

## Off-line and on-line assay of membrane protein with *o*-phthaldialdehyde by flow-injection with post-column reaction

### ABSTRACT

A rapid and convenient flow-injection absorption spectrophotometric procedure was developed for the determination of protein and peptide concentrations in discrete samples that contain biological membranes (subcellular particles) which scatter light. With some modification, the same system serves as a post-column reactor to determine protein and peptide concentrations continuously in the effluent of a separation device. The procedure is based on the well known determination of primary amines by reaction with a thiol and *o*-phthaldialdehyde. Although designed for preparations of biological membranes, the procedure is applicable to any solution in which the primary amino groups are predominantly those belonging to the proteins and/or peptides present; this includes many protein solutions which are turbid at acidic or neutral pH but clear at alkaline pH. The off-line flow-injection procedure for discrete samples and the on-line post-column reactor for flowing column effluents have been found especially useful for determining the distribution of membrane protein in the channel effluent after fractionation of subcellular particle preparations containing corn root mitochondria and microsomes.

Researchers dealing with subcellular particle preparations and other turbid protein-containing solutions are frequently limited in their choice of protein assay methods. This results from the necessity to solubilize the proteins in the sample [1,2], the presence, in many subcellular particle preparations, of reagents that interfere with one or another method of assay and, frequently, very low protein concentrations.

Such constraints were encountered in previous work on the fractionation of the subcellular particles of corn roots [3,4]. The fractionated material could not be assayed for protein by any Cu(I)-based method [5-7] because of the presence of the reductant 2-mercaptoethanol (ME), a reagent that is frequently added to subcellular particle (membrane) preparations to prevent oxidation. Coomassie Blue methods [8,9] were of limited utility

for two reasons. First, to ensure adequate exposure of protein in the corn root membranes to the reagent, it was desirable to add sodium dodecyl sulfate (SDS) to a final concentration in the assay mixture of 0.3-1%; however, these levels of SDS interfere with the Coomassie Blue assay [8]. Second, for convenience and speed, it was decided to utilize flow-injection methods (FIA) [10], but attempts to devise an FIA procedure with Coomassie Blue were unsuccessful because of the deposition of a blue film, presumably a protein-dye aggregate [8], on the detector windows. This problem also precludes on-line determination with Coomassie Blue. It was therefore necessary to consider other options.

Amino acids and peptides are frequently determined in chromatographic effluents by measuring the intensity of the fluorescence produced on

post-column reaction of their primary amino groups with *o*-phthaldialdehyde (OPA) and a thiol [11–13]. Church and co-workers [14,15], working with proteins and their digests, emphasized the advantage of measuring the absorbance of the amine–OPA–thiol adduct [16] rather than its fluorescence. In the presence of SDS, the molar absorptivities of all  $\alpha$ - and  $\epsilon$ -amino groups are similar [14], whereas the fluorescence yields vary widely. Further, Markwell et al. [1] have shown that, in the presence of SDS, membranes do not require pre-solubilization before their protein contents are determined. These considerations suggested that it would be worth examining the feasibility of developing an FIA system for membrane protein content utilizing the OPA reaction. Such a system is reported here, together with its adapta-

tion to make a reactor for on-line determination of the membrane protein content in flowing column effluents.

#### EXPERIMENTAL

Sodium dodecyl sulfate was either of technical grade from Matheson Coleman and Bell (Norwood, OH) or of laboratory grade from Fisher Scientific (Fair Lawn, NJ). Mercaptoethanol was of electrophoresis grade from Bio-Rad Laboratories (Richmond, CA). *o*-Phthaldialdehyde, dithiothreitol (DTT), *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), the sodium salt of HEPES and a stock solution of bovine serum albumin (BSA) containing 10 g of protein per 100

