THIN-LAYER TECHNIQUES FOR MAKING PEPTIDE MAPS*

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Peptide maps, or "fingerprints" have proved very useful in characterizing proteins by the pattern of peptides resulting from the application of chromatography and electrophoresis to a proteolytic digest. This technique was first described by INGRAM[1], and later improved by KATZ, DREYER AND ANFINSEN[2]. An outstanding example of the power of this method is the identification of a single amino acid as being responsible for the difference between normal and sickle cell anemia human hemoglobin[1].

BRENNER and co-workers[3,4] successfully applied thin-layer chromatography to amino acids and their derivatives. Thin-layer electrophoresis was previously used by HONEGGER[5] and by PASTUSKA AND TRINKS[6] for separating amines, amino acids, and organic acids. Because of the heat produced during electrophoresis, potentials of not more than 500 V were applied.

These results led us to the utilization of the combination of thin-layer chromatography and electrophoresis for the two-dimensional separation of peptides. Modifications of the chromatographic equipment and procedures used by BRENNER *et al.* were made. In the electrophoretic step we used 950 to 1.000 V, but additional cooling of the plates has been found absolutely necessary. The advantages of the thin layer technique lie in its rapidity and in the small amounts of material needed.

METHODS

Digestion of proteins

Digests of several proteins were prepared, including protamine and myosin which are cited as illustrations in this paper. The most convenient procedure is that of KATZ *et al.*[2], in which a volatile buffer is used. Trypsin and chymotrypsin are the most specific enzymes employed, but others may also be used. Trypsin and chymotrypsin were purchased from Worthington Biochemical Corporation, protamine sulfate from Nutritional Biochemicals Corporation. Myosin was prepared as previously described[7].

Thin-layer plates

Silica gel G proved to be the best carrier material for successive chromatography and electrophoresis. Surfaces coated with this material have good mechanical properties and allow excellent separations with distribution patterns similar to those found with paper sheets. Thin-layer plates are prepared according to BRENNER *et al.*[3],

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the best coating being obtained with a mixture of 20 g silica gel G in 80 ml water* The mixture is shaken for 1 min in an Erlenmeyer flask closed with a rubber stopper The plates, having standard sizes of 200 × 50 mm or 200 × 200 mm, are dried over night at room temperature since the use of plates dried in an oven leads to poorly reproducible results.

Solvent systems

In order to find the optimal solvent mixture of those listed in Table I, preliminary chromatographic runs with 200 mm × 50 mm thin layer plates are made. Cylindrical polyethylene jars, 210 mm × 90 mm, lined with filter paper soaked with the solvent

TABLE I

SOLVENT SYSTEMS FOR CHROMATOGRAPHY OF ENZYME DIGESTS OF PROTEINS

System	*Volume ratio*
A. Neutral systems	
1. 96% Ethanol–water	70:30
2. *n*-Propanol–water	70:30
B. Basic systems	
1. 96% Ethanol–34% ammonium hydroxide	70:30
2. *n*-Propanol–34% ammonium hydroxide	70:30
3. Chloroform–methanol–34% ammonium hydroxide	40:40:20
C. Acidic systems	
1. 96% Ethanol–water–acetic acid	70:20:10
2. *n*-Propanol–water–acetic acid	70:20:10
3. *n*-Butanol–water–acetic acid	80:20:20

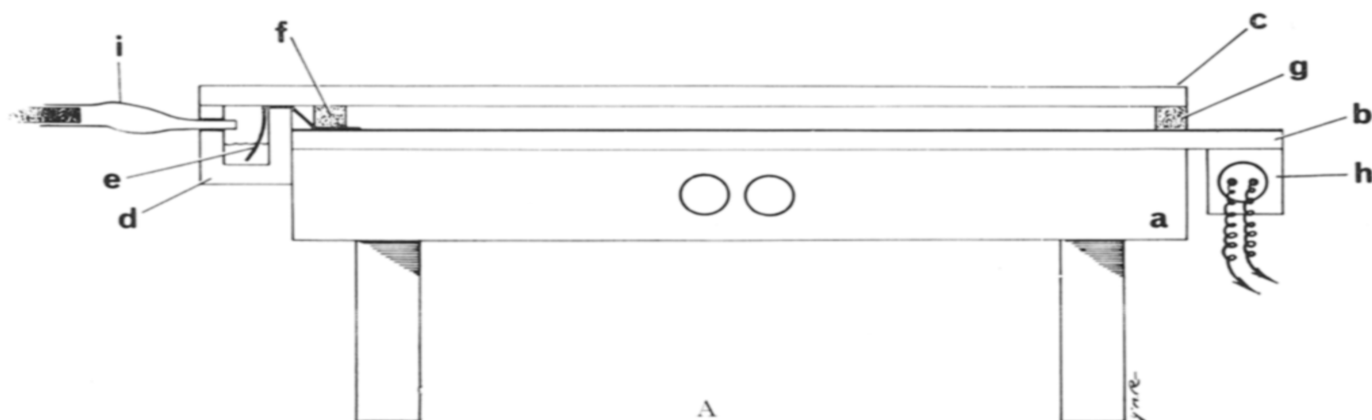
mixture and covered with glass lids are used. At the end of the run, which takes between 1 and 2 h, the plates are dried at 100° for 10 min and developed with ninhydrin spray. Chromatography, with the chosen solvent, for preparing the peptide maps is then carried out on 200 mm × 200 mm plates.

