

## Isoelectric Fractionation, Analysis, and Characterization of Ampholytes in Natural pH Gradients

### VIII. Continuous Recording of pH and Light Absorbance of Column Effluent after Isoelectric Focusing

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An experimental arrangement for getting continuous pH and light absorption records on effluents from electrofocusing columns is described. Extensive control measurements are reported, and conditions for getting reliable pH records are presented.

The analysis of the contents of the column after isoelectric focusing comprises concentration measurements in order to localize focused zones and pH measurements in order to determine the isoelectric points of separated components. Among unspecific methods for quantitative analysis of proteins, the UV light absorption method is most convenient and has found the widest application. According to the experimental procedure described by Vesterberg and Svensson,<sup>1</sup> the contents of the column are subdivided into a great number of fractions which are subjected to individual pH and extinction measurements. In order to retain as much as possible of the high resolution obtainable in the column, more than 50 and sometimes up to 100 fractions are taken. The analysis of so many fractions is, of course, a very time-consuming business. In this article, an experimental arrangement will be described by which it is possible to get continuous records of both pH and UV light absorbance of the effluent from the column after completed focusing.

#### EXPERIMENTAL

*Instrumental equipment.* An electrofocusing column of 110 ml capacity (manufacturer: Ingenjörfirman Consulta, Sköndalsvägen 106, Farsta, Sweden) was used. The density gradient mixer recently described by Svensson and Pettersson<sup>2</sup> and built in our workshop was used for preparation of constant density gradients in the column.

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A Beckman Expandomatic pH meter in conjunction with a Beckman 97633 capillary pH electrode assembly was used for the pH measurements. The latter consists of a reference electrode and a transparent epoxy block cast around a capillary glass electrode and a capillary glass tube (internal volume  $50 \mu\text{l}$ ). The pH meter was coupled to a Beckman  $10''$  linear potentiometric recorder.

A Vitatron Universal Photometer UFD (manufacturer: Vitatron N. V., Dieren, the Netherlands), equipped with a flow-through cell UFD 353, was used for the light absorption measurements. This cell has a horizontal optical path length of 10 mm and an internal volume of  $70.7 \mu\text{l}$ . Since the light filter for 280 nm supplied with the instrument did not function properly, it was replaced by a monochromator adjusted to the same wavelength. (Vitatron has subsequently delivered a much better filter.) The Vitatron photometer was connected to a lin/log integrating recorder from the same company.

For driving the effluent through the two cells at a constant rate, a pump giving a very even flow was required. Several commercial micro pumps were tried with only limited success. Finally, Mr. Sune Pettersson at our department designed and built a new type of peristaltic pump which proved to be very good for our purpose.

For effective temperature control a water thermostat in conjunction with a circulation pump was used. This thermostat could be set at  $+4^\circ\text{C}$  by use of a refrigerating machine as well as at  $+25^\circ\text{C}$  by means of an immersion heater.

*Chemicals.* Sucrose of analytical reagent grade from Mallinckrodt Chemical Works, St. Louis, Mo., USA, was used for making the density gradients, the denser solution in the gradient mixer containing 500 g of sucrose per litre of solution.

Carrier ampholytes (Svensson;<sup>3</sup> Vesterberg<sup>4</sup>) with the trade name Ampholine and delivered by LKB-Produkter AB, Bromma, Sweden, were used as electrolytes in the column for development of stable pH gradients. Around the electrodes special solutions were applied according to recommendations given by Vesterberg and Svensson.<sup>1</sup>

pH standard solutions were obtained from the Beckman Corporation and from P.-H. Tamm, Altuna, Sweden.

The recording system was tested on a sample of the fungal enzyme laccase, which was obtained from the Department of Biochemistry, University of Gothenburg.