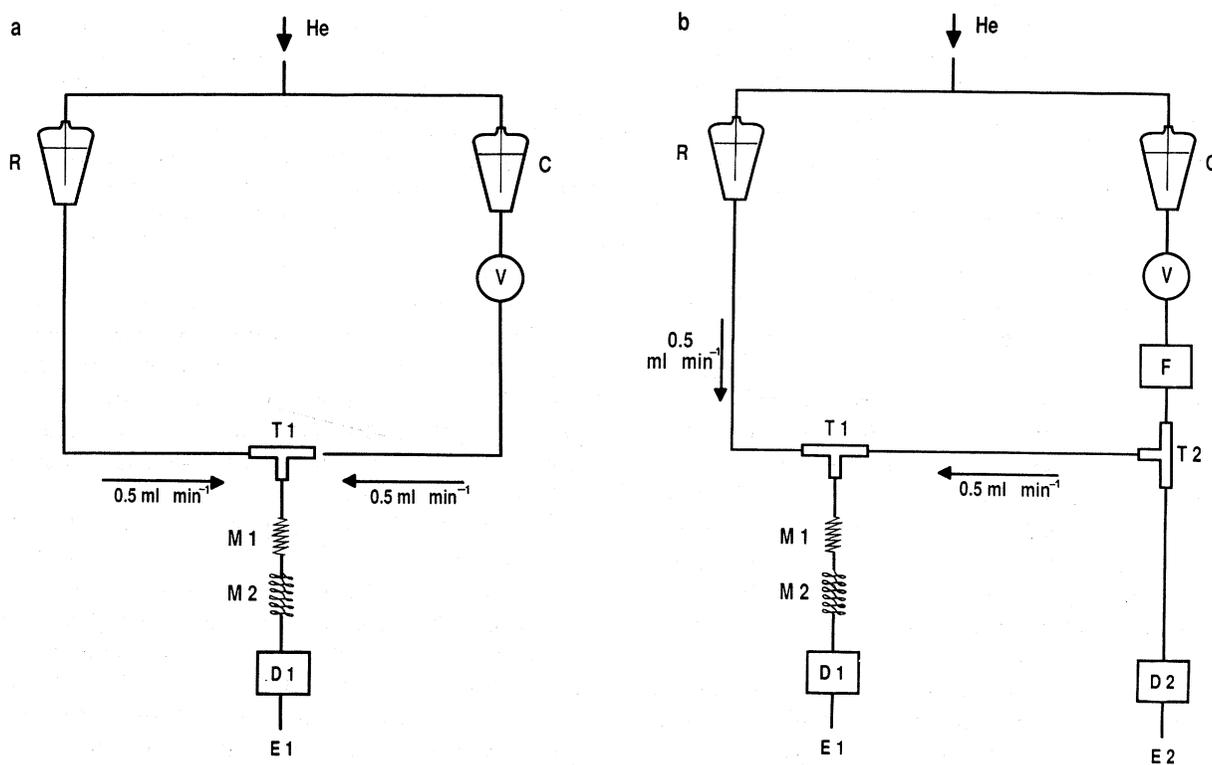


Fig. 1. (a) Schematic diagram of the FIA system for analysis of discrete samples (e.g., fractions) with the OPA reagent. The pressure applied to the reagent and carrier solutions by helium was 11 cmHg ( $1.5 \times 10^5$  dyn cm<sup>-2</sup>). Vessels R and C were raised to levels (at the tips of the gas inlet tubes) 55 and 45 cm, respectively, above the level of outlet E1. For specifications of tubing, see Table 1. (b) Schematic diagram of the post-column reactor system for on-line analysis of the effluent from a fractionator (F) with the OPA reagent. The gas pressure and the level of vessel R are as for (a). Vessel C was raised by 16 cm to a level 61 cm above E1. E2 is at the same level as E1. For specifications of tubing, see Table 2. R, reagent; C, carrier; V, injection valve; F, fractionator; T1, mixing tee; T2, stream splitter; M1, first mixing coil; M2, second mixing coil; D1, first detector; D2, second detector; E1 and E2, exits to waste.

ml were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical-reagent grade. Mitochondria were prepared from corn roots as previously described [3,4].

The sample carrier contained 300 mM sucrose, 1.0 mM ME, 1.0 mM DTT and 3.0 mM HEPES buffer (Na), pH  $7.38 \pm 0.15$  ( $25^\circ\text{C}$ ). The OPA reagent contained 2% (w/v) SDS, 11.9 mM OPA, 57.0 mM ME, 4.0% (v/v) methanol (MeOH) and 100 mM tetraborate buffer (Na). The pH was adjusted to  $10.75 \pm 0.10$  ( $25^\circ\text{C}$ ) with NaOH, except where stated otherwise.

