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HIGH-VOLTAGE ELECTROPHORESIS

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SUMMARY

The different principles of high-voltage electrophoresis and the various types of high-voltage electrophoresis-apparatus now commercially available are discussed. High-voltage electrophoresis offers a rapid and reproducible method for the separation and identification of small amphoteric molecules. Application of this method to the separation of amino acids in hereditary or acquired types of hyperaminoacidurias as well as alterations in indole, phenol, porphyrin, amine and vitamin metabolism are demonstrated. Other possible separations (inorganic ions, purines, pyrimidines or low molecular carbohydrates) will be mentioned.

Additional methods for the separation of complex low molecular mixtures by two-dimensional high-voltage electrophoresis and by combining thin-layer chromatography and thin-layer electrophoresis are discussed. These combinations are particularly useful in the separation of peptides, producing peptide or "fingerprint" maps in the case of proteolytic digests.

But not only low molecular substances can be separated by high-voltage electrophoresis or thin-layer electrophoresis; even relatively high molecular compounds such as hemoglobins give very clear-cut and definite bands thus making the recognition of hemoglobinopathias possible.

Quantitative evaluation of results can be achieved by densitometric or photometric procedures.

AN INTRODUCTORY SURVEY

Electrophoresis is based on the differential migration of charged particles in an electric field. Since migration rates within a given time are proportional to the field strength, it is desirable to be able to use as high a voltage as is experimentally possible in order to obtain an economy in time, a high resolving power and an easier detection of components present in low concentrations.

The definition of high-voltage technique implies the application of a potential gradient of, at least, 50 V/cm. To obtain the full benefit of this technique it is advisable, however, to aim at the use of potential gradients of 100 V/cm and more, though the difficulties in designing suitable equipment for this range become progressively greater with the increasing voltage.

Several designs of high-voltage equipment have been described in recent years.

Some are based on direct cooling of the paper strip by an organic solvent, immiscible with the sample and the aqueous background electrolyte solution, with and without cooling of the organic solvent as suggested by MICHL[1], HEILMEYER[2] and coworkers, TURBA *et al.*[3]. Others are based on cooling the paper strip by plates of metal, glass or plastic, which in turn are cooled by refrigerated brine or ordinary tap water. Designs incorporating one cooling plate, generally the bottom plate, have been described by BERBALK AND SCHIER[4], WERNER AND WESTPHAL[5], MICHL *et al.*[1a] and WIELAND AND PFEIDERER[12] last but not least, in great perfection by Camag. Designs incorporating two cooling plates (sandwich technique) have been proposed by MICHL[1] and by GROSS[6,7].

Which system is the best is still an open question and eepends mainly upon the substances to be separated, and the voltage to be applied. The latter point

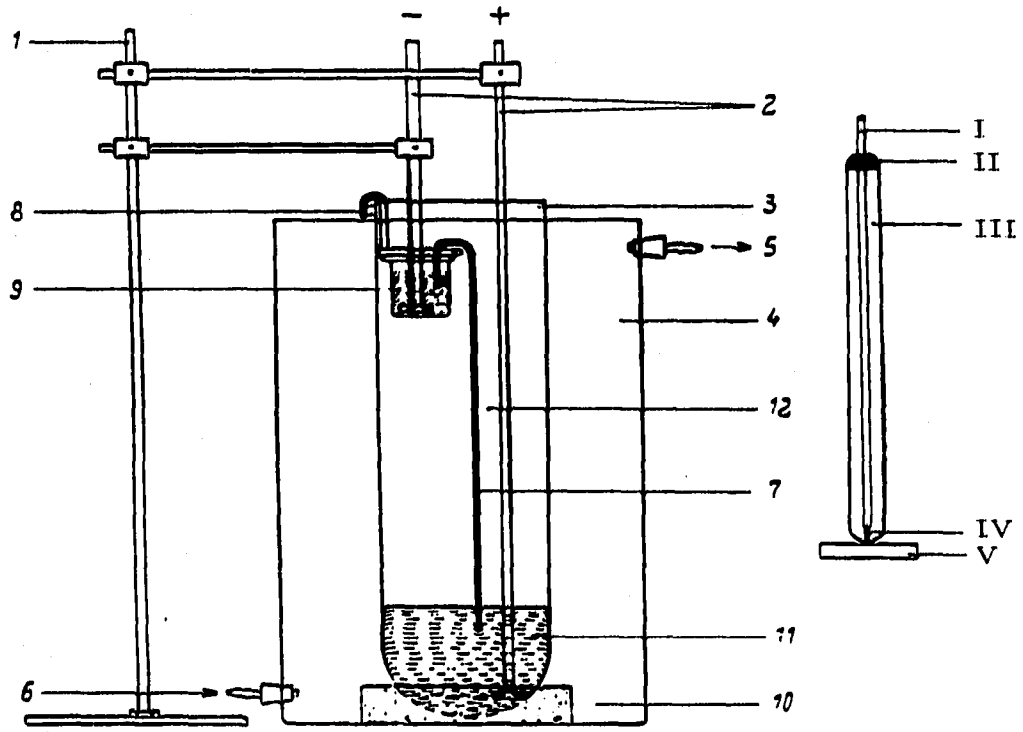


Fig. 1. HVE-laboratory equipment for direct cooling by an organic solvent. 1 = Electrode support; 2 = electrodes; 3 = glass tube; 4 = cooling jar; 5 = water outlet; 6 = water inlet; 7 = paper strip; 8 = holder for electrode vessel; 9 = electrolyte vessel; 10 = support for separation tube; 11 = electrolyte solution; 12 = organic solvent; I = copper wire; II = seal; III = glass tubing; IV = platinum wire; V = platinum electrode.

in particular is the more important one. The passage of electric current through the moist, conducting filter paper or any other stabilizing media is proportional to the voltage applied and causes generation of heat. The heat, according to Joule's law, increases with the square of the current. If the heat is not dissipated, the ensuing rise in temperature detrimentally affects the experimental conditions, since conductivity and, to a lesser degree, the pH of the background electrolyte solution as well as mobilities of the ions under investigation are all temperature-dependent. To obtain steady conditions it is essential that the generated heat be effectively dissipated so