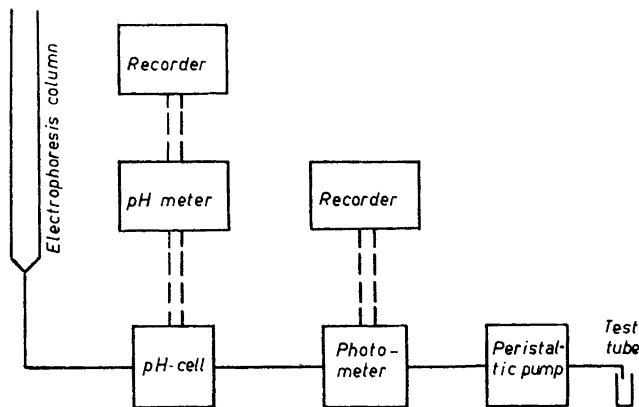


Fig. 1. Block diagram of the instrumental set-up for continuous recording of pH and UV light absorbance courses of the column solution.

*Experimental arrangement.* The various instruments mentioned above were put together as shown in the block diagram in Fig. 1. The capillary ducts between column, cells, and pump consisted of about 15 cm long polyethylene tubes of 1 mm internal diameter. The volume of such a piece of tubing is about  $120 \mu\text{l}$ , but the volume lag between the active glass membrane in the pH cell and the light absorption cell was about  $270 \mu\text{l}$ . At a rate of flow of  $0.8 \text{ ml/min}$ , as used in our experiments, this corresponds to a time lag of 20 sec between the two records.

Continuous recording of the pH of the effluent proved to be a delicate task. Difficulties of various kinds were encountered, and many precautions had to be taken before this recording could be brought to function properly.

First it was observed that pH readings depended slightly on whether the sample was stationary or flowing. In the manufacturer's manual this is interpreted as a hydrostatic pressure effect causing the sample solution to penetrate into the capillary region of the junction structure of the salt bridge between reference and glass electrodes, thereby affecting the liquid junction potential. This effect could be minimized by application of a sufficient hydrostatic pressure to the reference electrode and salt bridge. In the Beckman 97633 capillary pH electrode assembly a palladium annulus junction in the epoxy block permits electrical communication between the sample in the capillary tube and the reference electrode in an elevated remote reservoir. A Tygon tube links this junction with the reservoir, and by choosing a length of about 20 cm, the electrolyte solution can be maintained at a hydrostatic pressure great enough to give stable liquid junction potentials.

Secondly, an effective temperature control proved to be essential. The flowing solution and the pH cell must have the same constant temperature. For that reason, a final focusing was always performed at +25°C even if the isoelectric focusing was started and essentially completed at a lower temperature, since it was technically easier to perform pH measurements at room temperature.

Thirdly, accidental electrostatic charges on the instruments or parts thereof proved to cause serious disturbances in the form of inconsistent and false pH readings. According to normal practice, the pH meter and the recorder were grounded, but these precautions proved to be insufficient. When the pH cell was connected to the column, severe disturbances were encountered, and the pH records obtained were completely unreliable. The possibility of grounding the test solution instead of the instruments was also tried, but without success.

The necessity of shielding the pH cell grew increasingly evident, and experiments were then performed with the pH cell submerged into grounded and thermostatically controlled circulating water in an endeavour to accomplish both electrical shielding and temperature control by the same device. All such attempts were, however, unsuccessful. It seemed as if such arrangements were accompanied by a grounding of the test solution right through the epoxy resin. After these negative trials, it was decided to build a well-fitting brass box with hollow walls around the pH cell. Circulation of thermostatically controlled water through the hollow walls then secured the necessary temperature control, whereas grounding of the brass walls surrounding the cell secured an effective shielding. In order to avoid unintentional grounding of the flowing solution, the box was painted inside with an insulating coating.

