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Free-solution reactor for post-column fluorescence detection in capillary zone electrophoresis

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ABSTRACT

A post-column reactor for fluorescence detection in capillary zone electrophoresis is described. The reactor consists of a reservoir of *o*-phthaldialdehyde reagent which acts as the cathodic reservoir in which the electrophoresis capillary terminates. Zones from the capillary mix and react with the *o*-phthaldialdehyde reagent to produce a fluorophore which is detected just beyond the capillary tip. Qualitative and quantitative studies show convective forces as a major source of mixing within the reactor.

INTRODUCTION

Capillary electrophoresis (CE) is a class of separation techniques which uses an electric field, established between the ends of a capillary, to separate mixtures of compounds into discrete zones. The most common form of CE is capillary zone electrophoresis (CZE) in which the capillary is filled with buffered solution and the separation is based on the difference in mobility between the sample components. Detection of the zones can be accomplished by passing light through the capillary and measuring the increased absorbance due to the zone. However, because the internal diameter of the capillary rarely exceeds 100 μm, path length-dependent detection schemes such as UV absorbance have limited sensitivity. One alternative to UV absorbance is fluorescence detection. On-column fluorescence detection has been accomplished by using an arc lamp [1] or a laser [2,3] as the excitation source and focusing the light through the capillary wall to detect fluorescent sample components migrating within the capillary. The emitted fluorescence can arise from (1) the intrinsic fluorescence of the sample, or (2) the fluorescence of labels attached to sample components prior to separation, or (3) the fluorescence of labels attached to separated components after the separation. For the detection of proteins separated by CZE, intrinsic fluorescence is insensitive especially for proteins with few or no tryptophan residues [1]. Pre-separation labelling has produced impressive detection limits in the attomole range for amino acids [4], but this approach for the detection of proteins produces a number of labelled protein species for a given protein, each with a different number of tags and thus each with a different electrophoretic mobility. Furthermore,

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pre-separation labelling requires time for the labelling reaction and must contend with impurities and product stability. Post-separation, or post-column, fluorescent labelling mixes a non-fluorescent (fluorogenic) reagent with zones after the separation to produce a fluorescent product. Since the high efficiency of the CZE separation results in zone volumes on the order of tens of nanoliters, sufficient mixing of the zone with fluorogenic reagent while preserving the integrity of the zone is the most critical aspect of post-separation detection for CZE.

Several approaches to post-column fluorescent detection have been explored. A postcolumn coaxial reactor has been made by inserting the electrophoresis capillary into a larger reaction capillary and flowing *o*-phthaldialdehyde (OPA) reagent through the reaction capillary [5]. Reagent was mixed with effluent from the tip of the electrophoresis capillary and the fluorescent product detected a short distance downstream. An on-line connector for introducing OPA reagent directly into the capillary has been made by "drilling" reagent holes in the capillary wall with a CO_2 laser [6]. A more conventional approach has used two syringe pumps to mix basic buffer and fluorescamine reagent with the column effluent but required a third pump to offset the backflow in the capillary [7]. These post-column fluorescence detection schemes previously reported have certain limitations. For example, both the coaxial reactor and the on-line connector require careful assembly for good performance and all configurations described require plumbing of the reagent into the reactor.

This paper reports a unique type of reactor that simplifies post-column detection by terminating the electrophoresis capillary into a static solution of OPA reagent which acts as both the cathodic reservoir and a free-solution reactor. Characteristics of the reactor mixing dynamics will be presented.

EXPERIMENTAL

Free-solution reactor

The free-solution reactor, shown in Fig. 1, consists of a PTFA reactor body (RB) containing a reagent reservoir (RR) with a volume of approximately 10 ml. The electrophoresis capillary (EC) is mounted with epoxy in a 1.6 mm glass support

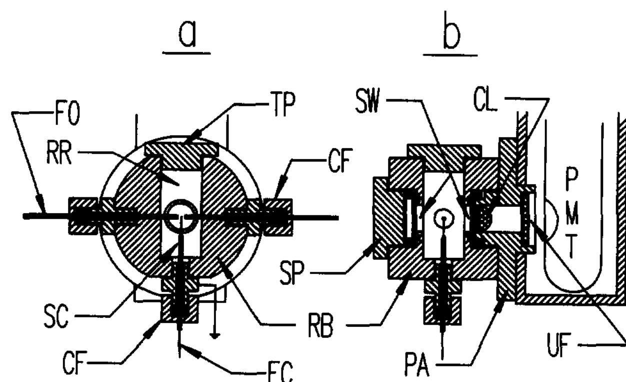

Fig. 1. Cross-sectional schematic of free-solution reactor. (a) Top view; (b) side view. For abbreviations, see text.