#### Off-line FIA

The design of the FIA system is shown schematically in Fig. 1(a). Reagent and carrier solutions (R and C) were pumped without pulsation by a combination of gas pressure (helium) and hydrostatic pressure. A standard Hoke-Phoenix (Englewood, NJ) pressure regulator was used to reduce the helium pressure from that in the tank to 4 psi. This was followed by a Hoke needle valve, Model R306A, which served as an on-off valve, and a Lexington Type 10 pressure regulator (Lexington Controls, Burlington, MA) to reduce the pressure further, to 11 cmHg ( $150 \text{ g cm}^{-2}$ ). The vessels (R and C) were 250-ml glass separating funnels. Although the pressure applied to them is small, as a safety precaution the separating funnels should be enclosed in plastic mesh. Fine control of the total pressure driving each of the two solutions was obtained by hydrostatic pressure, i.e., by adjusting the height of each solution with respect to the waste outlet. To provide the desired flow-rates of  $0.5 \text{ ml min}^{-1}$  for each solution, the reagent level was set at 55 cm and the sample carrier level at 45 cm. Outflow rates from vessels R and C were measured with simple ball flow meters (Cat. No. F-1100, size 1, Gilmont Instruments, Great Neck, NY). Each flow meter was calibrated for the fluid flowing through it. Flow-rate measurements were confirmed by use of a Thermal-pulse II liquid flow meter (Molytek, Pittsburgh, PA). The rate at which liquid issued from exit port E1 was determined by collecting the effluent over a measured time interval and weighing the liquid collected.

TABLE 1

Size of tubing used in the off-line FIA system

Location <sup>a</sup>	Tubing		
	Type <sup>b</sup>	AWG gauge <sup>c</sup>	Length (cm)
R-T1	SS	24	20
	+		
	T	20	87
C-V	SS	24	10
	+		
	T	20	87
V-T1	T	24	11
T1-M1	T	24	14
M1 <sup>d</sup>	T	24	9
M1-M2	T	24	15
M2 <sup>d</sup>	T	24	21
M2-D1	T	24	36
	+		
	T	20	7
D1-E1	T	20	39

<sup>a</sup> See Fig. 1(a). <sup>b</sup> SS, stainless steel; T, Teflon. <sup>c</sup> 20- and 24-gauge tubing have i.d. 0.81 and 0.51 mm, respectively. <sup>d</sup> See text for geometry.

From Poiseuille's law [17], it is apparent that the resistance to flow varies directly with the length of the conducting tubing and inversely with the fourth power of its inside diameter; it was therefore necessary to select these carefully. The outlets of the solution vessels (R and C) were connected to 0.020-in. i.d. stainless-steel tubing. All other tubing was of Teflon. Tubing dimensions are given in Table 1.

Connections between sections of stainless-steel and/or Teflon tubing were made with tightly fitting silicone-rubber tubing or with 1/4-28 plastic couplings. Connections between the glass separating funnels and the stainless-steel tubing were made as follows: a short length of Tygon tubing was connected to the outlet of the separating funnel and clamped into place with a miniature, worm-drive metal hose-clamp (Breeze Clamp, Saltsburg, PA). Four short lengths of plastic tubing of smaller and smaller size, and finally the stainless-steel tubing, were successively inserted

into the open end of the Tygon tubing. These were all clamped together with a second hose-clamp.

