Digitally Controlled Electrophoretic Focusing

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Proteins can be simultaneously separated and concentrated by applying a constant force and opposing this with a gradient in a second force. In this work, a constant hydrodynamic force is opposed by a gradient in the electric field which allows charged molecules to focus in order of their apparent electrophoretic mobilities. The electric field gradient is established and maintained using an array of electrodes whose voltages are individually monitored and adjusted by a computer-controlled circuit board. The computer-generated electric field gradient allows charged molecules to be focused without using a pH gradient. Since the proteins are not focused at their p*Is*, precipitates do not form, so focused concentrations in excess of 50 mg/mL are not unusual. In addition, since the field shape is dynamically controlled from the computer on a point-by-point basis, the field profile can be adjusted during a run to improve the resolution. In this paper, the column and controller are described together with experimental results and a model which illustrates the separating power and flexibility of this technique.

More than a decade ago, O'Farrell[1] described how proteins could be focused at the interface between two different gel filtration media packed into the upper and lower halves of an electrochromatography column. His results were soon replicated by others[2,3] who found that at least one protein, ferritin, could be concentrated beyond 100 mg/mL. This remarkable feat was tempered by the finding that his approach worked poorly with protein *mixtures* and would be difficult to scale up.[4] Nevertheless, O'Farrell had found a way to focus proteins in an electric field that did not require the use of a pH gradient. This suggested that other approaches to electrophoretic focusing might be found.

Recently, Koegler and Ivory[5] demonstrated that charged proteins could be separated and focused using an electric field gradient in an electrochromatography column. A fluted cooling jacket was used to form a linear gradient in the electric field which drove the proteins against a constant flow of buffer in a packed dialysis tube. This approach was slow and cumbersome and gave mediocre results, but it successfully illustrated an alternative focusing technique which we named electric field gradient focusing (EFGF).

Next, Greenlee and Ivory[6] showed that proteins would focus in the electric field gradient formed by an axial conductivity gradient and opposed by a constant flow of buffer. Greenlee's apparatus was far simpler to build and operate than was Koegler's. It was also surprisingly fast when run in free solution, reaching equilibrium in less than 10 min, and gave unexpectedly good results when filled with a 40-μm size exclusion (SEC) packing. The success of Koegler's and then Greenlee's approach to EFGF encouraged us to attempt focusing using an array of electrodes to establish the electric field gradient.

EXPERIMENTAL SECTION

Apparatus. The focusing chamber (Figure 1A) was formed from two blocks of 15 × 6 × 1.2 cm^3 Plexiglas and a 0.3-cm-thick Teflon spacer. The front block, which houses the separating column, has a 8 × 0.1 × 0.05 cm^3 trough machined into it. The rear block, which houses the 50 controllable electrodes, has a 6.4 × 0.3 × 1.5 cm^3 trough and the spacer has a 6.5 × 0.2 cm^2 slot machined through it. The trough in the front block is isolated from the spacer by a dialysis membrane and is packed with a 4.5-μm NovaPak Diol chromatography media (Waters). The rear trough and slot admit a recirculating buffer that may have the same composition as the running buffer, acts both as coolant and anolyte, and removes electrolysis products from the electrode array. Since the coolant is in contact with the column via a dialysis membrane, it can also be used to dialyze the running buffer to exchange salts or other low-molecular-weight solutes. The coolant inlet and outlet lines are visible at the top of Figure 1A.

Outside of the focusing chamber, the coolant buffer is circulated through a glass heat-exchange reservoir submerged in an ice bath. From here the coolant is introduced into the bottom of the focusing chamber and is passed over the electrodes at ~15 mL/s using a centrifugal pump (Cole-Parmer). A syringe pump controls the flow of the running buffer through the packed bed at 15−150 μL/h. The running buffer enters the column in the upper flow inlet on the front face and exits from the lower flow outlet on the front face (Figure 1A). All lines are PEEK with flangeless fittings; sample is loaded through a 10-μL loop on a six-port injection valve (Upchurch).