capillary (SC) and is held in the reactor body with a compression fitting (CF). Two 50 cm long, 600 μm O.D. optical fibres (FO, Highlight Fiberoptics custom bundle, numerical aperture = 0.22) enter from opposite sides of the reactor, at right angles to the electrophoresis capillary, and form an illuminated region above the capillary tip. The terminal 5 cm of the fibers are sheathed in 1/16-in. stainless-steel tubing to facilitate alignment and mounting in the compression fittings (CF) of the reactor body. A window in each side of the reactor body is formed by forcing a silica window (SW) against an O-ring by a threated ring (not labeled). The window at the side port (SP) allows visual inspection and positioning of the electrophoresis capillary and the fiber optics. The other window mates with the photomultiplier tube (PMT) adapter (PA), which attaches the reactor body to the PMT housing (Model 3150; Pacific Instruments, Concord, CA, U.S.A.).

Fluorescence excitation radiation from a high-pressure Hg arc lamp (102D; Ushio, Torrance, CA, U.S.A.) mounted in a lamp housing (Model 66002; Oriel, Stratford, CT, U.S.A.) was collected, collimated, filtered through an water IR filter (Oriel 6194) and a broad band UV black glass filter (Hoya UV-330), and focused (77800, Oriel) onto the two-fiber bundle (FO). The fiber optics carried excitation radiation into the reactor and illuminated effluent rising from the tip of the electrophoresis capillary. Fluorescence emission from within the illuminated volume of the reactor passed through the collection lens (CL, 16 mm focal length, Oriel) UV cut-off filter (UF, 420 nm, Oriel 52075), both housed in the PMT adapter, and onto a PMT (R636; Hamamatsu, San Jose, CA, U.S.A.) biased at −600 V (Model 206, Pacific Instruments).

Materials and methods

Mercaptoethanol, OPA, glycine, boric acid (Sigma, St. Louis, MO, U.S.A.), fluorescein (Aldrich, Milwaukee, WI, U.S.A.) and methanol were reagent grade. The OPA reagent was made by combining, in order, 13.4 mg OPA, 21.1 μl mercaptoethanol, 200 μl methanol, 10 ml 0.1 *M* borate buffer (pH 9.0). The borate buffer also served as the electrophoresis buffer. Test samples were either glycine or fluorescein in the concentration range of $1 \cdot 10^{-3}$–$1 \cdot 10^{-4}$ *M*. Electrophoresis capillaries were fused silica (Polymicro Technologies, Phoenix, AZ, U.S.A.), 50 cm × 50 μm I.D. The outer diameter of the capillary was nominally 360 μm. The outer diameter of the electrophoresis capillary tip within the free-solution reactor was approximately 330 μm since the polyimide coating was removed from this section. In some experiments, the outer diameter was reduced by etching a one centimeter section of the capillary tip in 48% hydrofluoric acid (water was forced through the capillary to prevent etching of the capillary bore).

OPA reagent was poured into the reagent reservoir of the free solution reactor through the top port (TP) and the lower compression fitting of the reactor was attached to electrical ground so that the reagent reservoir acted as the cathodic (−) reservoir of the electrophoresis system. A vacuum applied to the injection (anodic) end of the capillary filled the capillary with reagent from the reactor. The injection end then was placed in the anodic reservoir and the high voltage was turned on so that electroosmotic flow flushed the capillary with buffer from the anodic reservoir. Test sample was introduced into the capillary by electromigration (continuous introduction for qualitative studies, 2–4 s at 5 kV for quantitative studies). The sample zone emerged

from the tip of the electrophoresis capillary into the reagent reservoir, mixed with OPA reagent by diffusion and convection (no flow of reagent) and was excited shortly afterwards in the illumination volume created by the opposing optical fibers. Fluorescence emission was collected from the illumination volume, through the silica window (SW), and detected by the PMT, as described above. The signal from the PMT was digitized at 10 Hz (21-bit analog-to-digital converter and stored on computer disk. Peak characteristics such as area migration time, and efficiency were calculated using statistical moments between user-defined integration points.

