replicas of the electropherograms were made on a Fotopan DIA-T film, and measurements were carried out with photodensitometer VEB (Medizinische Gerätfabrik, Berlin, G.F.R.).

Elution of fractions from thin-layer electropherograms and analysis of the peptide-bound amino acids. The electrophoretic fractions revealing the differences in the amount or in quantitative proportion of the ninhydrin-positive substances were investigated with respect to peptide-bound amino acids. According to the pictures obtained on the paper replica stained with ninhydrin, the corresponding part of gel was carefully removed from glass plates, eluted with twice distilled water, and the pooled eluates were evaporated under reduced pressure to the proper volume. The substances with free α-amino groups were transformed into DNP-derivatives according to the method of SANGER[3]. The DNP-amino acids were extracted with ether, and DNP-peptides left in the water fraction were hydrolyzed in sealed tubes with 6 *N* HCl at 100° for 24 h. The HCl was removed under reduced pressure, and amino acids were analyzed by descending paper chromatography on Whatman No. 1 paper

strips using the solvent system *n*-butanol–acetic acid–water (143:13:43) and with ninhydrin as developing reagent.

RESULTS

Some examples from our investigations are given here to demonstrate the usefulness of thin-layer electrophoresis and chromatoelectrophoresis with Sephadex G-25 as supporting medium for examination of the amino acid and peptide composition of biological fluids.

Fig. 1 presents the patterns of ninhydrin-positive spots obtained during the

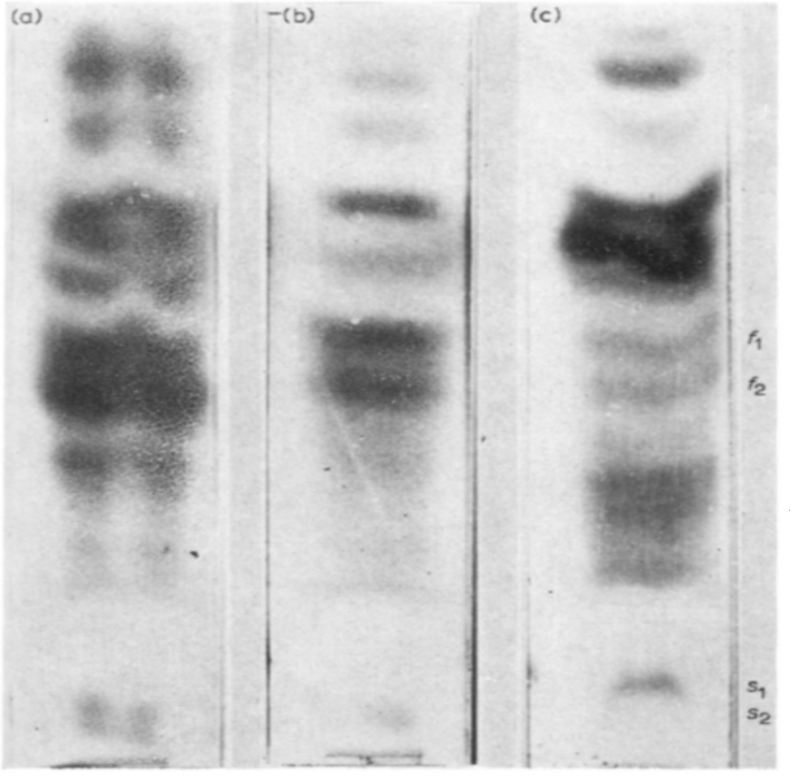

Fig. 1. The pattern of thin-layer Sephadex G-25 electrophoresis of the ninhydrin-positive substances present in 100 μl of urine from male mice hemizygous for the sex-linked lethal Mosaic (Ms) mutation. (a) Mice 10 days old; (b) mice 18 days old; (c) mice 6 months old. The sample of urine was deproteinized with ethanol. s_1 and s_2 = non-amino acid substances; f_1 and f_2 = fractions with various levels of constituents.

thin-layer electrophoresis on Sephadex G-25 of the urine of male mice hemizygous for the sex-linked lethal Mosaic (Ms) mutation[4]. Three groups of mice, 10 days, 18 days and 6 months old, were considered. The patterns (a, b and c) are similar in qualitative composition, but there is a striking difference in the two electrophoretic fractions (f_1 and f_2), the quantities of which decreased gradually with the age of immature animals. The main components of fraction f_1 were identified as serine, valine and leucine while fraction f_2 contains methionine, glutamic acid and glutamine. We suspect that the exogenous amino acids are especially responsible for the differences observed, and this hypothesis will be checked in a further investigation.