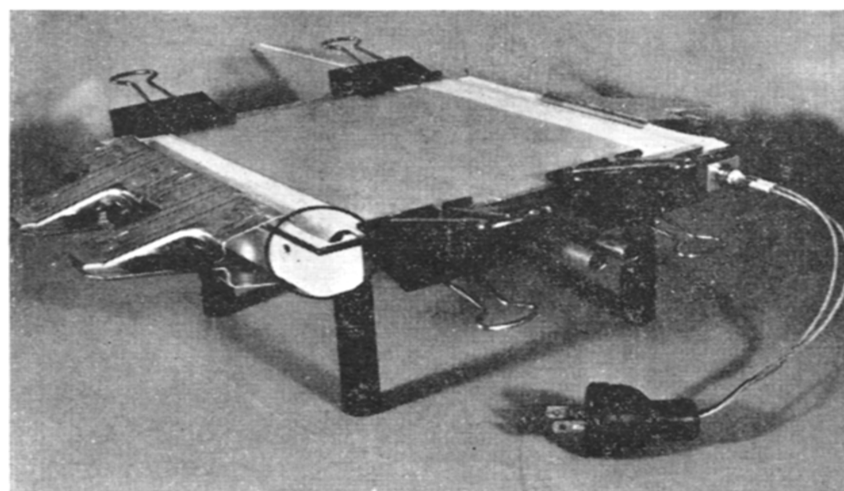
Modified Brenner-Niederwieser chamber (B-N chamber)

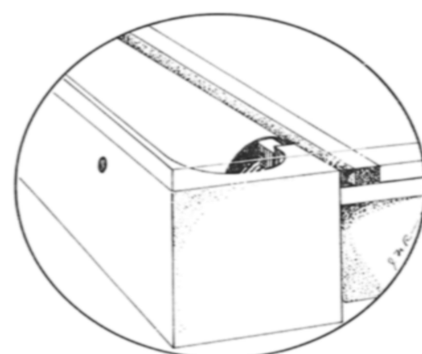
When longer runs are necessary for adequate separations, a chamber similar to that described by BRENNER AND NIEDERWIESER[8] is used, with some modifications. Their technique basically consists of a continuous flow of the solvent over the thin layer, upon which a second glass plate is placed as a cover. The solvent evaporates at the open end of the chamber. We have increased the distance between the two plates in order to prevent the filling of the space between the two plates with solvent condensed on the upper plate. A more rapid flow of the solvent is obtained with the use of an electric heating element at the edge of the chamber opposite the trough (Fig. 1).

A solvent trough of the dimensions given by BRENNER AND NIEDERWIESER, but made of high density polyethylene instead of stainless steel, is used. The edge over which the paper connection (wick) between the trough and the thin layer plate passes is 0.5 mm lower than the opposite edge. Whatman No. 4 paper is used for the wick in order to provide fast flow of the solvent, and it has a simpler (rectangular 175 mm ×

* The thin-layer equipment of the Brinkmann Instruments, Inc., Westbury, N. Y., was used.