Controller. The 50 chamber electrodes are made from 0.25-mm-o.d. platinum wire (Aldrich Chemical), are mounted in the rear Plexiglas block with a 0.05-in. pitch, and are connected to a SCSI ribbon cable using SMS-series microstrips (Samtec). Each of the SCSI leads is connected to its own printed-circuit (PC) monitor/controller board (Figure 1B) mounted on the wire wrap

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(2) Hunter, J. *Sep. Sci. Technol.* **1988**, *23*, 875.
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(4) Gobie, W.; Ivory, C. *Biotechnol. Prog.* **1990**, *6*, 21.
(5) Koegler, W.; Ivory, C. *J. Chromatogr., A* **1996**, *229*, 229−236.
(6) Greenlee, R.; Ivory, C. *Biotechnol. Prog.* **1998**, *14*, 300−309.

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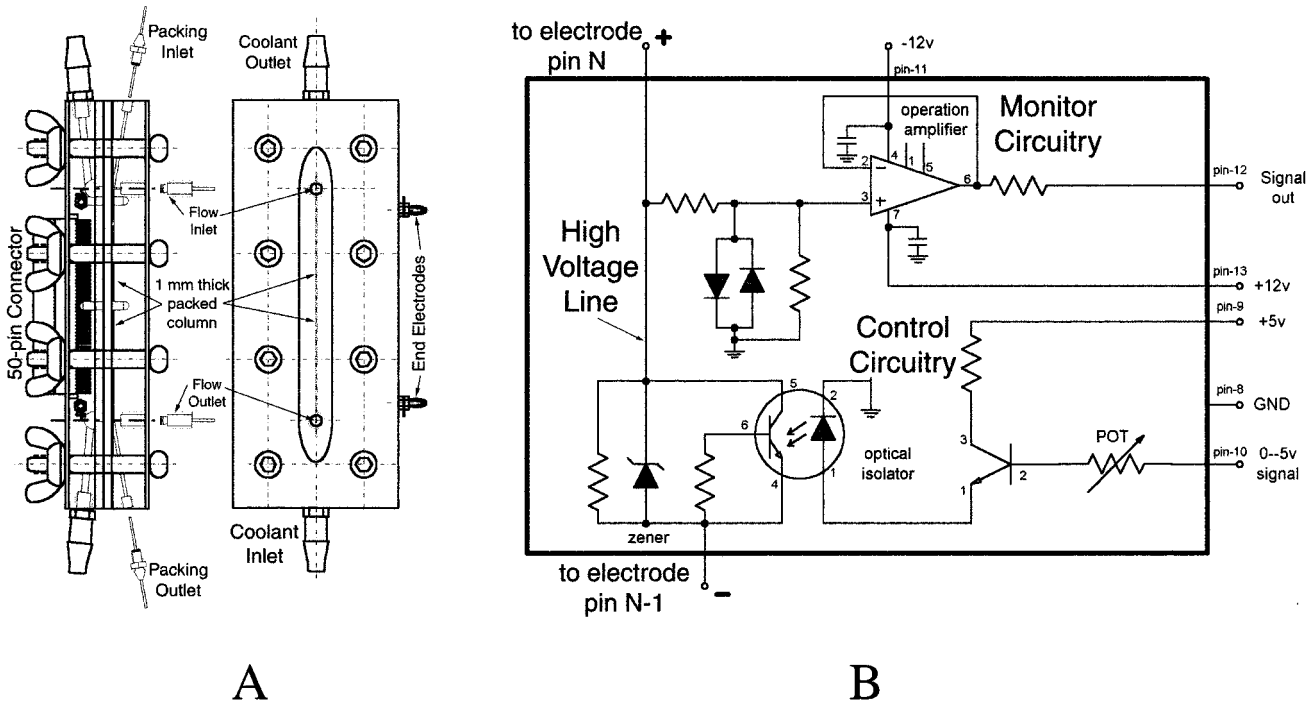

Figure 1. (A) Schematic showing front and side views of the DFGF chamber. In the side view note the 50-pin connector which supplies power to the 50 platinum electrodes that maintain the field gradient in the chamber. The 50-pin connector is 2.5 in. long. The packed section of the column, which is barely visible in the side view, extends from the inlet port to the outlet port, resides in the 0.05-cm trough in the front block, and is segregated from the recirculating coolant by a dialysis membrane. (B) Schematic showing the design of one of the 50 electrode controllers. The vertical lead on the left-hand side of the controller carries high voltage onto the controller board where it is registered by the OP-Amp in the monitoring section of the board. This signal is scaled-down, read by the computer, and compared with the programmed profile, and the effective resistance of the high-voltage line is adjusted by the optical isolator in the controller section of the board to match the programmed voltage profile with the measured profile.