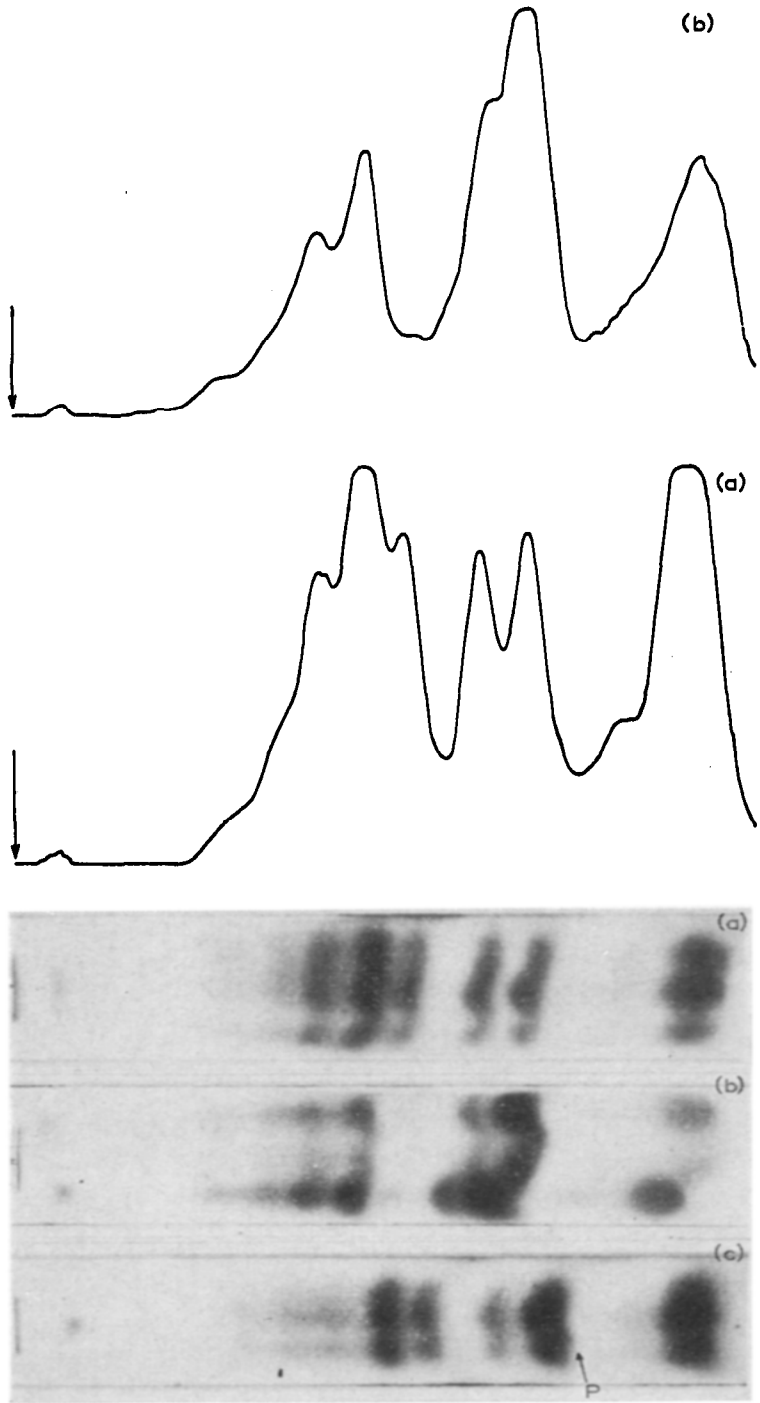

Fig. 2. The patterns of thin-layer Sephadex G-25 electrophoresis of urinary ninhydrin-positive substances and their corresponding photodensitograms. Urine is not deproteinized. Each sample analyzed corresponds to 100 μl of urine. (a) Urine of normal human adults; (b) collagenic urine of a patient suffering from lupus erythematosus disseminatus; (c) collagenic urine of a patient suffering from periarteritis. P = abnormal peptide absent in normal urine.