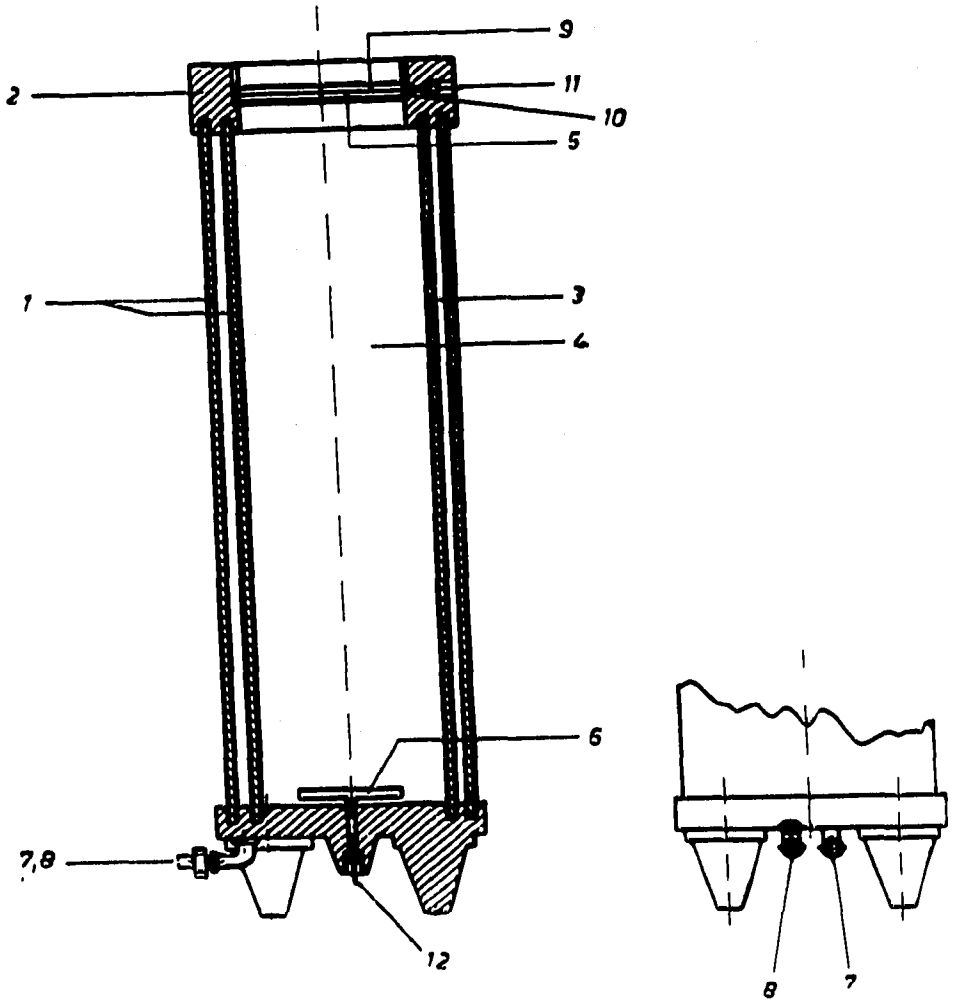


Fig. 2. HVE-design for preparative separations. 1 = Inner and outer plastic tubes (PVC); 2 = support ring for plastic tubes (Araldit); 3 = cooling space; 4 = separation room; 5, 6 = electrodes; 7, 8 = inlet and outlet for refrigerated brine; 9 = electrode vessel; 10 = contact spring clip; 11 = contact pins (HV-connections).

that there is no significant temperature rise during the experiment. The level of applied voltage is thus limited only by the efficiency of the cooling system.

Cooling can take place by evaporation, conduction or radiation, the latter playing no significant part in the designs discussed. Evaporation is the usual system, particularly in a simple apparatus of the "moist chamber" type found so useful in low-voltage electrophoresis. However, it does lead to serious complications above a potential gradient of 10 V/cm. It is thus desirable that heat dissipation should occur predominantly by conduction. Metal plates in close contact with the heated surface of the paper are excellent conductors of heat. The efficiency is, however, reduced by the need for electric insulation of the plates, by glass, polythene or other plastics. The heat-exchange mechanism in this case is complicated because the heat has to pass the narrow air gap between paper and insulating layer, the thickness of the layer, another air gap, and finally the distance from metal surface to coolant, before it can be carried away. By choosing the thinnest possible insulating layer, yet one of sufficient electric strength, and by application of sufficient pressure to ensure a close

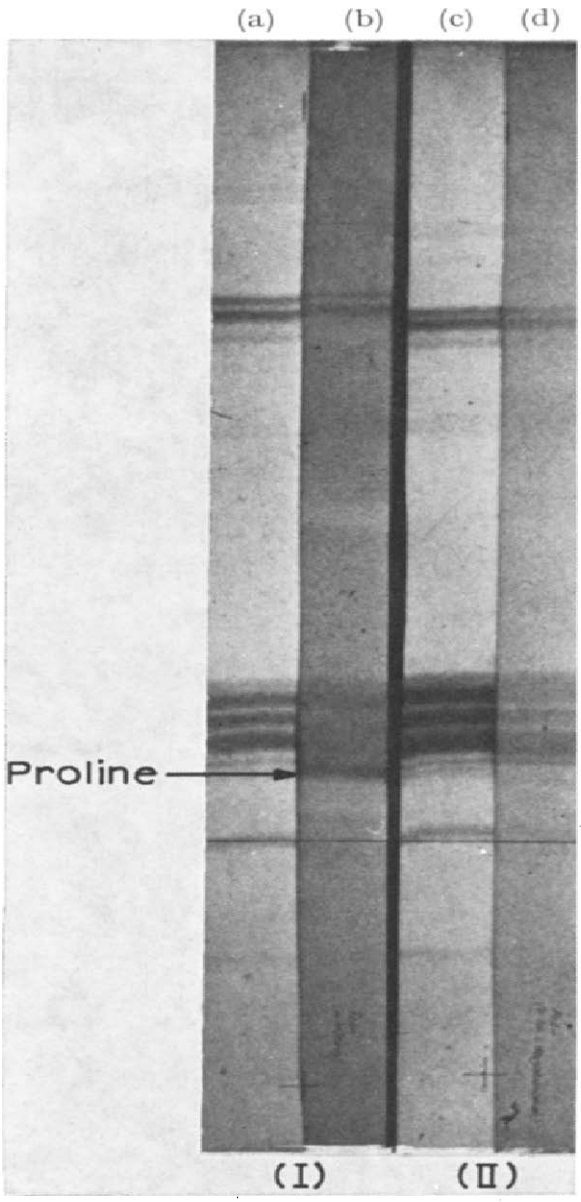


Fig. 3. Separation of (I) serum and (II) erythrocyte amino acids. pH, 3.6; potential gradient, 60 V/cm; time, 60 min. (a) Ninhydrin and (b) triketopiperideine (proline stains deep red, all the other amino acids blue-green to lavender).

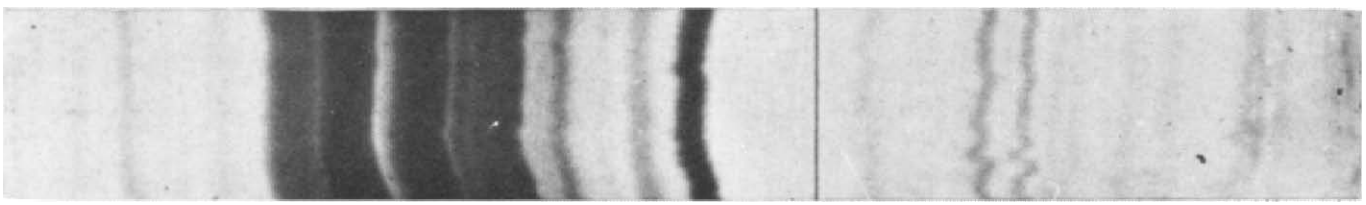


Fig. 4. Separation of neutral and acidic amino acids of serum. pH, 1.9; potential gradient, 100 V/cm; time, 45 min.

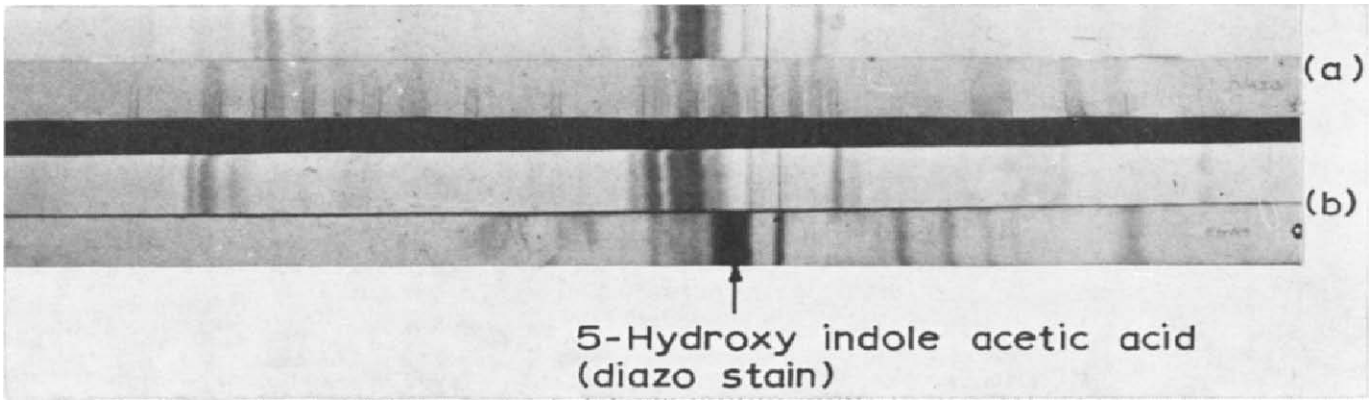


Fig. 5. Ninhydrin positive substances of (a) normal human urine and (b) of urine of a carcinoid patient.