RESULTS AND DISCUSSION

Qualitative studies

The mixing dynamics of the free-solution reactor were assessed by examination of sample emerging from the electrophoresis capillary tip into the static OPA solution, reacting with the OPA, and the fluorescent product illuminated by the optical fibers, as shown in Fig. 2. Two characteristic features of sample effluent were noted. The tapered, "flame"shaped effluent stream consisted of an outer region of high fluorescence and an inner region of lower fluorescence. Secondly, the width of the effluent stream at the capillary tip (*ca.* 330 μm) was many times the width of the

Fig. 2. Glycine emerging from the electrophoresis capillary into the reagent reservoir of the free-solution reactor, fiber optics illuminating fluorophore from side ($1 \cdot 10^{-3}$ *M* glycine continuous injection 30 kV).

effluent stream emanating from a 50 μm inner diameter capillary. One possible reason for the broad base of the effluent stream is due to distortion of the stream by diverging electric field lines. This, however, was ruled out when a pointed cathodic electrode, brought within 100 μm of the electrophoresis tip failed to change the general shape of the plume. Therefore, it is assumed that the effluent stream shape arises from density differences between the effluent and the surrounding reagent solution. Specifically, the effluent, warmed within the capillary by Joule heating, rises as it emerges from the capillary. The convective movement of solution from the tip of the capillary creates a slight pressure drop at the point the effluent emerges from the capillary tip and draws the sample radially outward across the face of the capillary tip to the outer edge. Although the broad base of the flame-shaped effluent stream can be accounted for by convection, the inhomogeneous regions of fluorescence within the flame may be due several factors. Firstly, convection may enhance mixing of the effluent with reagent at the edge of the capillary tip. Secondly, diffusion and reaction of reagent molecules with sample effluent molecules does not occur immediately upon sample exiting the capillary, especially at the broadened effluent stream base. To ensure the fluorescence inhomogeneities of the sample effluent stream were related to diffusion- and reactionlimited kinetics, and not anomalies in sample illumination or restricted flow paths, a sample of fluorescein (intrinsically fluorescent) was examined emanating from

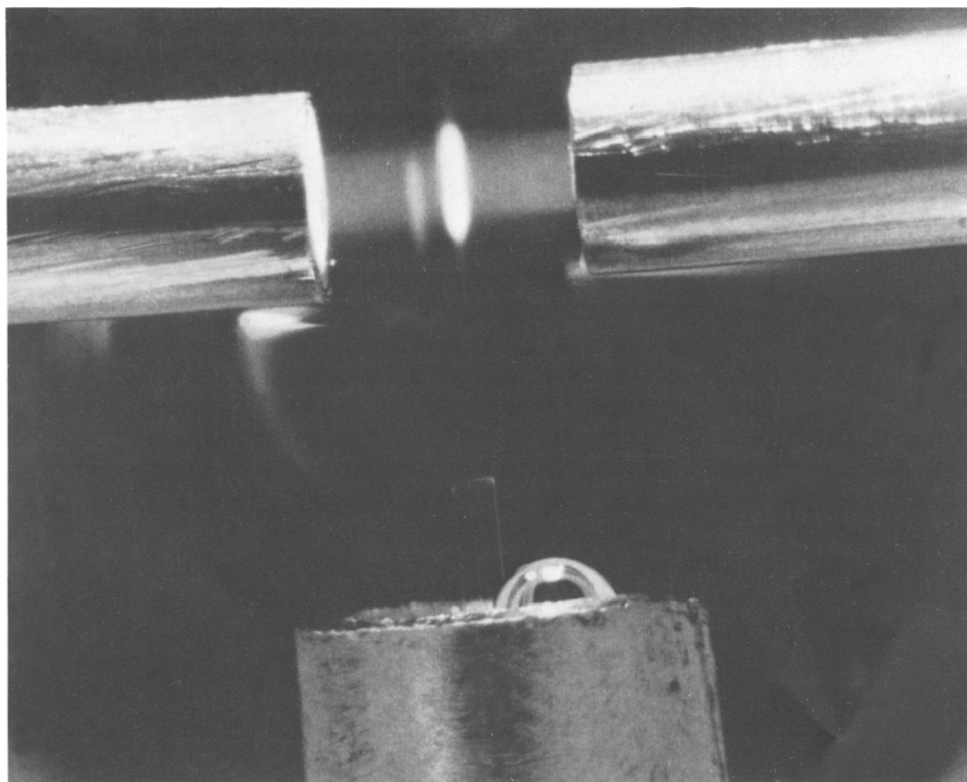

Fig. 3. Fluorescence of glycine sample stream migrating from an electrophoresis capillary approximately 1.9 mm below bottom of illumination region (same conditions as Fig. 2).