Fig. 2 shows the thin-layer electropherogram on Sephadex G-25 of ninhydrin-positive substances present in normal adult human urine (a) in comparison with the corresponding electropherograms of two cases of collagenosis (b, c). In this figure

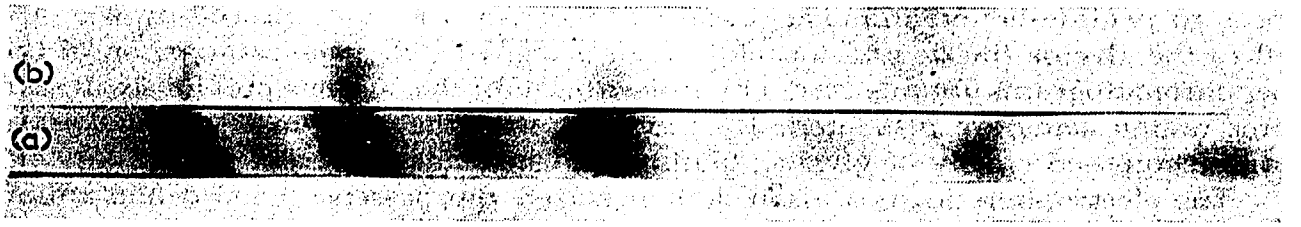

Fig. 3. The paper chromatograms of amino acids present in hydrolysates of a DNP-peptide. (a) Glycine electrophoretic fraction of periarteritic urine; (b) corresponding fraction of normal urine.

the photodensitograms of one normal and one of two pathological cases are also given as examples of the quantitation of the fractions. Besides the various proportions of the electrophoretic fractions in collagenic urine as compared with the normal one, the electrophoretic separation revealed one additional spot in the urine of the patient suffering from periarteritis nodosa. This substance shows the electrophoretic migration rate slightly greater than glycine but can be easily differentiated from this amino acid by its pink color with ninhydrin. After eluting the part of the gel containing this substance together with glycine, the fraction obtained was investigated with