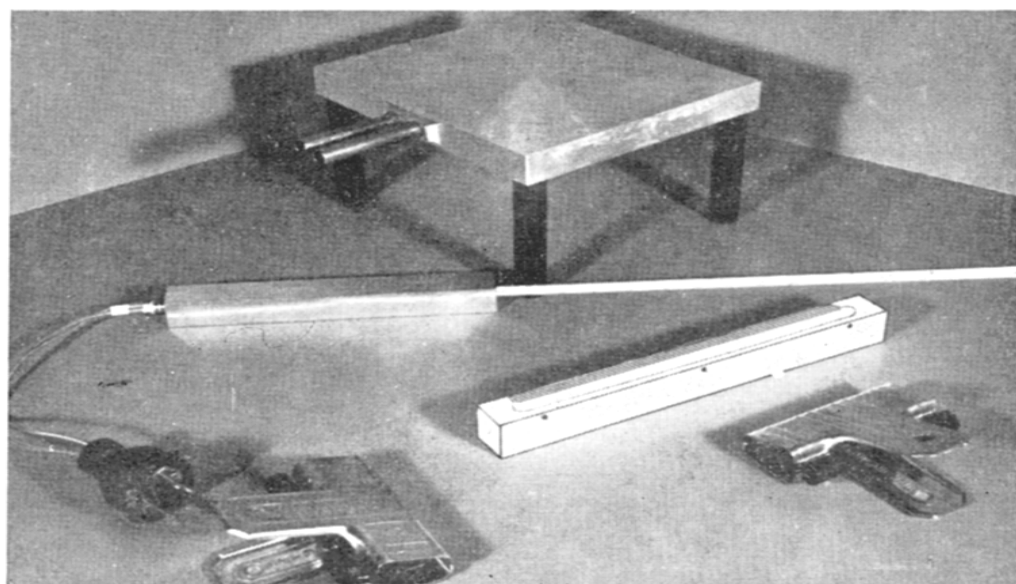


Fig. 1. Modified B-N chamber. A. Schematic drawing. (a) Aluminum platform with cooling coil inside. (b) Coated thin-layer plate. (c) Cover glass plate. (d) Solvent trough. (e) Paper connection (175 mm × 30 mm). (f) Teflon strip (200 mm × 50 mm × 30 mm). (g) Small teflon spacers (5 mm × 5 mm × 3 mm). (h) Heating element. (i) Pipette with polyethylene cathether. B. Assembled chamber with detailed view of the solvent trough. C. Disassembled chamber: Aluminum platform (185 mm × 175 mm). Heating element (185 mm × 20 mm × 15 mm) with inserted thermometer. Modified solvent trough.

30 mm) shape than in the original technique and is not folded. A teflon (200 mm × 5 mm × 3 mm) strip is fitted over the paper connection in order to obtain the proper distance between the two plates (3 mm). Two small teflon spacers (5 mm × 5 mm × 3 mm) are used between the two glass plates at the other end of the chamber. Both sides are sealed with scotch tape in order to prevent lateral evaporation. Cooling is provided by placing the chamber on an aluminum platform containing a cooling coil through which tap water is circulated. The heating element consists of an aluminum bar equipped with a heating cartridge* and a thermometer. The temperature is adjusted with a powerstat.