the capillary tip. This resulted in a flame-shaped plume which was homogeneous throughout (photograph not shown). Examination of the effluent stream beyond the electrophoresis tip (accomplished by moving the capillary veritically in the reactor) showed that the mixing of sample with OPA reagent was more complete; the effluent stream was homogeneous, as shown in Fig. 3. The fact that the effluent stays confined to a relatively narrow stream shows the impressive power of the convective forces within the reactor.

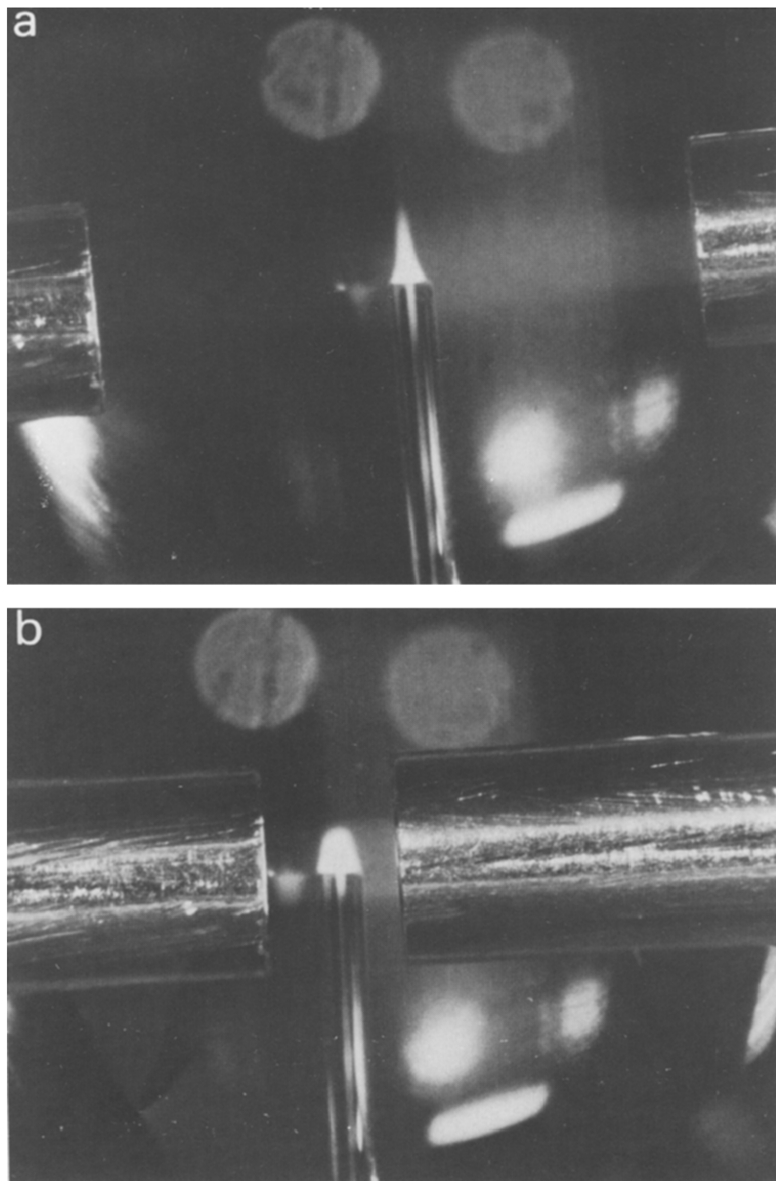

Fig. 4.