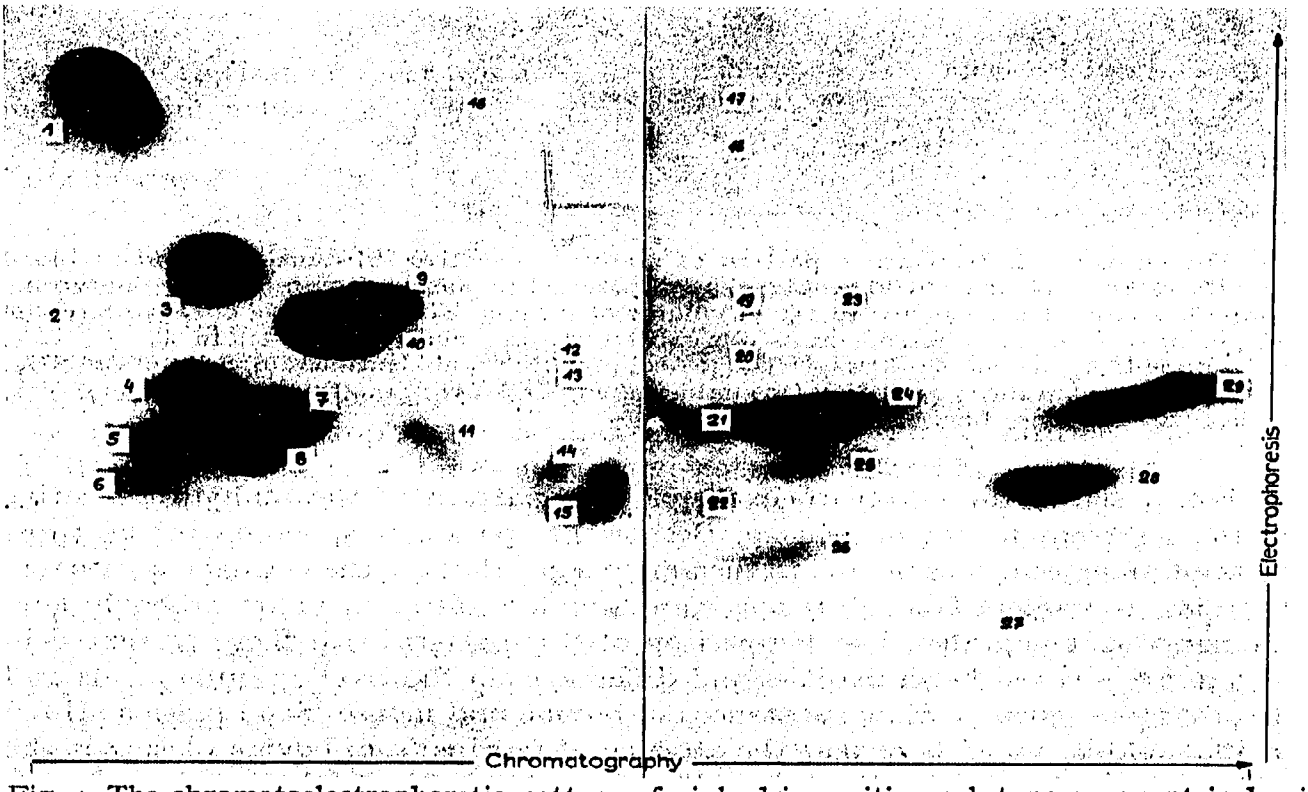

Fig. 4. The chromatoelectrophoretic pattern of ninhydrin-positive substances present in bovine blood plasma. Plasma deproteinized with Dowex 50 X8. The sample analyzed corresponds to a 170 μl volume of blood plasma. The distance of chromatographic development is *ca.* 40 cm; the electrophoresis is carried out on two plates each 24 × 36 cm. (1) His + Lys + Arg; (2) Cysteine; (3) Gly; (4) Ser; (5) $GluNH_2$; (6) Asp; (7) Thr; (8) Glu; (9) unidentified; (10) Ala; (11) Pro; (12) α-aminobutyric acid; (13) β-aminoisobutyric acid; (14) unidentified; (15) Tyr; (16) γ-amino butyric acid; (17–23) unidentified; (24) Val; (25) Met; (26) Try; (27) unidentified; (28) Phe; (29) Leu + Ile.

respect to peptide-bound amino acids. Simultaneously the same procedure was applied to the glycine fraction of normal human urine. In Fig. 3 the comparison of the paper chromatograms obtained from hydrolysates of both fractions previously devoid of free amino acids are presented. It is evident that the abnormal substance is a peptide composed chiefly of glycine, alanine and tyrosine.

The electrophoretic separation demonstrated the presence of two non-amino acid compounds (s_1 and s_2) in all samples of urine of different origin examined (see Fig. 1). Their migration rates are slower than that of taurine, and this is probably the consequence of their strong acidic nature. It was found that at least one of them contains a sugar residue.