motherboard. Each monitor/controller board is segregated into three areas: high voltage, monitoring, and control. The high-voltage area isolates the chamber electrode voltages, which can be as high as 600 V, from the relatively sensitive electronics used to measure and adjust the electrode voltages. The monitor area of each PC board scales down the electrode voltage by ~100× and sends this signal to a commercial thermocouple board which digitizes the signal before sending it to the computer. The computer scans all 50 electrodes, compares these readings with the programmed profile, and sends a digital signal to a set of 50 DACs which tell the optical isolators to adjust the effective resistance of high-voltage line to reduce the departure of the measured electrode voltages from the programmed voltage profile. A complete scan/control cycle of the 50 controllers is taken every second. Each of the 50 controllers is mounted vertically on a wire-wrapped motherboard; power to the controllers' motherboard is drawn from the computer. A 600-V power supply (Xantrex) provides current to the column's 50 high-voltage electrodes via the 50 voltage controllers.

Procedure. After the recirculating coolant has reached operating temperature and the packed column has been cleaned, e.g., with 7 M urea, and equilibrated with running buffer, 10 μL of protein solution is injected into the column, which has a packed volume of 28 μL exposed to the 50 controlled electrodes, using a standard sample loop. Before protein reaches the outlet, the controller is booted using a default voltage pattern and the power supply is brought up to a voltage in the range 200−600 V. The operator then selects the initial electric field gradient, and the computer program adjusts the electrode voltages until this gradient is attained, typically less than 5 min from a "cold" start.

RESULTS

Typical results with the chamber are presented in Figures 2−4, which are digitized images of naturally colored or artificially labeled proteins dynamically focused in an electric field gradient. In each of these figures, the flow in the packed section of the column is from top to bottom and the voltage gradient is greatest near the outlet, vanishes at the inlet, and is linear over the 2.5-in. length of the electrode section. In all of the experiments reported here, the first 49 array electrodes are anodes while the last electrode is a cathode set to ground and the electric field strength is linear

The proteins (all purchased from Sigma) and run conditions used in these experiments are listed in Table 1. Individual protein bands (Figure 2a−c) take 10−30 min to focus depending on the flow rate of the running buffer. The bands formed have roughly the baseline width predicted by the linear theory discussed in the next section of this paper and reach concentrations in the range of 5−50 mg/mL even without subtracting the nonaccessible volume of the packing. For example, in Figure 2a, the phycoerythrin band is less than 0.2 mm thick, 1.0 mm wide, and 0.5 mm deep and contains 2.5 μg of protein which translates to an apparent focused concentration of roughly 25 mg/mL.

When multiple proteins are run, as is the case in Figure 2d, it is sometimes difficult to set a linear field gradient where all of the proteins can be retained in the column and baseline separated

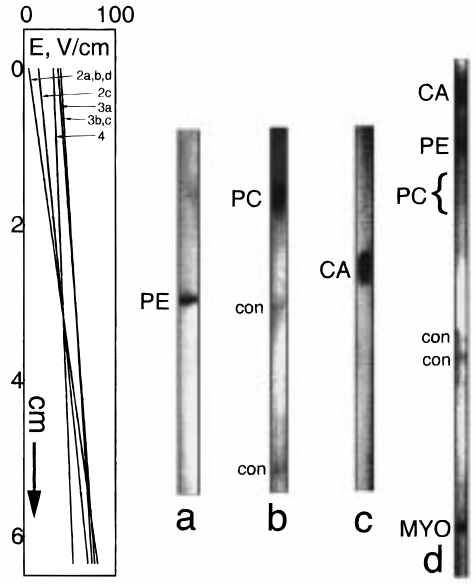

Figure 2. (A) Electric field profiles used in the experiments reported in this paper as noted in Table 1. In these experiments, the electric field profile was always linear; i.e., the electric field gradient, ∇E, is constant. (B) Digitized images of various focused proteins. (a) Phycoerythrin (PE), (b) Phycocyanin (PC) showing two contaminant (con) bands, (c) carbonic anhydrase labeled with Texas Red (CA), and (d) a cocktail of proteins including PE, PC, CA, and myoglobin (MYO) focused together in the column. See Table 1 for run conditions.