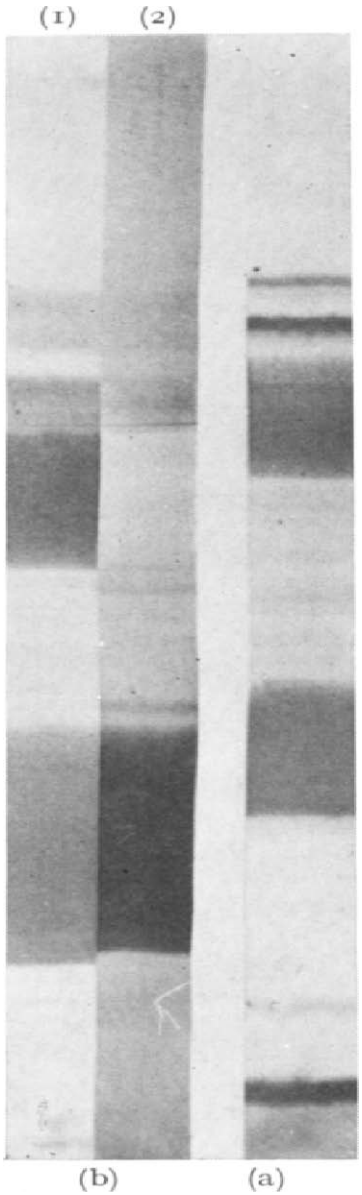


Fig. 6. (a) Normal urine and (b) urine of a patient with histidinaemia. 1 = ninhydrin; 2 = diazo compound.

contact to the cooling surface, a high degree of heat transfer efficiency can be achieved.

With evaporation reduced to a negligible minimum, shifts of liquid in the paper are due to electro-osmosis and capillary flow caused by small differences in temperature along the length of the strip. There is a certain non-uniformity of flow, with rate of flow increasing slightly from cathode to anode due to this composite effect. The liquid flow gradient is dependent on several factors such as pH, ionic strength, time, temperature, potential gradient and moisture content of the stabilizing medium. In the interest of a high reproducibility of results, it is advisable to minimize the hydrodynamic flow in the paper, although the inherent electrokinetic phenomenon of electro-osmosis cannot be eliminated. The choice of a suitable moisture content, mostly 130–140% of electrolyte solution on oven-dry paper, and the use of cellophane sheets in surface-cooled systems are helpful in this respect. The use of cellophane sleeves to restrict the influx of liquid from buffer vessels into the separating area greatly assists in stabilizing the liquid level in the paper for the relatively short duration of the high-voltage experiment.

Coming back to the question of which type of the presently available devices for high-voltage electrophoresis is best suited for routine separations of complex

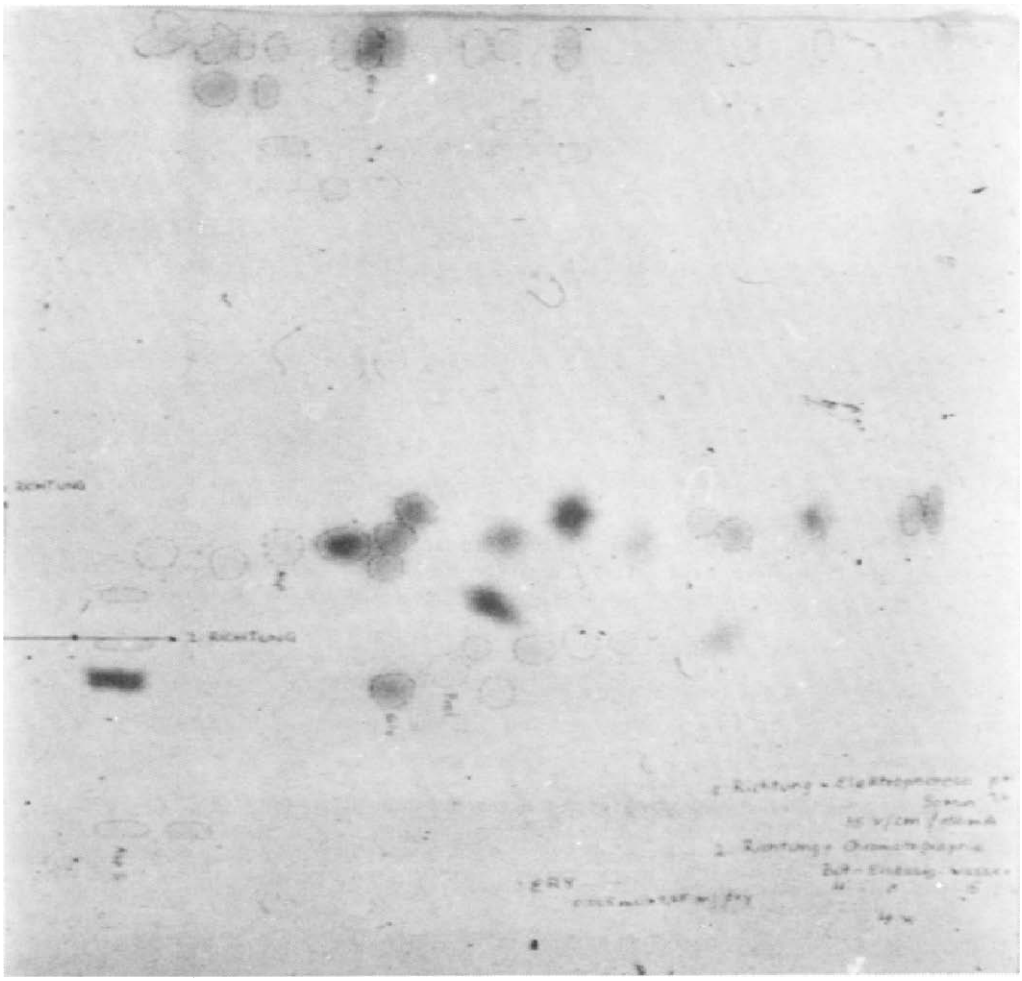


Fig. 7. Two-dimensional separation of amino acids from erythrocytes. First dimension: HVE; pH, 3.6; potential gradient, 45 V/cm. Second dimension: chromatography; solvent, *n*-butanol–acetic acid–water (4×).