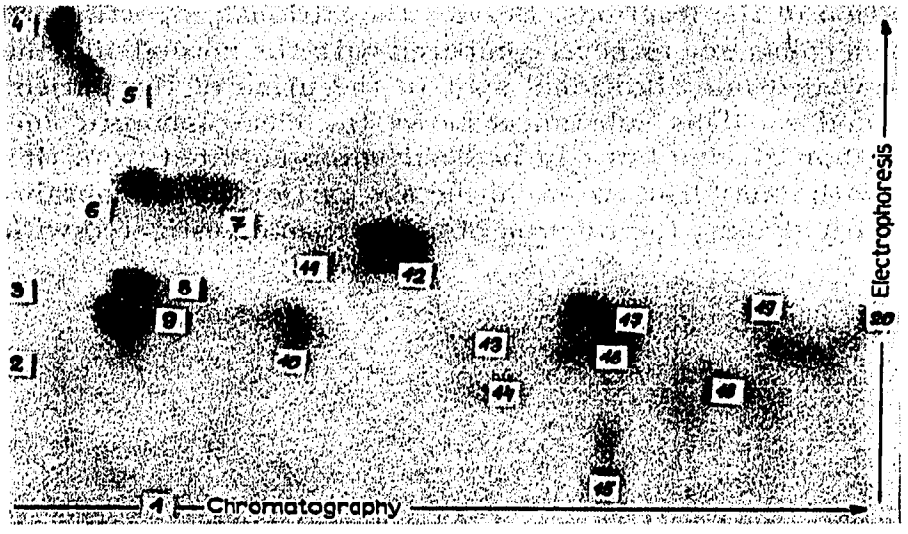

Fig. 5. The chromatoelectrophoretic pattern of ninhydrin-positive substances present in blood plasma of outbred male mice (wild genotype) 18 days old. The plasma deproteinized with acetone. The sample analyzed corresponds to 150 μl volume of plasma. The distance of chromatographic development is *ca.* 20 cm; electrophoresis was carried out on a plate 24 × 36 cm in size. (1) Tau; (2) unidentified; (3) Cystine; (4) Lys + His; (5) Arg; (6) Gly; (7) unidentified; (8) Ser; (9) $GluNH_2$; (10) Glu; (11) unidentified; (12) Ala; (13) unidentified; (14) Tyr; (15) Try; (16) Met; (17) Val; (18) Phe; (19) Ileu; (20) Leu.

Fig. 4 shows the chromatoelectrophoretic pattern of the ninhydrin-positive substances present in bovine plasma. Besides 19 spots corresponding to the three isomers of aminobutyric acid and to the amino acids that are the normal constituents of proteins, 10 unidentified substances were found. Some of them are probably low-molecular-weight peptides. The comparison of this pattern with those presented in Figs. 5 and 6 reveals the similarities and differences in the level of amino acids and some other ninhydrin-positive substances of bovine and mouse blood plasma. However, it should be noted here that the absence of taurine from bovine plasma is the consequence of only applying Dowex 50 X8 to deproteinization of the sample analyzed. The great number of unidentified spots observed on the chromatoelectropherogram of bovine blood plasma may also result from a longer chromatographic development.

Figs. 5 and 6 give a comparison of the composition of ninhydrin-positive substances of blood plasma originating from outbred male mice with the corresponding sample taken from male mice carrying the lethal Ms mutation. The most characteristic