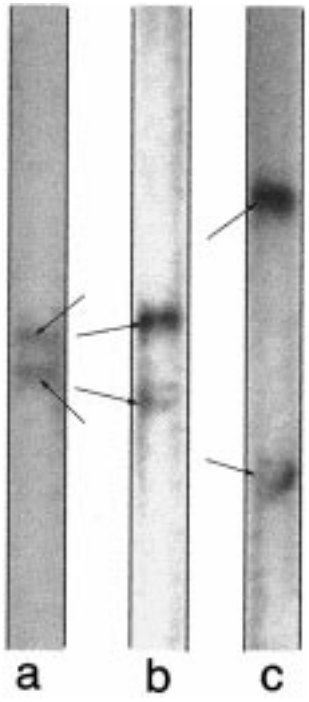

Figure 3. Digitized images of focused Sigma IEF-grade myoglobin in 10 mM tris−phosphate buffer. (a) At pH 8.8, 400 V, and $\nabla E = 6.9$, two bands ~0.5 mm thick are separated by ~0.5 mm. (b) Reducing the electric field gradient to $\nabla E = 5.9$ increases the resolution. (c) Reducing the pH in the coolant circuit to 8.3 further improves band separation.

at the same time. This is due in part to the wide variation in mobilities in this particular group of proteins and, to a greater extent, to the tendency of the concentrated protein bands to merge into isotachophoretic bands if they come too close to one another.

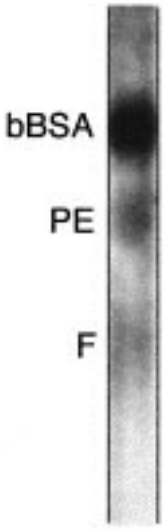

Figure 4. Digitized image of focused proteins including bovine serum albumin labeled with bromophenol blue (bBSA), PE, and ferritin (F) at pH 8.7 and $\nabla E = 3.7$.

Figure 3 shows how separation conditions can be modified by the operator during a run to improve resolution. In Figure 3a, IEF-grade marker myoglobin is separated into two bands. In Figure 3b, the electric field gradient has been reduced, and a few minutes later, the bands have moved further apart. In Figure 3c, the pH of the recirculating buffer/coolant has been lowered from 8.8 to 8.4 over a period of 30 min and the distance between the bands has increased further.

Figure 4 is a protein cocktail containing bovine serum albumin labeled with bromophenol blue (bBSA), PE, and ferritin and has been included to illustrate that other groups of proteins whose mobilities are similar can be baseline-separated with relative ease.

THEORY

Most, if not all, members of the family of electrophoretic focusing techniques can be described by the simple flux equation,

$$N_{p,x} = -D_p \frac{dc_p}{dx} + \left(\langle u_{p,x}\rangle + z_p \omega_p \frac{I_x}{\sigma}\right) c_p = 0 \qquad (1)$$

where $N_{p,x}$, is the molar flux of protein along the x-axis of the electric field. For focused protein bands, the flux is set equal to zero to indicate that the bands are stationary. Equation 1 is composed of a dispersive term, a convective term, and an electrophoretic term where c_p is the protein concentration, D_p is a diffusion or dispersion coefficient, $\langle u_{p,x}\rangle$ is the apparent hydrodynamic velocity along the x-axis, z_p is the protein charge, ω_p is the protein mobility, I_x, is the current density, and σ is the electrical conductivity. For proteins to focus, it is necessary that at least one of the terms in parentheses varies so that their sum forms a gradient which vanishes at a discrete point in the chamber and which pushes the protein toward that point regardless of its initial location. Focusing occurs at the point in the chamber where the sum of the terms in parentheses vanishes.

