Vol. 87, No. 4, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 27, 1979 Pages 1252-1255

ISOELECTRIC FOCUSING WITHOUT AMPHOLYTE

Simeon Pollack

Department of Medicine
Division of Hematology
Albert Einstein College of Medicine
Bronx, New York 10461

Received March 5, 1979

SUMMARY

We have studied the electrophoretic mobility of proteins in polyacrylamide gels when ampholyte and buffer were excluded from the system. We found, using a weak acid and base as anolyte and catholyte respectively, that a pH gradient was generated in the gel, andthat proteins focused as sharp bands.

INTRODUCTION

The ampholytes used in isoelectric focusing are potentially responsible for a variety of experimental problems. Our concern with their propensity for removing metal bound to metallo-ligands (1) led us to study the electrophoretic mobility of proteins in polyacrylamide gels when ampholyte and buffer were excluded from the system. Using a weak acid and base as anolyte and catholyte respectively, we found that a pH gradient was generated across the gel by an electric field and that proteins, contrary to theoretical prediction, focused as sharp bands. The position of the protein bands in the pH gradient correlated approximately with their isoelectric points.

MATERIALS AND METHODS

Hen's egg ovalbumin, whale myoglobin and bovine pancreatic ribonuclease were obtained from Sigma (St. Louis, Mo.). Hemoglobin was obtained from a distilled water hemolysate of human red cells.

3.6% gels (acrylamide: bis acrylamide ratio 30:0.8) were made as follows: 1.33 ml of acrylamide bis—acrylamide, 9.61 ml deionized water

ABBREVIATIONS

TEMED: N,N,N^{1}N^{1} tetramethylethylenediamine. TRIS: Tris(hydroxymethyl) aminomethane. MES: 2(N-Morpholino) ethane Sulfonic Acid

0006-291X/79/081252-04$01.00/0

(conductivity approximately 3 micromho at room temperature), 0.01 ml TEMED and 0.05 ml of 10% ammonium persulfate were mixed and aliquots transferred to cylindrical plastic tubes (inside diameter 0.3 cm, length 10cm) and permitted to gel. Electrophoresis was carried out in the MRA M 137 C apparatus (MRA corp., Clearwater, Fla.). The anodal bath consisted of 0.1 M acetic acid, and the cathodal bath of 0.1M Tris (pH 9.5), unless otherwise specified. A Buchler 3-1009 power supply (Fort Lee, N.J.) was used to generate 1 milliamp per gel at the beginning of the experiment. Voltage was increased, as current flow decreased, to a maximum of 800 volts. Samples, applied ½ hour after initiation of current flow, were deposited on the top surface of the gel (cathodal side) in a volume of 0.002 ml, 1/10 of which was 70% sucrose. Four micrograms of ovalbumin and whale myoglobin and bovine pancreatic ribonucleus and 2.1 micrograms of hemoglobin were used.

At the conclusion of the electrophoresis, pH of the gels was measured using a surface electrode (Micro Combination pH probe, Microelectrodes, Inc., Londonderry, New Hampshire). Gels were fixed and stained in a mixture of 25% isopropanol, 10% acetic acid, 0.05% Coomasie Blue; destaining was in 10% acetic acid, 15% isopropanol.

RESULTS AND DISCUSSION

Myoglobin, hemoglobin and ovalbumin focused as sharp bands after 18 hours of electrophoresis (Fig. 1). The pH gradient across the polyacrylamide gel is shown in Fig. 2. Whale myoglobin (major band pI=8.2), hemoglobin (pI=7) and ovalbumin (pI=4.7) (2,3) focused within 0.3 cm of their isoelectric pHs. In several experiments bovine pancreatic ribonuclease (pI=9.3) did not focus but spread diffusely in the extreme