The sample-injection valve was a Rheodyne 5020 low-pressure Teflon rotary valve. The valve as supplied by the manufacturer includes 7 cm × AWG 20 Teflon tubing attached to each port. A sample loop was made by attaching a length of AWG 20 Teflon tubing to the loop ports (1 and 4). On calibration, the injection volume was found to be 325 μl. To ensure adequate flushing, a load volume of 1.0 ml was always used to fill the loop. The sample-reagent mixing tee (T1) was a Chem-inert Kel-F tee connector (Supelco, catalog no. 5-8749). Further mixing of sample and reagent and appropriate delay periods for reaction were provided by two sets of coils. Coil 1 (M1) was made by wrapping five turns of 24-gauge Teflon tubing helically onto a 4 mm o.d. glass tube. Coil 2 (M2) was made by wrapping Teflon tubing of the same gauge onto two adjacent parallel 4 mm o.d. glass tubes, clockwise onto one and counter-clockwise onto the other, forming a series of six figure-eights. Coil M1 alone did not provide adequate mixing, as evidenced by an erratic detector output. The detector (D1) for the protein-OPA-thiol adduct was a Waters Model 450 or a Schoeffel Model 770 variable-wavelength transmittance/absorbance detector; these detectors are virtually identical. The wavelength was set at 340 nm [15] and the detector was operated in the absorbance mode. The output was recorded with a Hewlett-Packard Model 680 strip-chart recorder set on the 100 mV range.

#### *On-line monitoring (post-column reactor)*

The design in Fig. 1(a) was adapted [Fig. 1(b)] to on-line monitoring of the effluent from a fractionator (F). In this work the separation device was a field flow fractionator, in which suspended or dissolved particles are separated on the basis of particle mass and/or density [18,19]. The same basic design would apply to any separation device yielding an effluent stream, such as a chromatographic column. Tubing dimensions are given in Table 2. A Kel-F tee (T2) (Cat. No. 200-22, Rainin Instruments, Woburn, MA) served as a stream splitter. Detector D2 was a Schoeffel 770 absorbance monitor [D2, Fig. 1(b)] set at 254, 260 or

TABLE 2

Size of tubing used in the on-line assay system<sup>a</sup>

Location <sup>b</sup>	Tubing		
	Type <sup>c</sup>	AWG gauge <sup>d</sup>	Length (cm)
C-V	SS	24	10
	+		
	T	20	27
V-F	T	20	25
F-T2	T	20	35
T2-D2	T	24	24
D2-E2	T	24	25
	+		
	SS	30	10
T1-T2	T	24	11

<sup>a</sup> For dimensions of tubing in the region R-E1, see Table 1.

<sup>b</sup> See Fig. 1(b). <sup>c</sup> SS, stainless steel; T, Teflon. <sup>d</sup> 20-, 24- and 30-gauge tubing have i.d. 0.81, 0.51 and 0.25 mm, respectively.

280 nm, as desired. The output of this detector was recorded with a second Hewlett-Packard Model 680 strip-chart recorder set on the 100 mV scale. Vessel C was raised to a level (at the tip of the helium gas inlet) 61 cm above outlet E2, the latter being at the same level as outlet E1. This provided a flow-rate out of vessel C1 of 1 ml min<sup>-1</sup>, 0.5 ml min<sup>-1</sup> flowing to T1 and the remainder to D2. The pressure and tubing required would, of course, vary with the resistance to flow of the separation device.

Outflow rates from vessels R and C and exit port E1 were measured as described above for the off-line system. The flow-rate at exit port E2 was measured in the same way as that at port E1.

## RESULTS AND DISCUSSION

To facilitate mixing, equal volumes of sample and reagent were used, i.e., the reagent and carrier solutions flowed into the mixing tee [T1, Fig. 1(a)] at equal rates. The concentration of every component in the OPA reagent solution (Experimental) was therefore approximately twice that recommended by others, e.g., Church et al. [14], who used a reagent solution volume to sample volume

TABLE 3

Statistics of the off-line assay and variation of detector response with sensitivity

Protein Type	Concentration range (mg l <sup>-1</sup> )	n <sup>a</sup>	Sensitivity <sup>b</sup> (V absorbance <sup>-1</sup> cm)	s <sub>yx</sub> <sup>c</sup> (mV)	Slope <sup>d</sup> (V g <sup>-1</sup> l)	Apparent absorptivity <sup>e</sup> (absorbance cm <sup>-1</sup> g <sup>-1</sup> l)
BSA	0– 10	12	1	0.8	1.98 ± 0.05	1.98 ± 0.05
BSA	0– 50	12	0.5	0.5	1.13 ± 0.01	2.27 ± 0.02
BSA	0– 200	6	0.25	2.1	0.51 ± 0.01	2.06 ± 0.04
BSA	0– 1000	6	0.05	2.0	0.086 ± 0.002	1.70 ± 0.04
Mit <sup>f</sup>	0– 140	8	0.25	1.2	0.49 ± 0.01	1.96 ± 0.04
BSA	All data	36	0.05 <sup>g</sup>	1.2	0.0877 ± 0.0008	1.75 ± 0.02
BSA + Mit	All data	44	0.05 <sup>g</sup>	1.1	0.0878 ± 0.0008	1.76 ± 0.02