Assembling the chamber

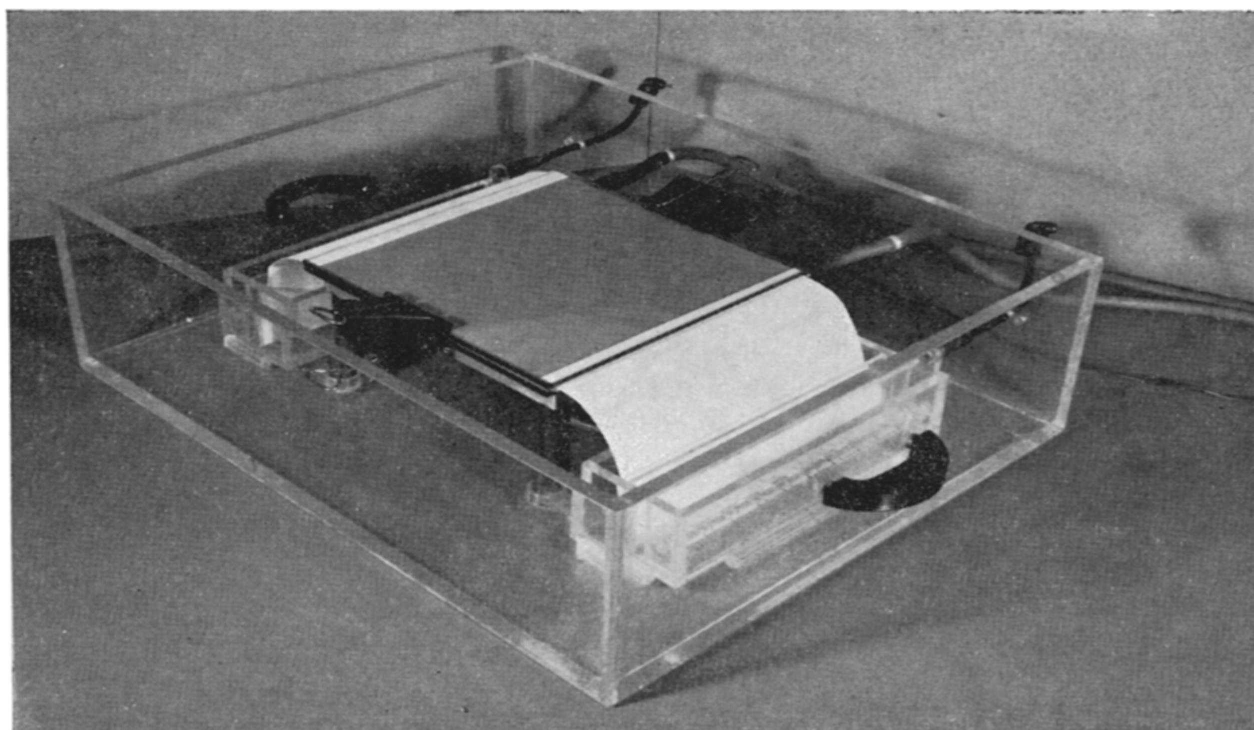
The chamber is assembled in an inverted position. The cover glass is placed on the aluminum platform and the open surface of the solvent trough with its wick place is put opening downward on the plate and clamped into position. The teflon strip is laid under the free end of the wick parallel to the solvent trough, and the other two teflon spacers are arranged at the opposite corners of the plate. Then the silica gel surface of the thin-layer plate is placed on the spacers. The two plates are clamped together along one edge and pressed together by hand along the opposite edge while being sealed with scotch tape. The taped edge is then clamped and the other edge similarly taped and clamped. The entire chamber is then inverted on the aluminum platform, as shown in Fig. 1. The heating element is clamped to the thin-layer plate, the cooling water turned on, and the temperature of the heating element adjusted to 100°. The solvent mixture, 20 ml, is added through a small hole in the trough using a pipette with a polyethylene catheter on the tip. Because the opening of the trough is not covered by the paper wick, in contrast to BRENNER's technique, the evel of the solvent can be monitored during the run and additional solvent may be ntroduced if necessary.

Electrophoresis

The electrophoresis chamber shown in Fig. 2 consists of two electrode vessels with platinum electrodes, a platform (190 mm × 190 mm) the thin-layer plate and cover plate. Connections of Whatman No. 3 paper link the electrode vessels to the thin-layer plate, and the cover plate is held in position with clamps. To avoid corrosion, all metal surfaces are coated with a clear acrylic paint. A power supply with an output of 0-1000 V and 0-200 mA is used**. Preliminary electrophoretic runs are made as follows: various amounts of the peptide mixture are spotted on a line in the middle of a 200 mm × 200 mm thin-layer plate, allowing at least 10 mm between spots. Buffer is evenly sprayed on the plate, which is then laid on the aluminum platform.

The electrode vessels are then joined by the paper bridges to the silica gel layer, the glass cover plate is brought into position, and the two plates are clamped. Tap water provides sufficient cooling permitting the application of 950–1000 V and currents of 30–80 mA. After a 30 min run, the plate is removed, dried at 100° and sprayed with ninhydrin reagent. These preliminary results decide the optimal sample concentration and time of the run. The direction of peptide migration during preliminary electrophoresis determines whether the spots for preparing the peptide maps should be

* C35 standard Hotwatt Heating Cartridge, Hotwatt, Inc., Danvers, Mass.
** Power supply 3-1009, Buchler Instruments, Inc., Fort Lee, N. J.