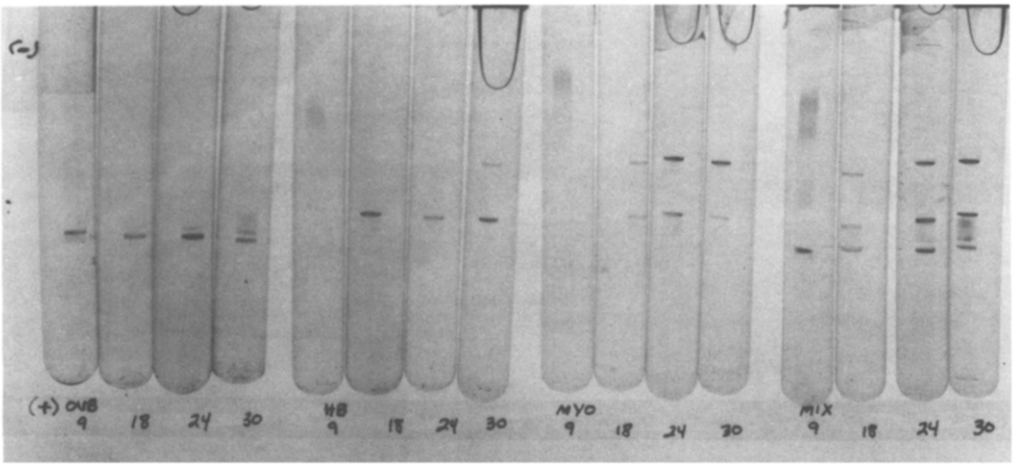

Fig. 1: Whale myoglobin, human hemoglobin and hen's egg ovalbumin were subjected to electrophoresis for 9,18,24 and 30 hours. The anodal band which appears in the hemoglobin samples at 30 hours is probably methemoglobin (3).

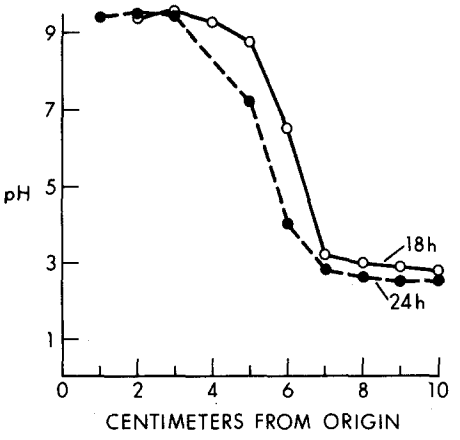

Fig. 2: The pH of gels on which the mixture of myoglobin, ovalbumin and hemoglobin were electrophoresed for 18 hours and 24 hours is shown.

cathodal end of the gel. There was cathodal drift of focused bands with the passage of time.

Although the major whale myoglobin band was well separated from hemoglobin, the minor whale myoglobin band (pI=7.7) was not separated from the hemoglobin. We attempted to improve discrimination by using increasingly weak acids as anolytes, anticipating a shallower pH gradient. 0.1M MES (pH 3.7), 0.1M boric acid (pH 5.0) and finally deionized water (initial pH 6.2) were tried. None separated the minor myoglobin band from hemoglobin. In other experiments we tested anolytes and catholytes to which small amounts of electrolyte had been added (NaCl or KCl in concentrations ranging from 0.001M to 0.01 M). There was no focusing at all in these latter experiments, nor was there any focusing when the spontaneous pH of the Tris or acetic acid was altered by the addition of acid or base respectively.

The failure to discriminate between proteins with isoelectric points less than one pH unit apart makes it unlikely that the present technique of isoelectric focusing will have much practical application. However, it might be useful, in special circumstances, when ampholyte

is interdicted, or when concentration of protein in a gel is required as a preliminary to another separation.

Our observations cast some light on the process of isoelectric focusing per se. The "law" of pH monotony states that the pH increases monotonically from anode to cathode. That law and the assumption that a zone of pure water separating two ampholytes would be incompatible with pH monotony has led to the conclusions that a single ampholyte could never concentrate (except at the anode or cathode), and that two ampholytes could be separated from one another only when a third with intermediate pI was between them (4,5). These latter conclusions, as shown by our experiment, are incorrect. Presumably, proton concentration in a zone of otherwise pure water need not be 10^{-7} in the presence of an electric field. The cathodal drift of focused protein bands in our experiment is reminiscent of the cathodal drift also seen when orthodox isoelectric focusing is done with ampholytes. Explanations of cathodal drift have frequently implicated ampholyte (6). The demonstration that cathodal drift can be independent of ampholyte suggests an alternative explanation, that proton flux, which is greater than OH^- ion flux at equivalent concentrations, eventually overwhelms whatever buffer capacity exists in the system.

REFERENCES

(1) *Righetti, P.G., Drysdale, J.W. (1976) Isoelectric Focusing, page 543-549, North Holland Publishing Col, Amsterdam.*

(2) *Radola, B.J. (1973) Biochim Biophys Acta 295,412-428.*

(3) *Drysdale, J.W., Righetti, P., Bunn, H.F. (1971) Biochim. Biophys. Acta 229, 42-50.*

(4) *Svensson, H. (1967) Prot. Biol. Fluids 15, 515-522.*

(5) *Svensson, H. (1961) Acta Chem. Scandinavica 15, 325-341.*

(6) *Righetti, P.G., Drysdale, J.W. ibid page 525-526.*