<sup>a</sup> The number of data points in the set. <sup>b</sup> Sensitivities of 1, 0.5, 0.25 and 0.05 V absorbance<sup>-1</sup> cm are given by sensitivity dial settings of 0.01, 0.02, 0.04 and 0.2 a.u.f.s., respectively. The sensitivity dial setting (a.u.f.s.) refers to the absorbance per cm of a solution which gives a detector output of 10 mV. <sup>c</sup> Standard error of the estimate. The recorder range was 100 mV, except for the lowest range of protein concentration (0–10 mg l<sup>-1</sup> BSA), for which it was 50 mV. <sup>d</sup> Slope ± standard error of the slope. <sup>e</sup> Apparent absorptivity ( $\epsilon$ ) is defined here as the absorbance per cm (path length) produced by a protein solution of unit concentration (1 g l<sup>-1</sup>). It is given by  $\epsilon = m/S$ , where  $m$  and  $S$  are slope and sensitivity, respectively. <sup>f</sup> Mitochondrial preparation. For statistical purpose, 1 g of mitochondrial protein is defined as the amount of mitochondrial protein equivalent to 1 g BSA in the off-line assay. <sup>g</sup> For pooling, data were normalized to  $S = 0.05$ .

ratio of between 100 : 1 and 20 : 1 for their test-tube assay procedure. A flow-rate for each solution [R and C, Fig. 1(a)] of 0.5–1 ml min<sup>-1</sup> was found to provide a suitable incubation period of 20–40 s between mixing of sample with reagent and measurement of absorbance; the half-time for reaction of the reagent with proteins at 25°C is ca. 11 s [14]. If both flow-rates exceed 1 ml min<sup>-1</sup>, peak

heights are reduced because of reduction of the incubation period. The lower flow-rate (0.5 ml min<sup>-1</sup> for each solution) was preferred because of its compatibility with our on-line analysis procedure (see below). The flow-rate of the fractionator [F, Fig. 1(b)] effluent is 1 ml min<sup>-1</sup>, so that half of the effluent is used for on-line protein analysis; the remainder can be monitored directly [D2, Fig. 1(b)] and collected fractionally for other uses. Fractionator effluent flow-rates of less than 1 ml min<sup>-1</sup> yield a reduced detector response because the flow from T2 to T1 is then reduced to less than 0.5 ml min<sup>-1</sup>, which reduces the protein concentration in the mixing coils and detector.

Figure 2 shows continuous recordings of absorbance at 340 nm ( $A_{340}$ ) after injection of BSA samples of various concentrations. The maximum value,  $A_{\max}$ , i.e., the peak height, was used as a measure of concentration. The time between injection and maximum absorbance was identical for all samples. A rare spurious peak was easily identified as not being the adduct peak because it did not appear at the time characteristic of the adduct.

The fractionator effluent in our work contains 0.3 M sucrose. Sucrose (0.3 M) was therefore incorporated in all the samples assayed, including the standard BSA solutions, so that the sample

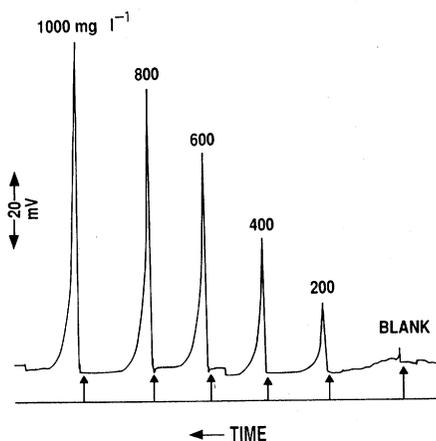


Fig. 2. Detector response (mV) on flow-injection analysis of samples of BSA, 0–1000 mg l<sup>-1</sup>. Load volume, 1 ml; injection volume, 325  $\mu$ l; detector sensitivity, 0.05 V absorbance<sup>-1</sup> cm (0.2 a.u.f.s.).

and carrier solutions would be of similar density and viscosity. Large differences in density and viscosity between sample and carrier were found to affect the adduct peaks substantially, including  $A_{\max}$ . Application of the method to samples taken from a density gradient effluent would therefore be precluded, unless all samples and the carrier were supplemented with an ingredient, e.g., sucrose, to bring them to approximately the same density and viscosity.