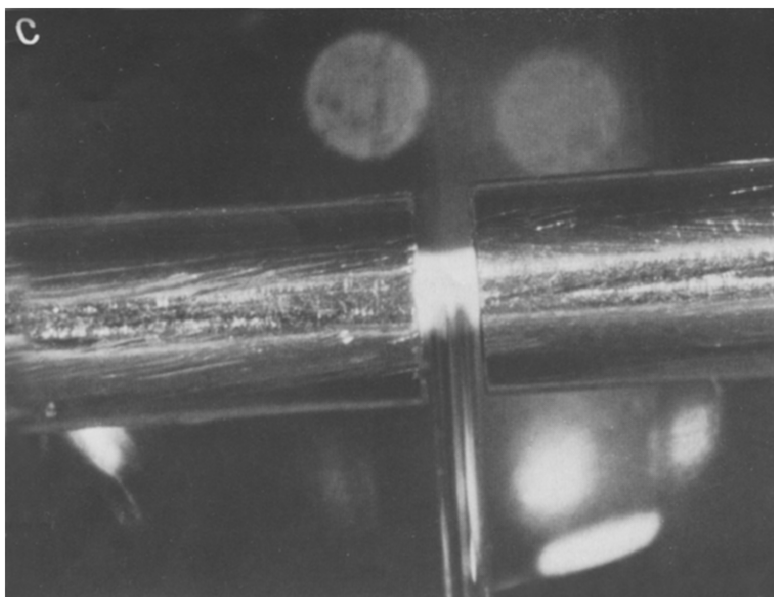

Fig. 4. Shape of a fluorescein sample stream as a function fiber optic separation distance: (a) 4.5 mm, (b) 1.0 mm, (c) 0.5 mm (30 kV continuous injection of $1 \cdot 10^{-4}$ *M* fluorescein).

A fortuitous advantage of the convective stream of the effluent is to rapidly remove reacted sample from the illumination volume. However, the convective forces limit how close the fiber optics can be positioned relative to the electrophoresis capillary. As the distance between fiber optics decreases, distortion of the capillary effluent occurs, as shown in Fig. 4. Measuring the area and width of a detected zone as a function of the gap between the optical fibers, the minimal gap between the optical fibers which did not significantly perturb the zone was between 1 and 2 mm (data not shown). The illumination region formed between the fibers at this distance is much wider than the capillary effluent stream. Consequently, the portion of illumination volume not contributing to sample excitation produces a significant signal due to background fluorescence of the reagent solution and scattering of light within the reactor (because of the large volume of the reservoir, the reagent background primarily arises from impurities in the OPA reagent and not residual sample). To reduce the background signal while not perturbing the capillary effluent, the optical fibers were fitted with moveable apertures, as shown in Fig. 5, capable of forming a vertical slit (note: only background radiation from either side of the effluent stream is masked, not that which comes from the front and back of effluent stream). The effect of the spatial filtering, shown in Fig. 6, is to reduce the background noise and drift.

Quantitative studies

Since the exiting effluent stream expands to the outer diameter of the capillary, significant mixing and zone broadening occurs. In an effort to reduce this effect, the outer diameter the capillary tip was reduced by etching in hydrofluoric acid. Table I shows that zone broadening does decrease with a smaller outer diameter capillary,

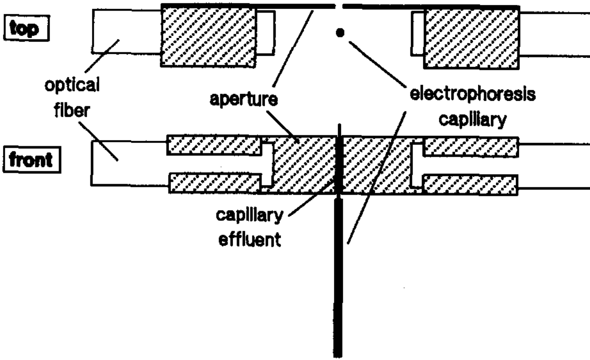

Fig. 5. Top and front schematic of optical fibers modified with apertures (aperture located between electrophoresis capillary and collection lens).