Setting the sum of the terms in parentheses in eq 1 equal to zero, it is seen that focusing may be accomplished in at least five different ways: (a) in a pH gradient with $u_p = 0$, proteins focus at

Table 1. Run Conditions for Proteins in Figures 2−4[a]

figure	proteins (Sigma)	catalog no.	pH	∇E (V/cm^2)	flow (μL/h)	applied voltage (V)	protein mass loaded (μg)	load protein conc (mg/mL)
2a	(*R*)-phycoerythrin (PE)	P 0159	7.0	13.0	44	300	2.5	0.25
2b	(*R*)-phycocyanin (PC)	P 1536	7.0	13.0	42	300	5.0	0.50
2c	carbonic anhydrase (CA)	C 6653	7.0	9.3	40	300	5.0	0.50
2d	carbonic anhydrase	C 6653	8.0	13.0	39	300	4.4	0.44
	(*R*)-phycoerythrin	P 0159					2.8	0.28
	(*R*)-phycocyanin	P 1536					2.5	0.25
	myoglobin (MYO)	M 9267					5.0	0.50
3a	myoglobin	M 9267	8.8	6.9	100	400	10.0	1.0
3b			8.8	5.9				2.0
3c			8.4	5.9				3.0
	bovine serum albumin (bBSA)		8.7	3.7	138	300	2.0	0.20
	(*R*)-phycoerythrin	P 0159					2.0	0.20
	ferritin (F)	F 4503					3.0	0.30

[a] Conditions: 10 mM tris−phosphate buffer on 4.5-μm NovaPak-Diol packing.

the point where the net charge on the protein vanishes, i.e., $z_p = 0$, as is the case with isoelectric focusing (IEF); (b) in a gradient in $u_{p,x}$ with z_p, I, and σ held constant, which corresponds to O'Farrell's[1] counteracting chromatographic electrophoresis; (c) in a gradient in ω_p with $u_{p,x}$, z_p, I, and σ constant, e.g., focusing a protein in a urea gradient. With u_p held constant, proteins can be focused by (d) forming gradients in I, as was done by Koegler and Ivory,[5] or (e) forming gradients in σ, as was done by Greenlee and Ivory.[6] Both of these approaches generate gradients in the electric field similar in many respects to the gradients generated by the instrument described in this paper.

Setting $I_x = I_{0,x} + xI_{1,x}$ to form a linear gradient in the current, the focal point is found at

$$x_f = -\left(\frac{\langle u_{p,x}\rangle}{z_p\omega_p I_{1,x}} + \frac{I_{0,x}}{I_{1,x}}\right) \qquad (2)$$

and, integrating eq 1, the concentration is given by

$$c_p = \frac{M_T}{W}\sqrt{\frac{z_p\omega_p I_{1,x}}{2\pi\sigma D_p}}\exp\left[-\frac{z_p\omega_p I_{1,x}}{2\sigma D_p}(x - x_f)^2\right] \qquad (3)$$

which yields a Gaussian distribution in the focused band. The standard deviation, χ, of the peak around the focal point is then

$$\chi = \sqrt{\sigma D_p / z_p\omega_p I_{1,x}} \qquad (4)$$

where M_T is the total mass in the focusing chamber and W is the perimeter of the chamber. Note that focused bands are made thinner by low conductivities and steep current gradients. Conversely, resolution, R

$$R = \frac{1}{2}\sqrt{\frac{\langle u_{p,x}\rangle^2\sigma}{D_p I_{1,x}}}\left|\frac{1}{\sqrt{z_{p,1}\omega_{p,1}}} - \frac{1}{\sqrt{z_{p,2}\omega_{p,2}}}\right| \qquad (5)$$

is improved by reducing the gradient, raising the conductivity, and increasing the velocity of the running buffer. The simple linear model presented above does a good job of predicting protein location and baseline width when bands are completely resolved. However, since it ignores nonlinear coupling between the electric field and the ions in solution, it cannot accurately describe overlapping or contiguous bands. A more detailed version of this model that can handle these situations is given by Koegler and Ivory.[7]

Table 2. Simulation Electrophoretic Mobilities

fast peak	-1.65×10^{-5} cm^2/V•s
	-1.60×10^{-5} cm^2/V•s
	-1.30×10^{-5} cm^2/V•s
	-1.10×10^{-5} cm^2/V•s
slow peak	-1.00×10^{-5} cm^2/V•s

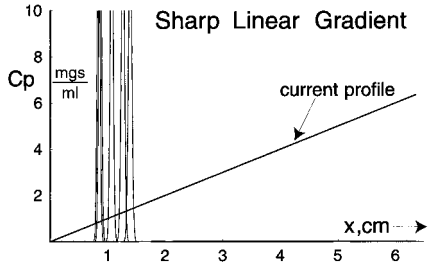

Figure 5. Five simulated proteins focused in a sharp linear current gradient which goes from zero current at the inlet, $x = 0$, to ~6.5 mA at the column outlet, $x = 6.35$ cm. Note that the two fastest peaks overlap near $x = 0.8$ cm.