The dependence of  $A_{\max}$  on pH was found to be maximum in the pH range 10.2–11.3. A pH in the middle of this range, viz., 10.75, was selected as optimum. Hare [13] found a pH of 10.5 to be suitable for amino acid analysis, and Trepman and Chen [20] showed that the adduct of OPA and ME reacts with alanine at a maximum rate at pH 10.5–11.

By linear least-squares regression analysis (Table 3), peak height ( $A_{\max}$ ) was shown to be a linear function of protein concentration ( $c_p$ ) in the range 0–1000 mg l<sup>-1</sup> for BSA and 0–140 mg l<sup>-1</sup> for mitochondrial protein. The range for mitochondrial protein probably extends beyond 140 mg l<sup>-1</sup>, but more concentrated solutions were not available. To optimize the precision, the sensitivity,  $S$ , of the detector was adjusted according to the range of protein concentration in the set of samples to be measured, i.e., the highest sensitivity which would give a detector output of < 100 mV for the most concentrated sample was selected. A separate calibration graph was obtained for each of the four ranges (Table 3). The apparent absorptivity,  $\epsilon$  (Table 3, footnote e), was ca. 2 for all four sensitivity settings (last column of Table 3), but varied from one sensitivity setting to another. This may have resulted from the relatively low recorder input impedance loading the detector output, from slight differences in flow-rates between runs and from imprecision in the relative sensitivity values obtained by different dial settings.  $A_{\max}$  vs.  $c_p$  was linear on every sensitivity setting. In the regression analysis (Table 3), the relative standard error of the estimate of the detector response ( $A_{\max}$ ) on protein concentration ( $c_p$ ) for BSA (all data) was 1.2% of full-scale (100 mV) and was the same for mitochondrial protein. The average relative standard error of the slope (relative to the slope) was

2.1%. There was no degradation of precision at the highest sensitivity setting.

The fraction of amino groups “seen” by the assay system can be calculated as follows. An apparent absorptivity ( $\epsilon$ ) of 2 (see above) means that the detector sees 2 absorbance cm<sup>-1</sup> for an injected sample concentration of 1 g l<sup>-1</sup> BSA, but the sample is diluted in half by the reagent solution. Disregarding dispersion, the absorptivity related to the protein concentration in the detector is therefore 4 absorbance cm<sup>-1</sup> (g BSA)<sup>-1</sup> l. The absorptivity of the adduct referred to the amino group concentration is  $6 \times 10^3$  absorbance cm<sup>-1</sup> (mol NH<sub>2</sub>)<sup>-1</sup> l [14]. Knowing that 10<sup>5</sup> g of BSA contains 88 lysine residues [21], the detector must have “seen”, at the peak maximum, ca. 75% of the free amino groups.

The linearity of  $A_{\max}$  vs.  $c_p$  obtained with mitochondria is attributable to the presence of SDS and to the high pH (10.75), both of which rupture organelles, denature proteins and expose their amino groups. Sodium dodecyl sulfate [15] and high pH also stop proteolysis, which would yield falsely high values.

For protein concentrations greater than 1 g l<sup>-1</sup>, peak height is no longer linear with  $c_p$ , a plateau being reached at about 4 g l<sup>-1</sup>. However, in the range 1–4 g l<sup>-1</sup> protein,  $w_{1/2}$ , the peak width at half-height, varies linearly with  $c_p$  (Fig. 3). For  $c_p = 4\text{--}8$  g l<sup>-1</sup>,  $w_{1/2}$ , although not linear with  $c_p$ , varies monotonically with it. Thus, in the range 1–8 g l<sup>-1</sup>,  $w_{1/2}$  furnishes an estimate of  $c_p$ . This estimate can then be used to calculate the factor by which the sample should be diluted with carrier for a valid peak-height measurement.