After extensive trials, we finally concluded that disturbance-free and reliable pH records could only be obtained by grounding the pH meter, the recorder, the brass housing surrounding the pH cell, and the circulating water in the mantle of the column. The effectiveness of the latter grounding had to be secured by dissolving sodium chloride in the circulating water and by placing a grounded copper sheet in the water reservoir of the thermostat. Disturbances persisted, however, even with these extensive precautions.

Disconnection of both cables between power supply and platinum electrodes in the column led to some improvement, but complete success was not obtained until all liquid contact between the electrodes and the solution in the column was disrupted prior to the recording procedure. The necessity of this last step is easily understood by recalling that, at the end of the run, the anolyte is saturated with oxygen, the catholyte with hydrogen. These gases also stick to the electrodes in the form of bubbles. Consequently the electrodes and the liquid in the column form together a galvanic element with a considerable electromotive force which of course interferes violently with the pH measurement.

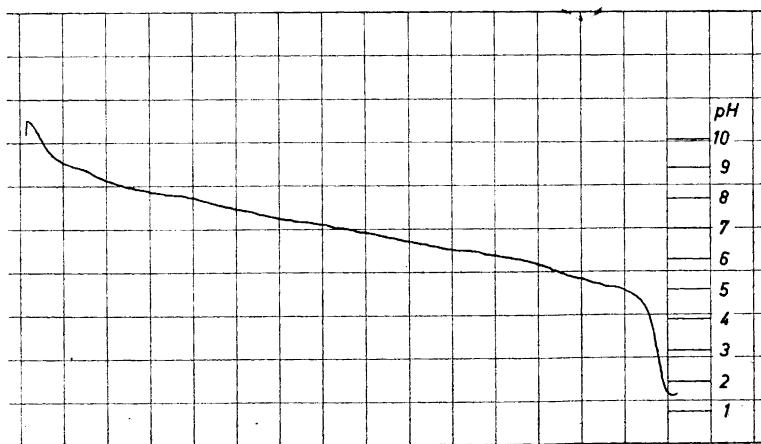
The continuous recording of UV light absorbance involved only minor problems. Because of the steadily decreasing density and refractive index of the flowing solution from the column, a vertical flow-through cell would have been much more suitable than the horizontal cell in the Vitatron equipment. However, it was not found necessary to reconstruct the instrument since the density and refractive index differences prevailing within the small cell volume did not appear to cause any detectable disturbances. The

formation of gas bubbles proved to be a much more serious problem. Gas bubbles, once formed somewhere in the capillary tubes or in the cell due to the heating effect of the radiation, were effectively entrapped in the cell due to its horizontal orientation. Since the column solution after electrolysis is necessarily saturated with hydrogen at one end and with oxygen at the other, the tendency toward bubble formation is very pronounced, and something had to be done about it. We finally decided to cool down the flow-through cell by surrounding it with a well-fitting brass housing with hollow walls through which cold water was circulated. In this way, the flowing solution became unsaturated with gases, and subsequently we never had any trouble with gas bubbles.

*Procedure.* Equal volumes of one dense solution containing sucrose and 3/4 of the carrier ampholytes and one less dense solution containing the protein sample and 1/4 of the carrier ampholytes were transferred to the two compartments of the density gradient mixer described by Svensson and Pettersson.<sup>2</sup> The effluent from the mixer was fed into the electrolysis column. After introduction of the special electrode solutions according to Vesterberg and Svensson,<sup>1</sup> the column was slowly cooled down to a working temperature of +4°C. Isoelectric focusing was effected by electrolysis during 24–48 h. The temperature of the circulating water was then slowly raised to +25°C, and a final focusing was performed at this temperature during 3–4 h. The thermostat water, grounded as described above, was then directed also through the hollow brass box surrounding the pH cell.

Before emptying the column, the pH meter was calibrated with the aid of a number of standard buffer solutions. These were put into the thermostatic bath and were pumped through the pH cell at the same rate (0.8 ml/min) as was subsequently used for the column solution. The pH meter was standardized against one of the buffer solutions. Then the others were pumped through in succession, and their pH values were obtained as straight parallel lines on the chart paper as shown in Fig. 2.