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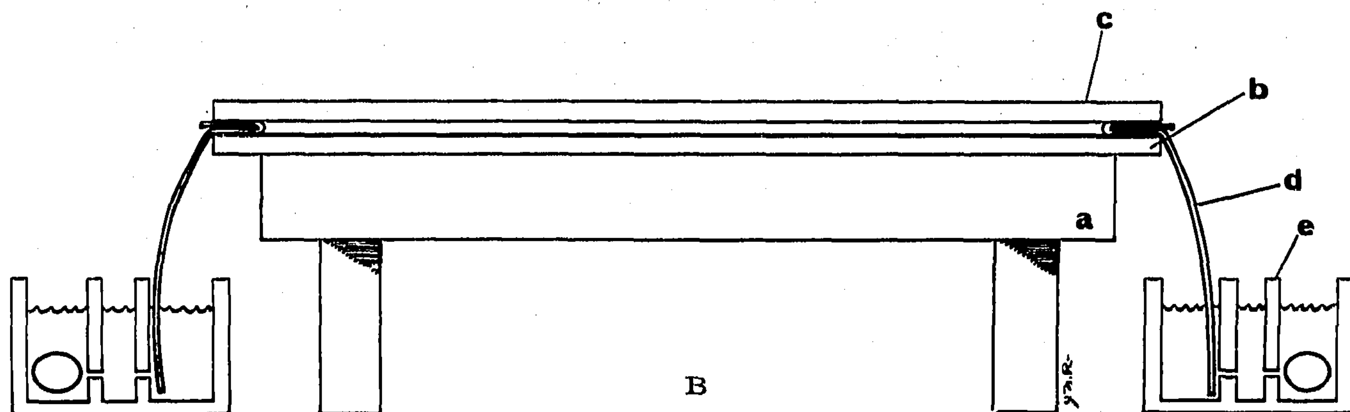

Fig. 2. Electrophoresis chamber. A. Assembled chamber with electrical connections and rubber tubings leading to the cooling coil. B. Schematic drawing. (a) Aluminum platform (190 mm × 190 mm). (b) Coated thin-layer plate. (c) Cover plate. (d) Paper connection. (e) Electrode vessel.

applied at a corner or at the middle of an edge of the plate. Usually migration occurs towards the cathode, permitting corner application for chromatography. A good buffer for electrophoresis consists of 1 ml of pyridine plus 10 ml of glacial acetic acid made up to 500 ml, pH 3.5. Ammonium acetate and ammonium carbonate buffers at low ionic strength are also satisfactory.

Peptide maps

Peptide maps are prepared on thin-layer plates (200 mm × 200 mm) by successive chromatography and electrophoresis as described above. The preliminary chromatographic runs indicated the choice of solvent and also suggested whether ascending

chromatography or the B-N chamber would be more feasible. Ascending chromatography, when feasible, saves considerable time, requiring 1–2 h as opposed to 10–15 h in the B-N chamber. Sample application is made with a micropipette. The amount of peptide mixture applied varies from 0.05 to 0.5 mg. To obtain highly concentrated deposits, repeated application can be made and the spots, not to exceed 4 mm in diameter, are dried with a hair dryer (excessive heat will damage the coating of the plate). Following chromatography, the plate is heated for 10 min at 100°, after cooling sprayed with the selected buffer, and subjected to electrophoresis in the direction perpendicular to that in which the chromatography was carried out. With the pyridine–acetic acid buffer at 950–1000 V and 30 mA. electrophoresis is completed in 1 h. The plate is then dried at 100° and the peptide spots developed with ninhydrin, or by using the chlorine *o*-tolidine test[9,10]. The locations of the spots should be marked with a pencil immediately after applying the reagent because the color diffuses and fades rapidly.

RESULTS AND DISCUSSION

Fig. 3 shows a peptide map of a tryptic digest of protamine. Tryptic digests of myosin yield a much larger number of peptides, of which more than 60 were separated on a thin-layer plate (Fig. 4).

The thin-layer techniques we have described for making peptide maps result in a saving of time as compared to more conventional methods, and also permit the use of smaller amounts of proteolytic digest and relatively simple equipment. In contrast, peptide maps on paper require complicated apparatus: large chromatography jars, high voltage power supplies, and electrophoresis chambers with organic solvents as cooling liquids. In cases of difficult peptide separation, the B-N chamber with the modifications described above is recommended. However, by selecting an appropriate solvent system most separations can utilize ascending chromatography and thus