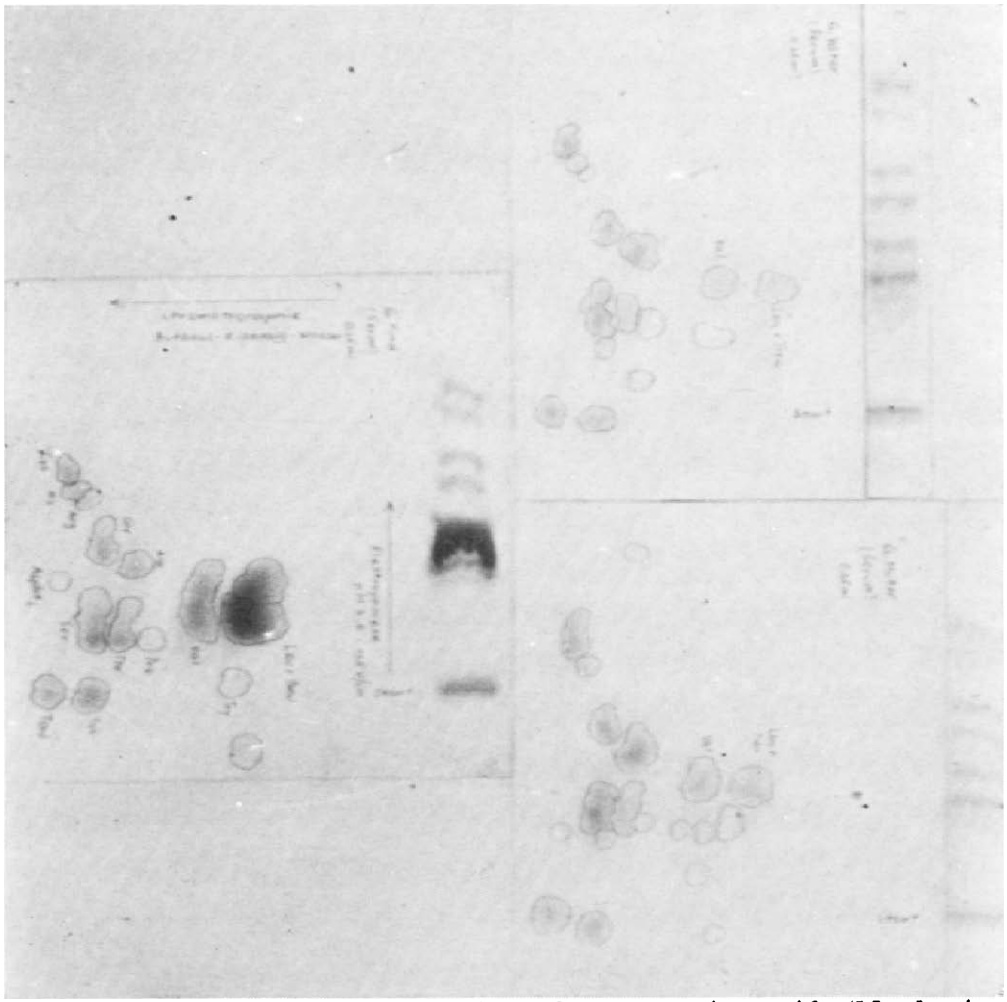


Fig. 8. Two-dimensional separation of serum amino acids (Maple sirup disease, leucine fraction extremely elevated).

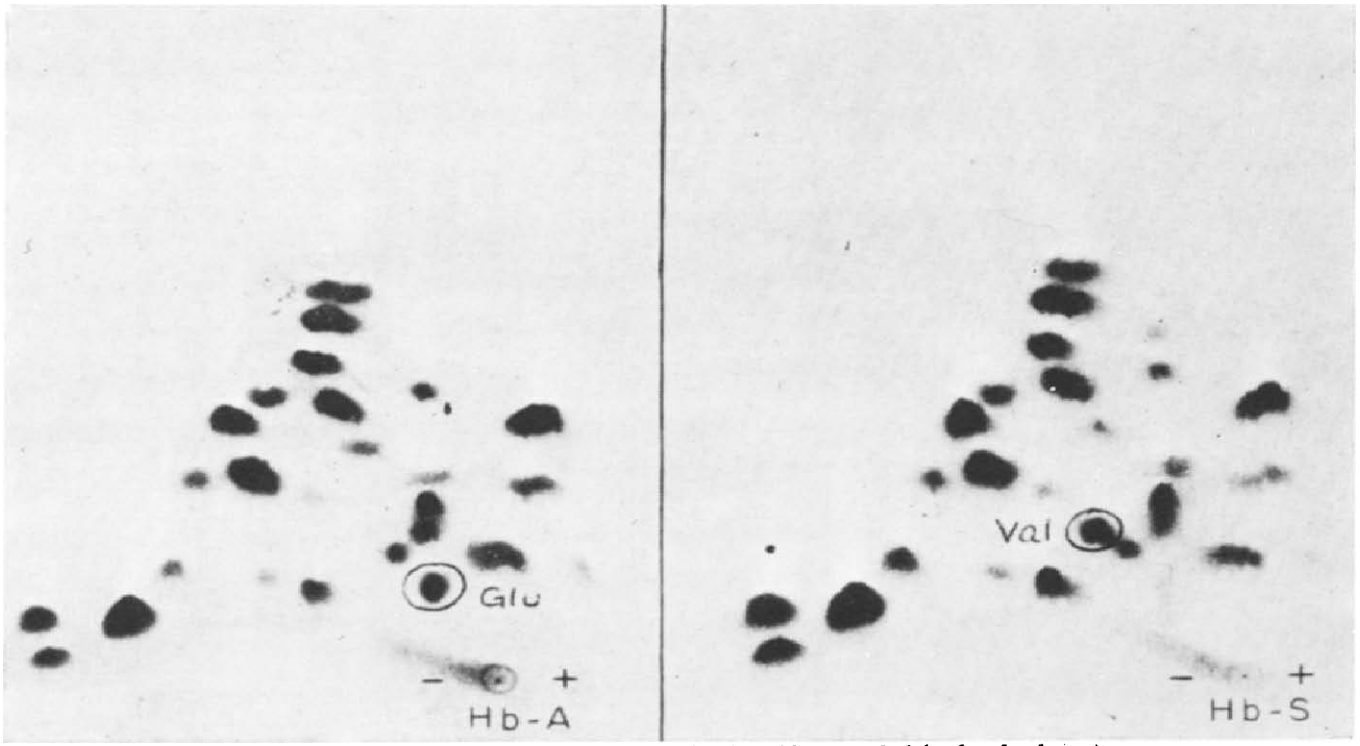


Fig. 9. Two-dimensional separation (fingerprinting) of hemoglobin-hydrolysates.

mixtures in biological materials, I would prefer the designs using single or double cooling plates, in spite of the fact that most of our work with high-voltage electrophoresis in the past fifteen years was performed with liquid-heat exchange in an organic solvent. The reason for this is simply, that these cooling devices only permit heat dissipation of about 0.3 W/cm², wheareas solid heat exchangers are far more effective, thus permitting application of higher voltages. The solvent design is the simplest and easiest to construct and has proven to be satisfactory in many laboratories. The limitations are the relatively low-cooling capacity and the fact that the solvent must be immiscible with the background electrolyte and must not affect the