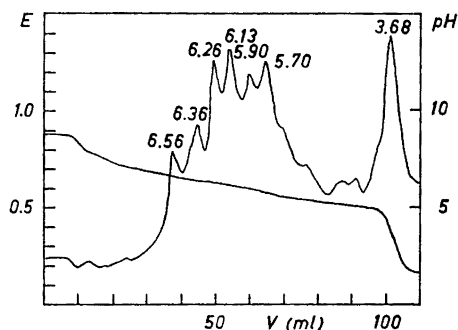
The electric current was switched off, and both cables from the power supply were removed. After closing the central gas-escape tube in the column at the bottom, the solution enclosed therein was removed by suction through a plastic capillary tube going down to the bottom stopper. The solution round the upper electrode was likewise removed. The column was then attached to the pH cell through a piece of capillary tubing, and



*Fig. 2.* Photograph of a pH recorder chart obtained after steady state electrolysis of Ampholine pH 5–8 at a concentration of 2 g/100 ml. The calibration lines seen to the right were obtained with a series of standard buffer solutions. The steep pH decline between 5 and 1.5 is due to the absence of buffering protolytes in this region. Comparatively large errors persist in this pH region, but within the nominal pH range 5–8 of the Ampholine preparation, the pH record represents the true pH course with an accuracy of  $\pm 0.03$  pH units (see text).

draining and recording were started. The pH recorder then gave the monotonically increasing or decreasing pH course, whereas focused protein zones appeared as peaks in the light absorption record. After about 2 h and 20 min, the column was empty, and the records were taken out and superposed with a mutual lateral shift corresponding to 20 sec, the time lag between the two flow cells. Both records could then be traced on a superimposed transparent paper sheet. Combined records of the type shown in Fig. 3 were thus obtained. They allow a most convenient determination of the isoelectric points of all protein components by drawing vertical lines from the light absorption peaks to the pH curve.

Fig. 3. Continuous pH and light absorbance courses of effluent from an electrofocusing column after isoelectric focusing of the enzyme fungal laccase B. The figures at the UV absorption peaks are the isoelectric points of some of the separated enzyme components, obtained by vertical transfer from the peaks to the continuous pH record.



*Control of continuous records by conventional sampling.* In order to test critically the reliability of the continuous records, the effluent from the peristaltic pump was subdivided manually into 0.8 ml fractions (see Fig. 1). The use of a time-controlled fraction collector for this purpose was tried, but abandoned since it was difficult to avoid disturbances in the continuous records from the relays of the collector.

The pH's and, in some experiments, the UV light absorbances of each or every second fraction were measured at the same temperature and with the same equipment as in the continuous measurement.

In some experiments, the pH's of fractions were measured by way of the recorder, which was then standardized and calibrated as already described. In other experiments, the recorder was not used, the individual pH's being read directly on the pH meter, which was then standardized against Beckman and P.-H. Tamm buffers with pH's within  $\pm 0.5$  pH unit from the pH's of the actual fractions.

The fractions were pumped into the cell, one after the other, alternately with portions of rinsing water. For each fraction, the pump was stopped during measurement. No significant differences were found between control runs done with and without use of the pH recorder.

Five electrofocusing experiments with extensive control of the continuous records were carried out using different Ampholine preparations and concentrations. What concerns the pH records, the results can be summarized as follows.

With an Ampholine preparation covering 3 pH units at a concentration of 2 %, the continuous pH records were found to be correct within a margin of  $\pm 0.03$  units, which must be regarded as very satisfactory, particularly since the deviations didn't show any systematic trend. With an Ampholine preparation covering the pH range between 3 and 10, used at the same concentration, the continuous pH records were found to be in error by as much as 0.07 units in the steepest parts of the pH course. These deviations were systematic in the sense that the response of the pH meter lagged behind. Experiments with an Ampholine concentration of only 1 % displayed greater errors in the continuous pH records, especially in the great pH range between 3 and 10, but also in narrower pH regions comprising only 3 units.