Simulation. The linear model can be used to explore the advantages of electronically controlled focusing, specifically, by adjusting the field parameters to enhance resolution during a run. Consider the case, for instance, in which five recombinant protein isoforms with the electrophoretic mobilities given in Table 2 are focused near the top of the DFGF chamber (Figure 5). These proteins might first be moved as a unit to the center of the chamber, e.g., by increasing the flow rate, and then spread over the entire length of the column by expanding the electric field so that the fastest peak is near the chamber inlet and the slowest peak is near the outlet (Figure 6). By flattening and reducing the electric field gradient, the three low-mobility peaks could be eluted from the chamber while the two fastest peaks are retained. After switching to step changes in the electric field the remaining two

(7) Koegler, W.; Ivory, C. *Biotechnol. Prog.* **1996**, *12*, 822−836.

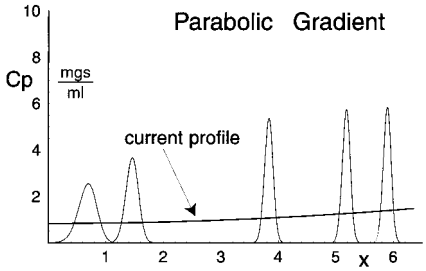

Figure 6. Complete separation of the fast proteins while keeping the slower peaks apart by flattening the front of the gradient and steepening the rear. .

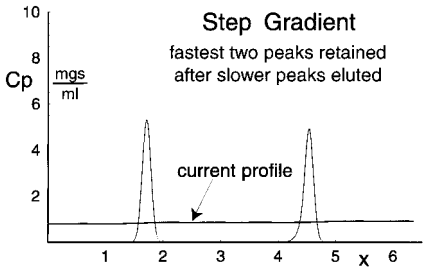

Figure 7. Step gradients to sharpen peaks and set their positions precisely. In this example, the two small step changes in the electric field located at $x = 1.5$ and 4.5 cm allow the fast proteins to remain separated and tightly focused.

peaks, whose mobilities differ by ~3%, can be completely separated and individually eluted from the chamber (Figure 7).

CONCLUSIONS

These results demonstrate that it is possible to establish and manipulate an electric field gradient by using a computer-controlled electrode array. In combination with a continuous flow of buffer, this gradient can be used to simultaneously separate and focus proteins as well as other charged molecules at concentrations in excess of 50 mg/mL in a packed-column format.

It should be noted that DFGF cannot replace IEF as an analytical technique: it cannot work at the isoelectric point (p*I*) because the proteins' mobilities vanish at that point. However, it does effectively extend the pH range over which focusing can take place to include native buffers as well as non-native, denaturing, and reducing conditions. A resultant advantage is that focusing can be accomplished away from a protein's p*I*, thus avoiding the precipitates that often form near the isoelectric point and making it preferable to IEF as a preparative technique.

Though all of the work presented here was done using linear electric field gradients, the software can be revised to allow point-by-point adjustment of the field including reversing the field to aid in elution of fractionated bands, isolating and mobilizing a single protein band, or stepping the gradient to improve processing capacity. In addition, since the electronic controller and the DFGF technique is largely independent of chamber capacity, there is no reason it cannot be applied to other types of electrophoresis equipment operating at larger or smaller scales.

Finally, this work was done with colored and labeled proteins to avoid the cost of installing on-line detectors during early trials with the column. However, there is no reason optical or other detectors could not be mounted on the chamber to provide real-time monitoring of the separation. This would open up the possibility of having the computer detect the various peaks, optimize the separation by locally adjusting the field gradient to tease refractory proteins apart, and then pull off those peaks that were selected by the operator either before or during a separation. This is planned for future work.

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