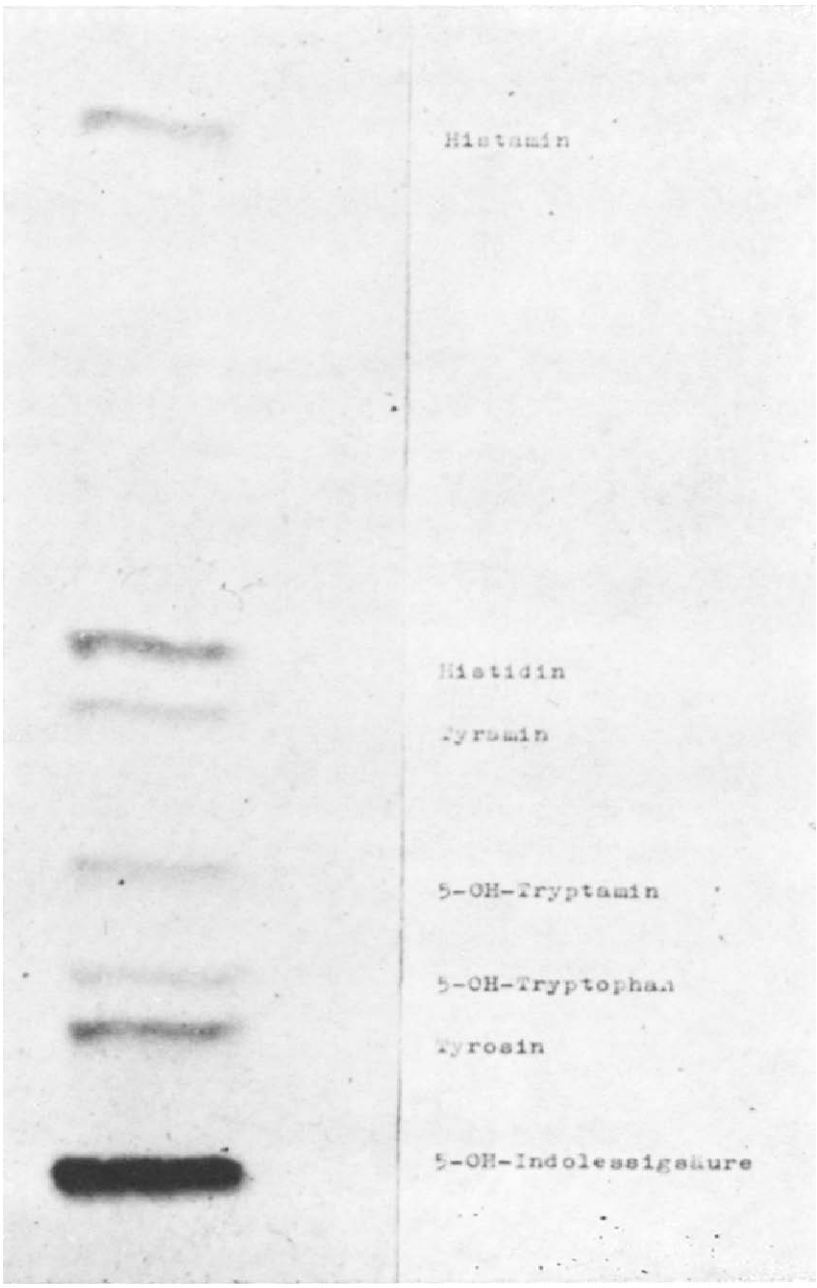


Fig. 10. HVE separation of indoles and biogenic amines. pH, 3.6; potential gradient, 60 V/cm; time, 40 min.

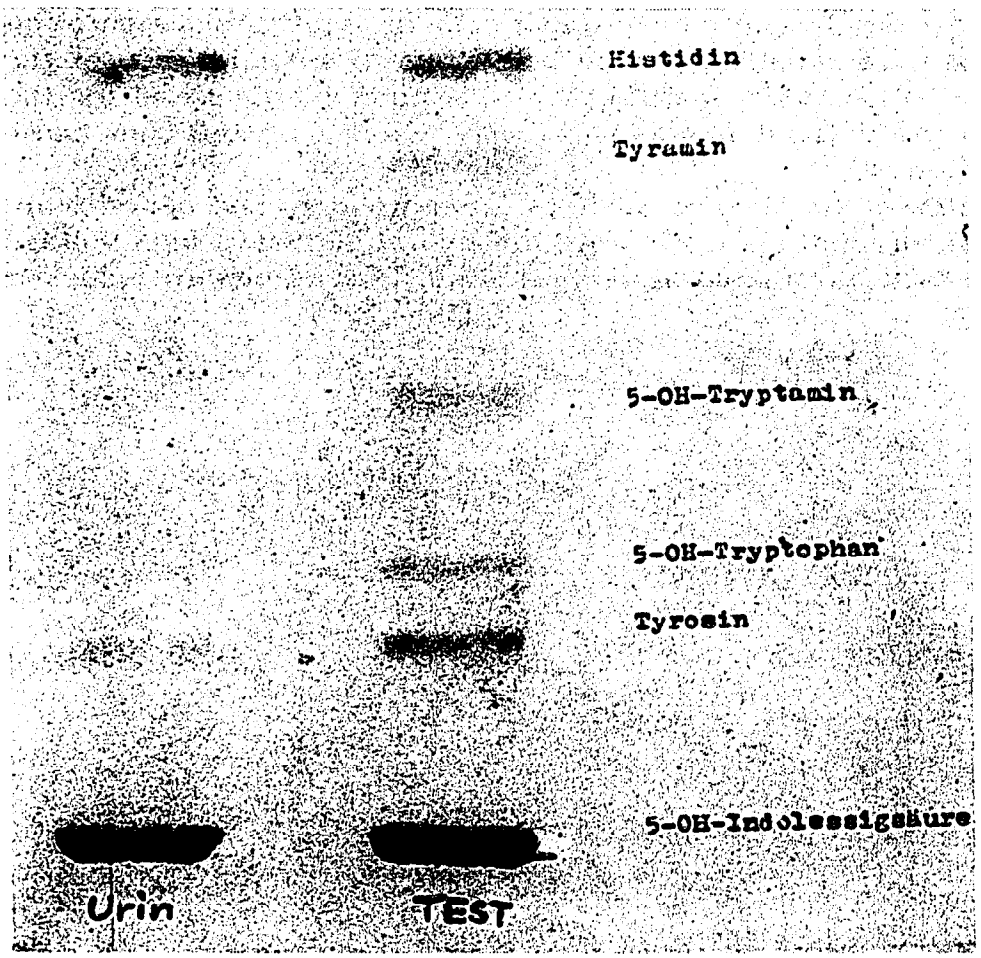


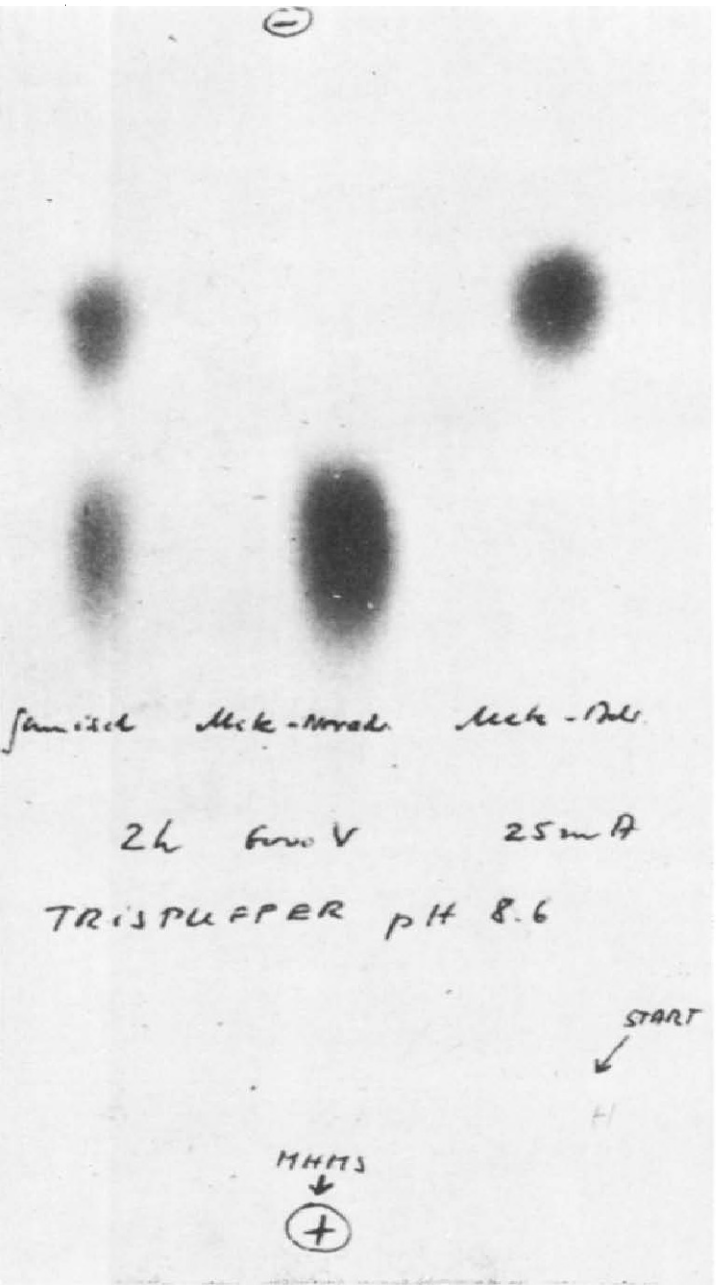
Fig. 11. HVE separation of test indoles and those in urine of a carcinoid patient.

compounds to be separated. There is also the complication of unavoidable evaporation of solvent with possible toxic and fire hazards. Another reason for preferring solid cooling systems is that one can use practically all types of stabilizing media, for instance cellulose acetate films, glass fiber, agar, granular starch and starch gels, as well as polyacrylamide, Sephadex or agarose. These media offer the possibility even for the separation of higher molecular mixtures such as enzymes and other protein components which are normally not separable on paper by high-voltage electrophoresis. This leads to the question of when to use high-voltage electrophoresis and whether it is advantageous over other separation procedures. It is impossible to quote all the papers of the last ten years describing successful separations of various mixtures by this method.