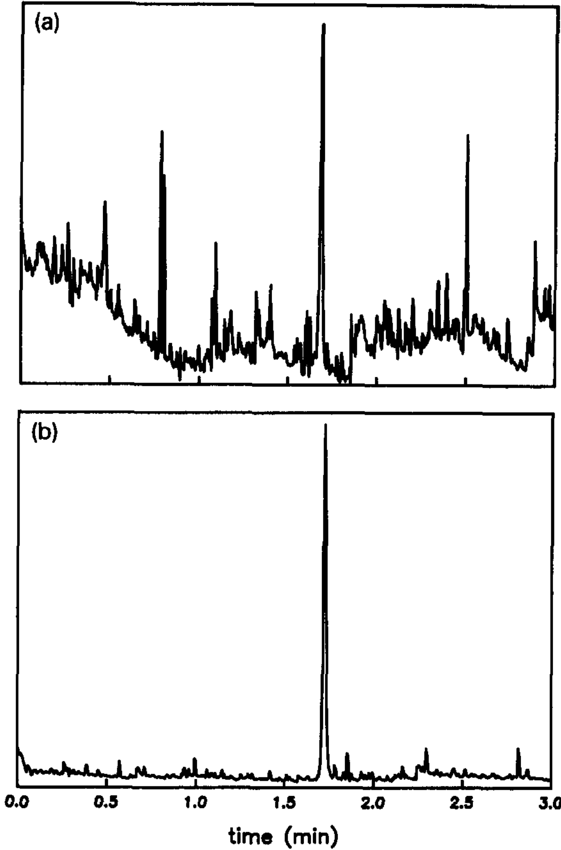

Fig. 6. Effect of apertures on background signal. (a) No aperture, (b) aperture with an approximately 200-μm wide slit. Conditions: sample, $1 \cdot 10^{-3}$ *M* glycine; injection, 2 s at 5 kV; capillary, 110 μm O.D. at tip; optical fiber gap, approximately 1.2 mm.

TABLE I

EFFECT OF ELECTROPHORESIS CAPILLARY OUTER DIAMETER ON PEAK AREA AND EFFICIENCY

Conditions: electrophoresis capillaries, 50 cm × 50 μm I.D. (outer diameters below 328 μm made by etching outside of capillary with hydrofluoric acid); capillary tip position, level with bottom edge of illumination volume; sample $1 \cdot 10^{-3}$ M glycine in 0.1 M borate buffer; reagent reservoir, OPA reagent; sample introduction, 2 s at 2.5 kV; run voltage, 30 kV (44 μA); data represent average of three runs (except 328 μm O.D. data, n = 1).

Capillary tip outer diameter (μm)	Peak area	N
328	2 943 000	21 000
110	201 000	42 000
66	132 000	110 000

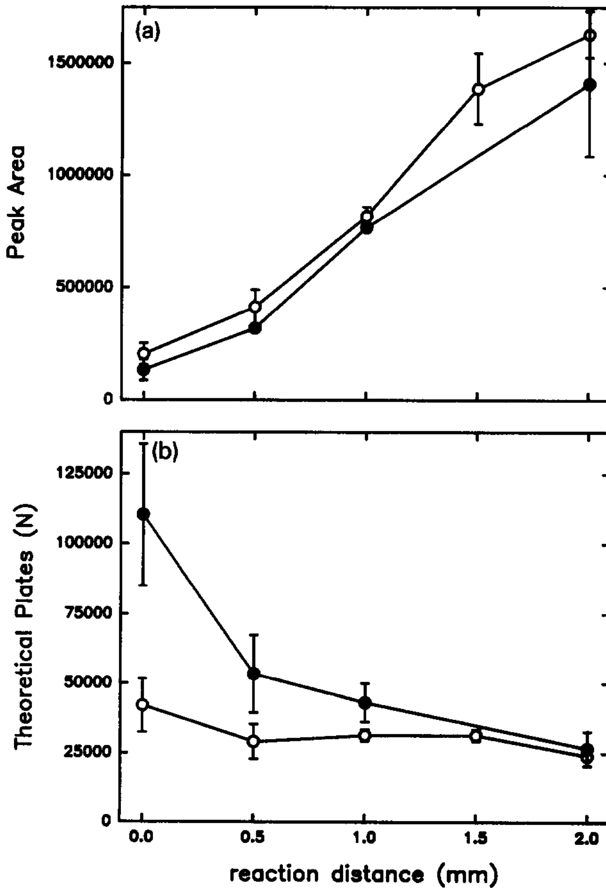

Fig. 7. The effect of reaction distance on (a) peak area and (b) peak efficiency (theoretical plates, N) for different outer diameters of the electrophoresis capillary: ○ = 110 μm O.D.; ● = 66 μm O.D.; sample, $1 \cdot 10^{-3}$ M glycine (triplicate runs for each data point); reaction distance was increased by translating the capillary vertically relative to the fiber optics.

but less mixing also decreases the fluorescent signal (peak area). Qualitative examination of the effluent stream emanating from the reduced outer diameter capillaries showed signal increasing as the effluent rose above the capillary tip. Thus, a study was made of the effect of reaction distance, distance from capillary tip to illumination region, on peak area and broadening. Fig. 7 shows that as reaction distance increases, the greater mixing and/or reaction time causes an increase in the signal. However, as with the larger outer diameter capillary tip, more mixing produces more zone broadening. Note how the peak areas for these capillaries at a 2 mm reaction distance are beginning to approach the peak area for the normal outer diameter capillary (see Table I).