Plots of UV light absorbance against fraction number showed a very fine agreement with the continuous records in all instances. This proves that the liquid transport from

absorption cell through pump to test tubes has no detectable effect on a concentration record. This conclusion can of course be extended to the liquid transport from column to pH cell. In vertical parts of the capillary ducts, the density gradient can be expected to counteract remixing of zones liable to occur due to the parabolic velocity profile characterizing laminar flow. Remixing due to axial diffusion in a capillary pipeline is negligible due to the very small cross-sectional area and the very small concentration gradient. The diffusional remixing occurring in the column during more than 2 h must, however, give rise to some remixing, particularly for zones focused in the upper part of the column, which are eluted last.

The rate of flow used by us was about the same as that recommended by Vesterberg<sup>5</sup> for a fraction-cutting procedure.

#### DISCUSSION

The results reported above prove the possibility of obtaining reliable continuous pH records from electrofocusing columns containing Ampholine covering 3 pH units at a concentration of 2 % if the rate of flow through the pH cell does not exceed 0.8 ml/min. Experiments with Ampholine preparations covering greater pH ranges and experiments with an Ampholine concentration of only 1 % reveal that the exactitude of continuous pH records diminishes with an increasing pH gradient and with a decreasing concentration of carrier ampholytes. The rate of flow has not been varied in our experiments, but it appears as self-evident that a low rate of flow favours an exact recording of pH. With due regard to the fact that the speed of response of a glass electrode to pH changes has been found to increase with the buffering capacity of the test solution (Perley<sup>6</sup>), our experiments thus seem to justify the conclusion that the reliability of continuous pH records depends on only two factors: the buffering capacity of the flowing solution and the rate of change of its pH, that is, the derivative  $d(\text{pH})/dt$ , where  $t$  denotes time. It appears plausible to assume direct proportionality between the allowable time derivative of pH and the concentration  $c$  of the carrier ampholytes, which are responsible for the buffering capacity:

$$d(\text{pH})/dt = k c \quad (1)$$

where  $k$  is a constant. This equation can also be written:

$$\frac{d(\text{pH})}{dV} \frac{dV}{dt} = k c \quad (2)$$

where  $V$  denotes volume. If the symbol  $v$  is used for the rate of flow  $dV/dt$ , the equation can be solved for the allowable rate of flow:

$$v = \frac{k c}{d(\text{pH})/dV} \quad (3)$$

If, finally, the pH gradient  $d(\text{pH})/dV$  is approximated by the ratio between total pH range and total volume, one gets the equation:

$$v = \frac{k c V}{\Delta\text{pH}} \quad (4)$$

The numerical value of  $k$  can be derived from our result that 0.8 ml/min is an allowable rate of flow for an ampholyte concentration of 2 g/100 ml and for a pH range of 3 units within a volume of 100 ml. This gives  $k=1.2$  ml pH units per gramme and minute. Thus one has for the allowable rate of flow:

$$v = \frac{1.2 c V}{\Delta\text{pH}} = \frac{1.2 m}{\Delta\text{pH}} \text{ ml/min} \quad (5)$$

where  $m$  is the amount of carrier ampholytes in grammes. The time required for a complete record becomes:

$$t = \frac{\Delta\text{pH}}{1.2 c} = \frac{\Delta\text{pH} V}{1.2 m} \text{ min} \quad (6)$$

with g and ml as mass and volume units, respectively. For an unchanged ampholyte concentration, this time is independent of the size of the column. At least a couple of hours will thus be necessary even for small columns.

Continuous recording of pH and light absorption has many advantages compared to discrete measurements on fractions. It gives with less work more detailed information. The solution is also protected from contact with air.

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