Extensive work has been put into high quality resolutions of complex mixtures of amino acids and lower peptides in protein hydrolysates and biological fluids. Separations of mixtures of ninhydrin reacting substances in human plasma, urine and cerebrospinal fluid have been numerous, and were of decisive help in discovering hereditary defects in amino acid metabolism. The clear-cut and highly reproducible separations easily permit the recognition of qualitative and quantitative alterations of the normal amino acid spectrum. Some pictures may help to illustrate that. Of special importance in those separations is the fact that desalting of the biological

material is not necessary as desalting occurs automatically in high-voltage separation procedures. Inorganic ions are rapidly removed before significant migration of the organic substances takes place. This desalting effect is particularly useful for the separation of carbohydrates without loss of substance, where inorganic ions cause severe migration disturbances.

Even better and almost complete separations of amino acids and peptides are possible by using two-dimensional electrophoresis, or a combination of high-voltage electrophoresis and a suitable chromatographic procedure. Best results are achieved



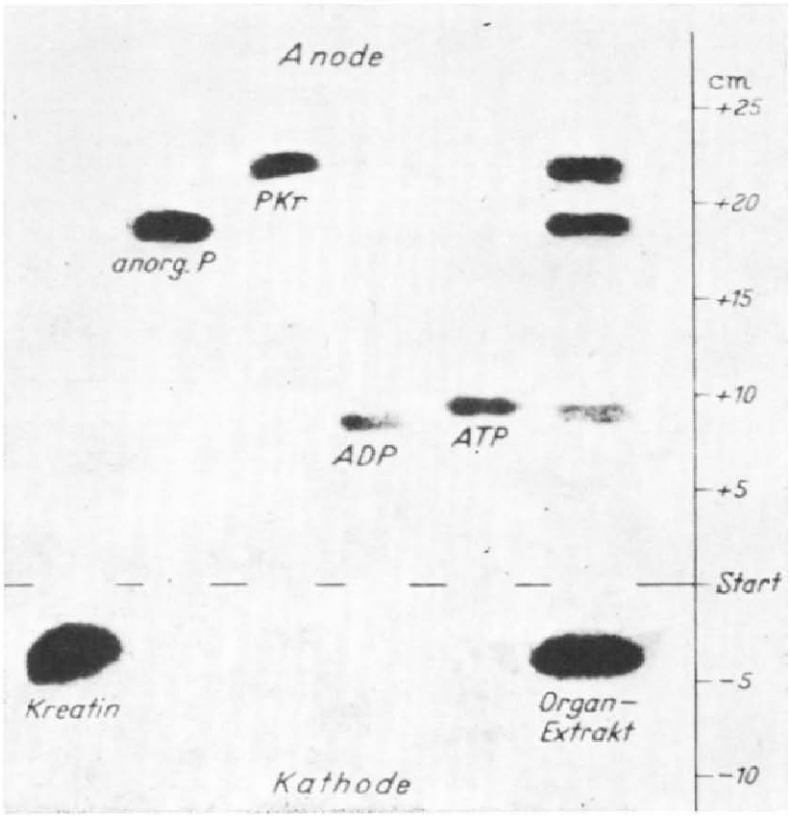


Fig. 12. Separation of metanephrines. pH Tris-buffer, 8.6; potential gradient, 100 V/cm.

Fig. 13. Separation of adenosine-phosphates and phosphocreatine in tissue extract. (With kind permission of THORN *et al.*, *Biochem. J.*, 330 (1958) 385.)

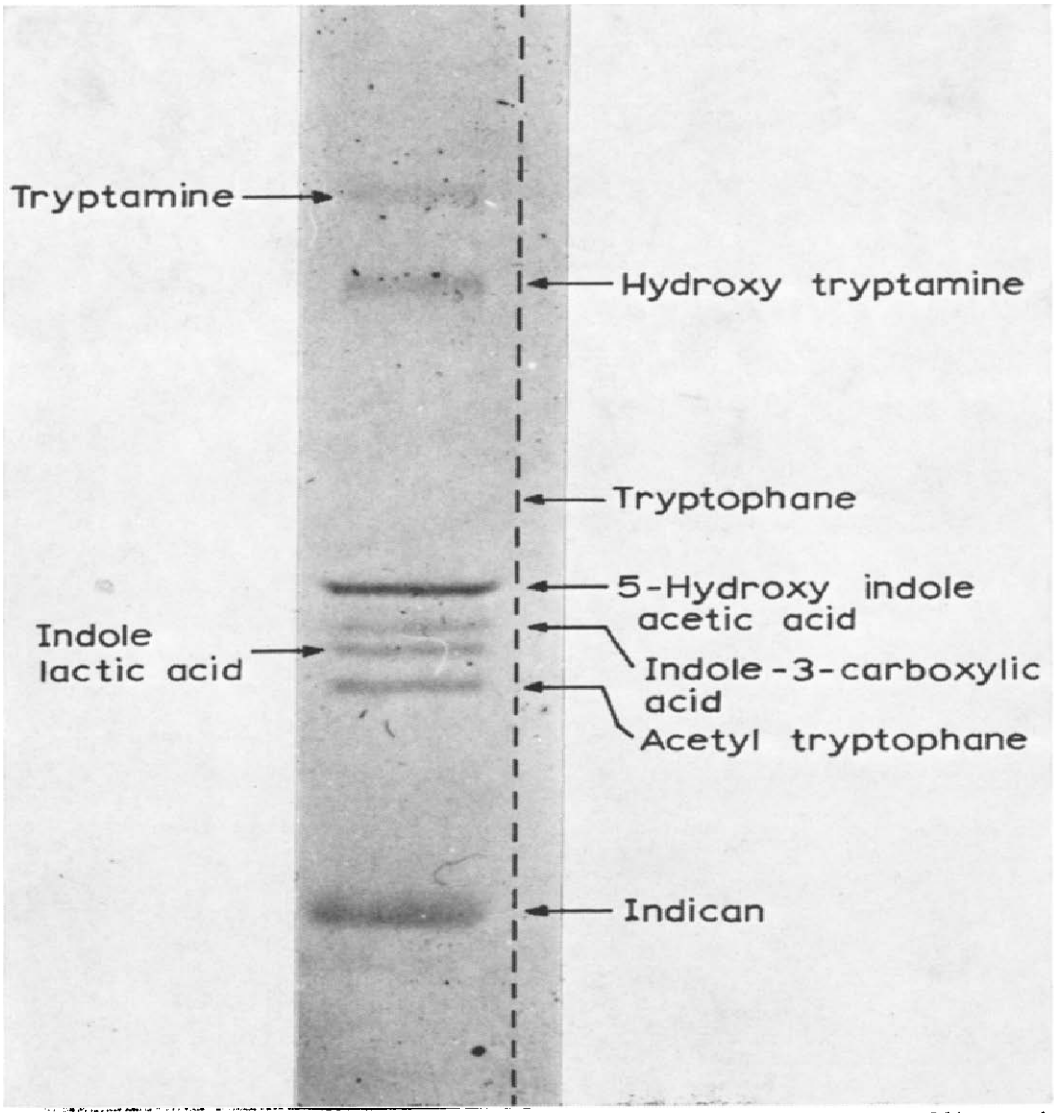


Fig. 14. Separation of indoles. pH, 3.5; potential gradient, 100 V/cm; time, 13 min.