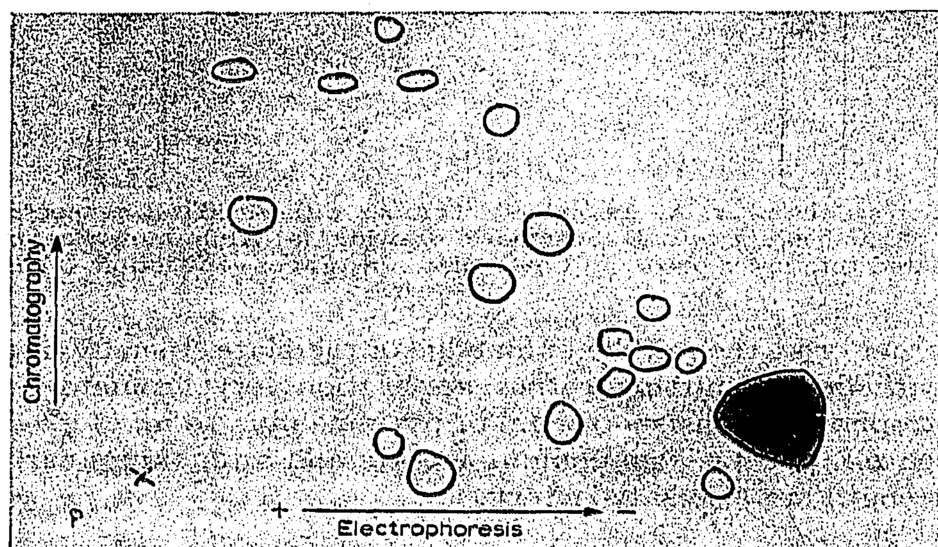

Fig. 3. Peptide map of the tryptic digest of protamine sulphate. Enzyme to substrate ratio = 1:100. Buffer: 0.1 *M* ammonium carbonate, pH 8.5. Incubation time: 24 h. First dimension: chromatography. Solvent system: Chloroform–methanol–ammonium hydroxide (40:40:20). Time: 60 min. Second dimension: electrophoresis. Buffer: pyridine–glacial acetic acid–water (1:10:489 ml). Current: 980 V, 30 mA. Time: 1 h.

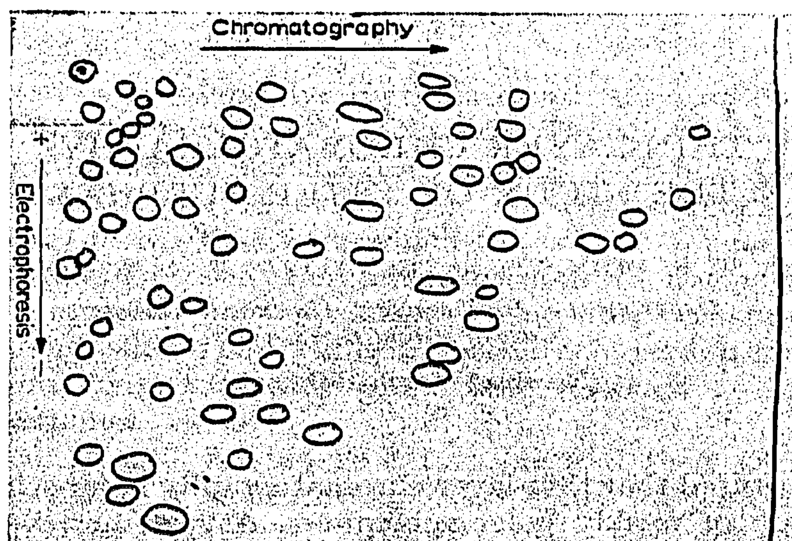

Fig. 4. Peptide maps of the tryptic digest of myosin. Same experimental conditions as in Fig. 3.

allow faster runs. Under such conditions, eight peptide maps can easily be prepared in a day. These techniques might be useful not only for structural analysis of proteins but also for checking the purity of fractions obtained by preparative column chromatography of peptide mixtures.

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The modified B-N chamber and the electrophoresis chamber were built in the Retina Foundation workshop under the supervision of Mr. R. THOMPSON.

SUMMARY

A method for preparing peptide maps is described which makes use of thin layer techniques instead of employing paper sheets. In short preliminary runs, the optimal experimental conditions (solvent systems, buffers, time, and sample concentrations) are explored and optimal conditions chosen for the preparation of the peptide maps. Chromatography of about two hours is followed by electrophoresis at 950–1000 V. The peptide mixture is applied in amounts of 0.05 to 0.5 mg per peptide map. Eight peptide maps are easily prepared per day.

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