Another approach to controlling the convection-induced distortion of the capillary effluent is to flow solution coaxial to, and in the direction of, the effluent. To study this effect independent of the sample-reagent mixing and reaction kinetics, fluorescein was used as the test sample and borate buffer as the "reagent" solution and the coaxial flow solution. The qualitative effect of the coaxial flow is to narrow the sample effluent stream, as shown in Fig. 8. The results, shown in Table II, indicate a reduction in the zone broadening with increasing flow rate due to confinement of the capillary effluent into a smaller region, similar to a sheath flow cuvette. The decrease in peak area can be accounted for by dilution, as opposed to the effect of decreased residence time in the detector window, since peak height and area decrease with increasing flow-rate.

A free-solution reactor for post-column fluorescence detection for CZE has been shown to simplify post-column detection process by reducing the assembly, positioning, and plumbing requirements of previous approaches. However, this system of

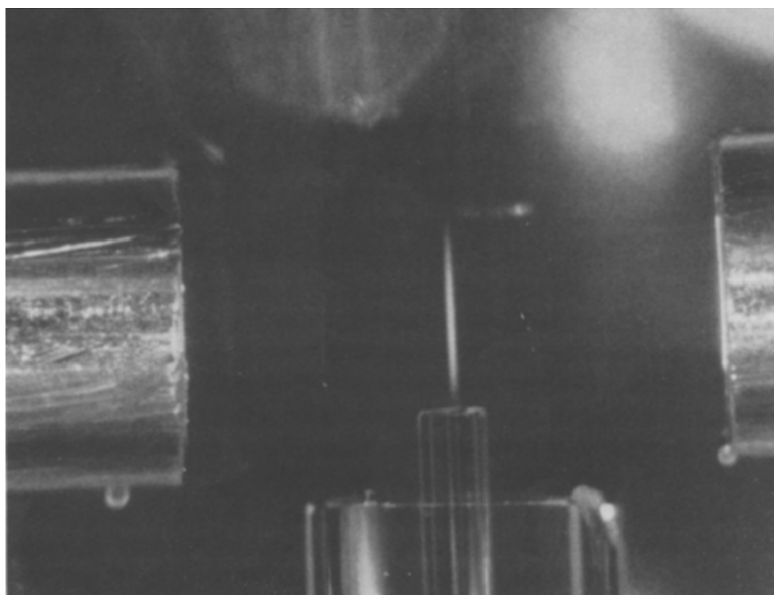

Fig. 8. Shape of a fluorescein sample stream with 1.35 ml/min flow of borate buffer coaxial with electrophoresis capillary (30 kV continuous injection of $1 \cdot 10^{-4}$ M fluorescein).

TABLE II

EFFECT OF FLOW-RATE IN FREE-SOLUTION REACTOR

Conditions: electrophoresis capillary, 50 cm × 50 μm I.D. (320 μm O.D. at tip); sample, $1 \cdot 10^{-4}$ *M* fluorescein; reagent reservoir, borate buffer; flowing solution, borate buffer; sample introduction, 4 s at 2.5 kV; run voltage, 30 kV (44 μA); data represents average of two runs at each flow-rate.

Coaxial flow-rate (ml/min)	Peak area	Peak height	*N*
0.0	2 986 000	0.161	57 500
0.01	1 140 000	0.163	69 500
0.1	692 000	0.100	112 500
1.0	146 000	0.031	190 000

detection results in zone broadening due to convective forces and high background fluorescence due to the large illumination volume. The convective forces seen in this reactor suggest that similar forces may be operative in other post-column reactors, such as the laser-drilled "tee" [6] and the coaxial capillaries [5], where warm sample effluent mixes with cooler reagent. The convection-induced streaming of capillary effluent also suggests a way to confine the sample components once they have left the capillary in order to do additional chemical and physical manipulations (*e.g.* base hydrolysis, fraction collection).

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