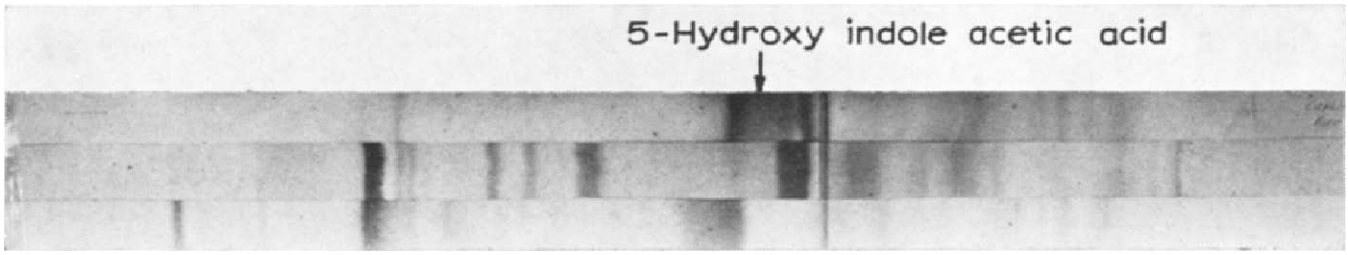


Fig. 15. Separation of carcinoid urine.

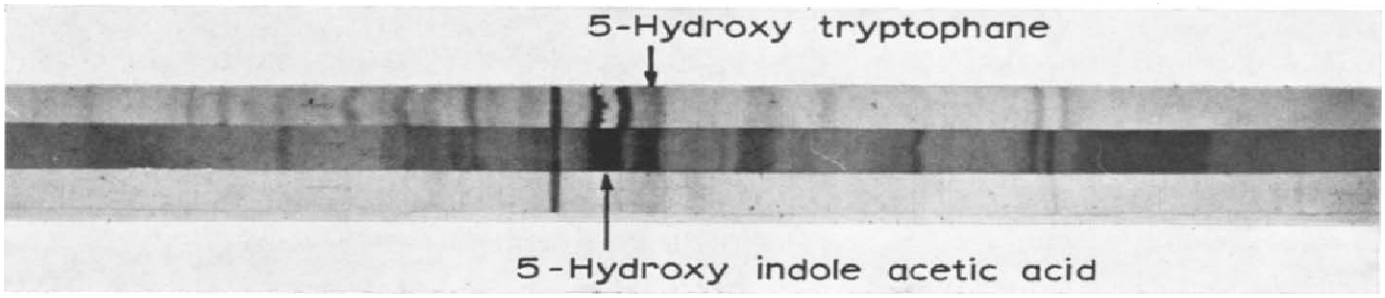


Fig. 16. Separation of 5-hydroxytryptophane and 5-hydroxy-indole acetic acid in a case of a typical carcinoid syndrom (urine). pH, 3.6; potential gradient, 100 V/cm; time, 90 min.

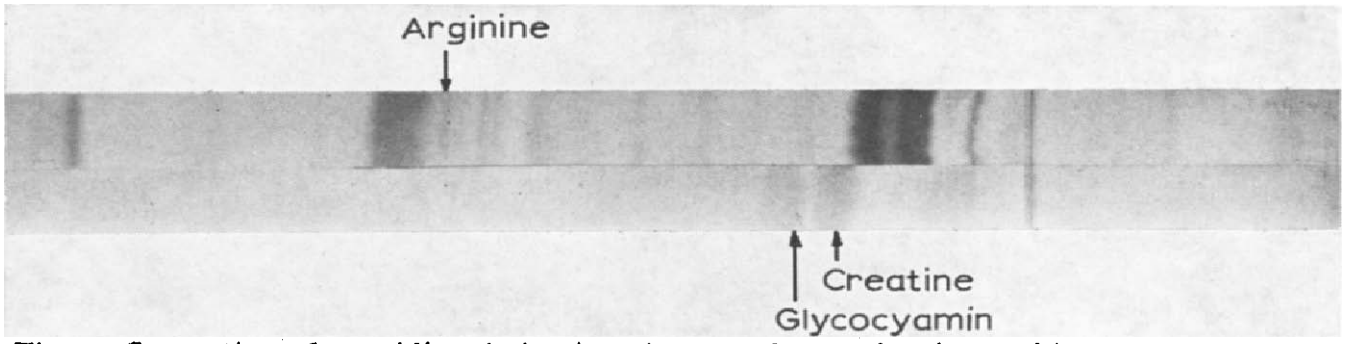


Fig. 17. Separation of guanidine derivatives (serum of muscular dystrophia).

Fig. 18. Separation of flavines and derivatives. pH, 5.1; potential gradient, 100 V/cm; time, 90 min.

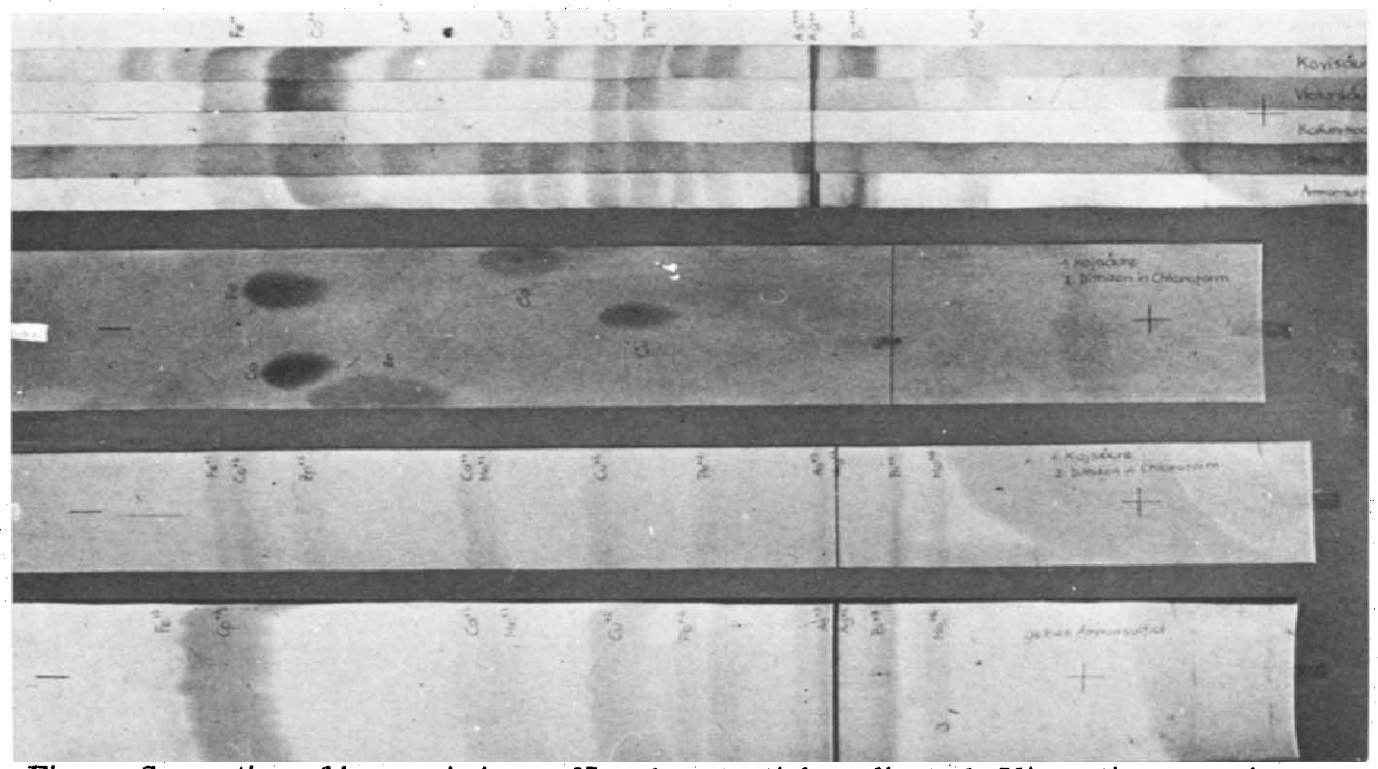


Fig. 19. Separation of inorganic ions. pH, 3.6; potential gradient, 60 V/cm; time, 90 min.