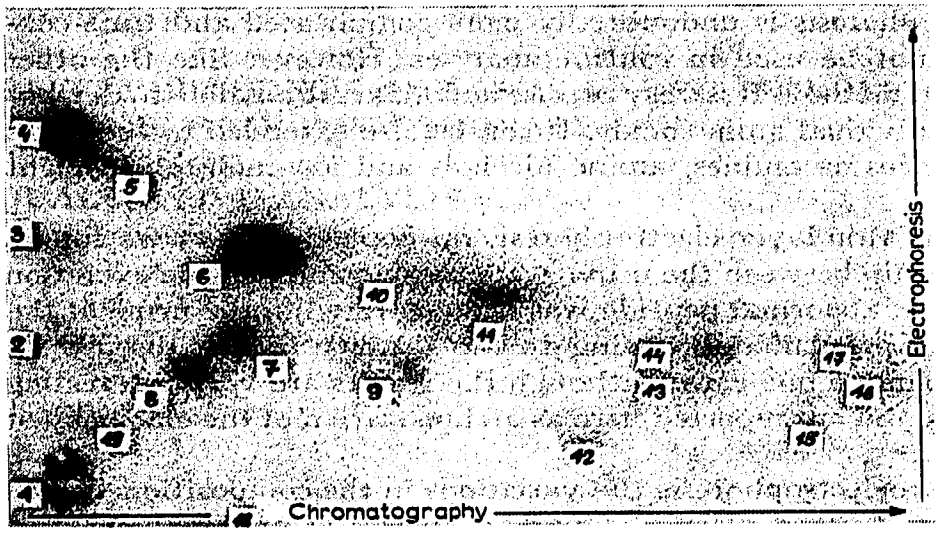

Fig. 6. The chromatoelectrophoretic pattern of ninhydrin-positive substances present in blood plasma of 18-day-old male mice hemizygous for the sex-linked lethal Mosaic(Ms) mutation. The deproteinization, volume of sample and conditions of chromatographic and electrophoretic separation are the same as those given in Fig. 5. (1) Unidentified; (2) Cystine; (3) Cysteine; (4) Lys + His; (5) Arg; (6) Gly; (7) Ser; (8) $GluNH_2$; (9) Glu; (10) unidentified; (11) Ala; (12) Tyr; (13) Met; (14) Val; (15) Phe; (16) Leu; (17) Ile; (18) Tau; (19) Asp.

feature of the last sample is the absence of tryptophan as well as a lower level of some other amino acids.

DISCUSSION

The results given in this paper showed that Sephadex G-25 (fine) is a good supporting medium for thin-layer electrophoresis of ninhydrin-positive substances present in biological fluids. The regeneration of the gel is very simple, and the patterns obtained are reproducible. It should be noted here that our attempts to apply Sephadex G-25 to thin-layer chromatography of the same substances were unsuccessful. Also the thin-layer electrophoresis carried out under the same conditions on Sephadex G-10 (fine) as well as on Bio-Gel P-2 have completely failed.

Thin-layer electrophoresis on Sephadex G-25 (fine) combined with photodensitometric analysis is especially useful in examining the amino acid and peptide composition of human urine with physiological level of proteins. In pathological cases connected with proteinuria or in some mammalian urine with normally high levels of proteins, *e.g.* in rats[5] and in mice[6], a very simple method of deproteinization with alcohol is sufficient. Thin-layer electrophoresis can be recommended for rough but rapid analysis of the amino acid and peptide composition of the urine. For this purpose the paper replicas should be made and the ninhydrin-positive spots developed on them at 60° for 10 min. After making the replica the plate can be used for performing a different color test or for a ninhydrin test developed at room temperature for 24–48 h because only in this way is it possible to obtain the gel surface suitable for taking the photographs of the pattern obtained.

The electrophoretic fractions can be very easily eluted by water and be further investigated as was the case in our examination of collagenic urine.

The chromatoelectrophoresis is undoubtedly more complicated and time consuming and therefore cannot be used in routine analyses. However like the other two-dimensional separation methods it is very precise and makes it possible to demonstrate the differences in individual amino acids. It can be also extended to the other ninhydrin-positive substances as amines, amino alcohols and low-molecular-weight peptides.

While employing the thin-layer electrophoresis on Sephadex G-25 we found, among others, the relationship between the urinary amino acid and peptide excretion and the age of mice. Also an abnormal peptide was demonstrated in the urine of the patient with periarteritis. Unfortunately owing to the scarcity of this disease we have only been able to examine one case. Although the results are very interesting they cannot be generalized and are reported here as an illustration of the efficiency of the method employed.

By means of chromatoelectrophoresis the variations in the composition of ninhydrin-positive substances of blood plasma of various species of mammalians, and even different genotypes of the same species, were found. The details of the differences involving the amino acid and peptide composition in urine and blood plasma between Ms mutant and control mice are given in separate papers[7,8].

REFERENCES

1 J. CHUDZIK AND A. KLEIN, *J. Chromatog.*, 36 (1968) 262.
2 H. ROSEN, *Arch. Biochem. Biophys.*, 67 (1957) 10.
3 F. SANGER, *Biochem. J.*, 39 (1945) 507.
4 H. KRZANOWSKA, *Mouse News Letter*, 35 (1966) 35.
5 A. K. ROY, O. W. NEUHAUS AND CH. R. HARMISSON, *Biochim. Biophys. Acta*, 127 (1966) 72.
6 L. F. WICKS, *Proc. Soc. Exper. Biol. Med.*, 48 (1941) 395.
7 A. KLEIN AND J. STYRNA, *Acta Biol. Cracov. ser. Zool.*, in press.
8 A. KLEIN AND K. SITARZ, *Acta Biol. Cracov. ser. Zool.*, in press.