by either two-dimensional thin-layer electrophoresis or thin-layer electrophoresis and thin-layer chromatography. Such a technique, for instance, was applied to the separation of peptides produced by the tryptic digestion of human hemoglobins and other proteins. Hemoglobin digests give characteristic patterns of peptides, commonly called "fingerprints". Up to eighty peptides could be separated on one sheet by an improved method. A few pictures may illustrate such separations. The qualitative and quantitative analysis of biogenic amines by high-voltage paper electrophoresis was described in detail by FISCHER AND BOHN[8] and by FERENCIK and GROSS[7]. The

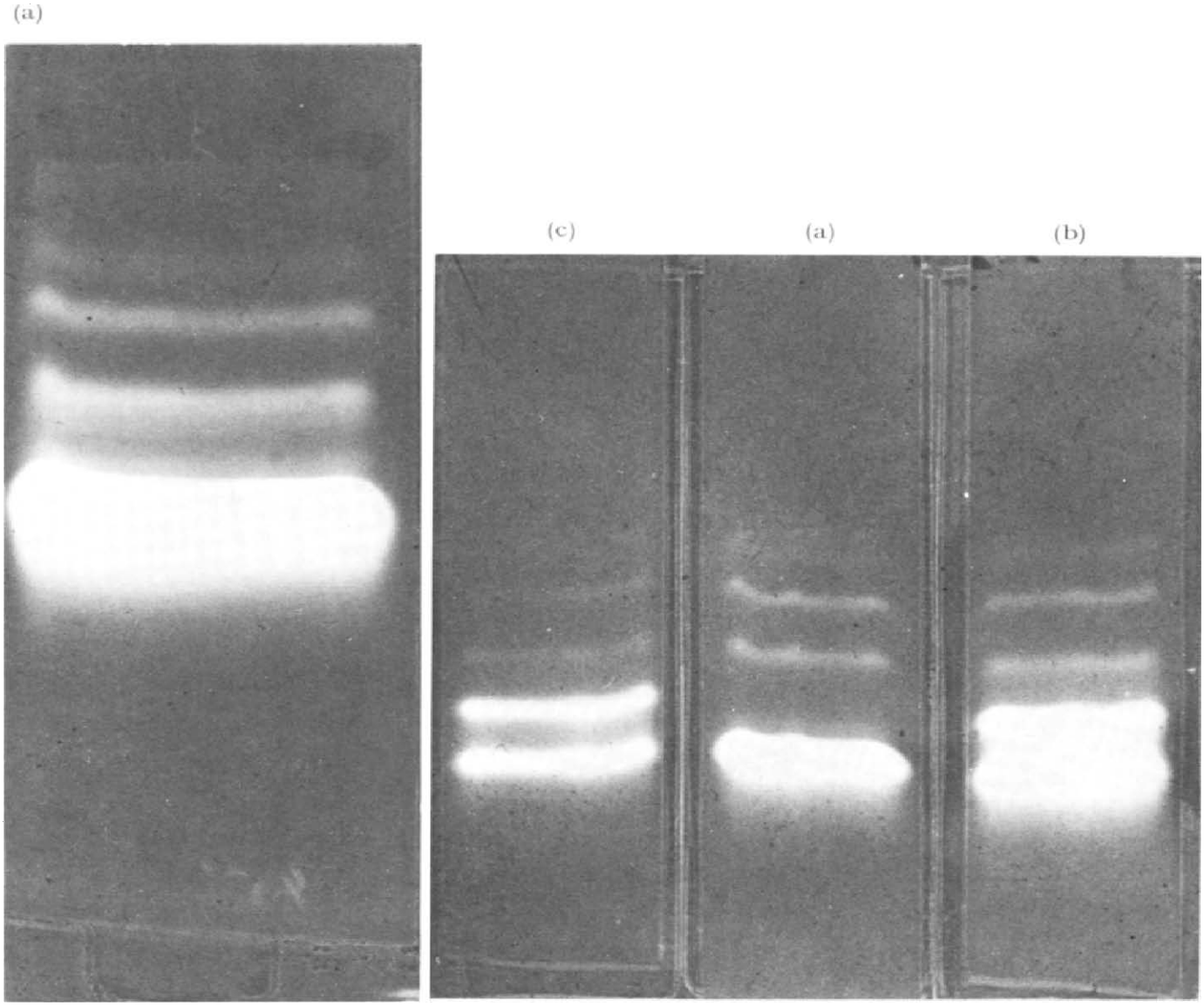


Fig. 20. Separation of hemoglobins. pH Tris-buffer, 8.6; potential gradient, 60 V/cm; time, 45 min. (a) normal hemoglobin; (b) and (c) sickel cell anemia (heterogenic type) in two brothers.

separation of some of these clinically important metabolites is demonstrated in Figs. 11–13. Considerable success was achieved in separation of nucleic acid components, nucleosides and nucleotides by FOSTER[9], BURKE[10], THOMAS and others. Electrophoretic techniques proved very useful for the separation of guanidine compounds and of phenolic acids and indole compounds in biological materials. Some examples may illustrate this.

Special attention is drawn to the separation of vitamins and their metabolites in blood. A very efficient separation of alkali and alkali-earth metals was demonstrated GROSS[6,7] by means of a suitable high-voltage technique.

Complex mixtures of metaphosphates and condensed phosphates were resolved by SANSONI AND BAUMGARTNER[11]. GROSS has also shown, that at 100 V/cm many of the heavy metal ions could be separated in 30 to 45 min as anionic complexes of

organic acids, whereas zinc (II) and other ions could be separated in the cationic form in 10 to 15 min.

For carbohydrate separations, a complexing agent is necessary, which gives the neutral substances an electric charge. Defined spots are obtained either when there is a rapid interconversion between complex and substance or when the equilibrium is displaced strongly towards the complex. The former is preferable as it permits greater differences in mobility between individual substances. High-voltage electrophoresis of neutral carbohydrates is usually performed in borate, hydrogen sulfite, arsenite, basic lead acetate, molybdate and sodium hydroxide buffers.

In our experience, germanate of pH 10.7 gives the best resolutions of complex carbohydrate mixtures. Germanate gives complexes with 1,2-*cis*-diols in furanosides and pyranosides and with open chain 1,2-diols with a *threo*-configuration. The same type of complex is formed with borate but the complexes between germanate and furanosidic 1,2-*cis*-diols seem to be especially strong.

Unfortunately no single buffer, not even germanate, gives a satisfactory separation of all the sugars likely to be encountered in biological specimens. But with two runs in buffer solutions of different pH practically all the monosaccharides and disaccharides can be separated. Electrophoresis on kieselguhr G gives much better results than on paper.

Electrophoresis has also been performed on substances of higher molecular weight and very clear separations of hemoglobins can be obtained either on starch block or on cellulose acetate.

From these few examples you can see that high-voltage electrophoresis is a very useful and practically unlimited tool for the separation of complex synthetic and biological mixtures. With the designs now available it is technically easy to perform, the results are very definite and reproducible, but of course, as in any other chemical or biochemical procedure the limits of the method must be kept in mind.

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