A pair of fractograms obtained with the on-line system in Fig. 1(b), which includes a post-column reactor (T1–E1), is shown in Fig. 4. In this example a preparation containing the mitochondria and microsomes of corn roots was fractionated [F, Fig. 1(b)] as previously described [3,4]. Monitoring such a preparation only in the usual way, by direct measurement of absorbance [detector D2, Fig. 1(b)] at 254, 260 or 280 nm, can be misleading, because suspensions of this kind scatter light, in addition to absorbing it. Further, different subcellular particles scatter light to different extents. In the post-column reactor [T1–E1, Fig. 1(b)], the

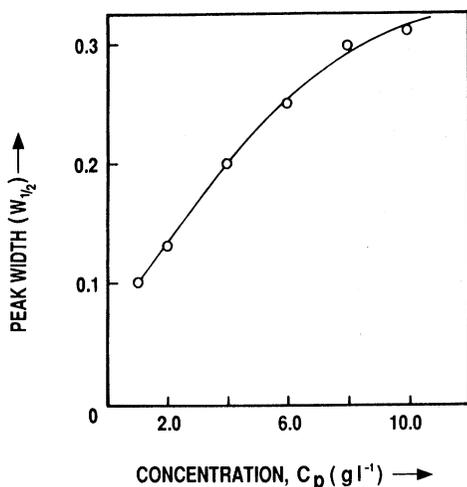


Fig. 3. Peak width at peak half-height as a function of protein concentration.

SDS and high pH of the OPA reagent [R, Fig. 1(b)] ensure exposure of the protein  $NH_2$  groups, which react with the OPA and ME in the reagent.

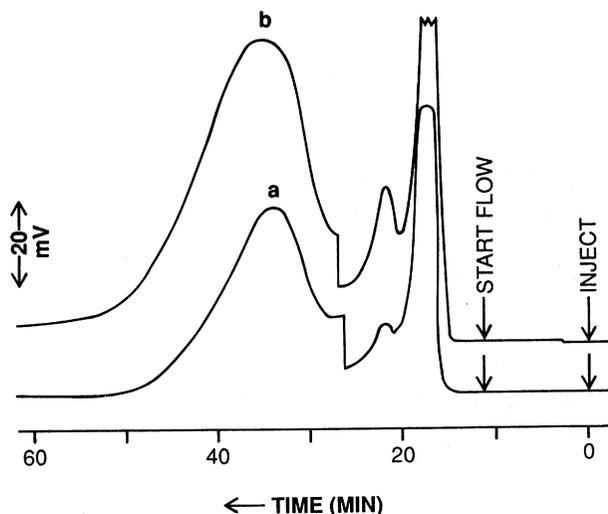


Fig. 4. Fractograms of (a) protein concentration and (b) absorbance at 260 nm of the effluent from a field flow fractionator as observed on-line by detectors D1 and D2 in Fig. 1(b). The sample was a preparation made from corn roots containing all subcellular particles except cell walls, nuclei and ribosomes. Note the changes in detector sensitivity: in (a) at ca. 26 min, from 0.025 to 0.1 V absorbance $^{-1}$  cm; in (b) at ca. 28 min, from 0.025 to 0.05 V absorbance $^{-1}$  cm. Sensitivity settings of 0.025, 0.05 and 0.1 V absorbance $^{-1}$  cm correspond to 0.4, 0.2 and 0.1 a.u.f.s., respectively.

The concentration of the adduct is then measured with detector D1, which therefore provides a measure of the primary amine content of the sample. Since the amino groups present in such preparations are predominantly those of protein origin, the result obtained is a fractogram of subcellular particle protein (Fig. 4, curve a). Although the absorbance fractogram (curve b) in Fig. 4 is similar to the protein fractogram (curve a), the ratios of the absorbances obtained with the two detectors [D1 and D2, Fig. 1(b)] vary with retention time. This is attributable to variations in light scattering by different subcellular particles (in detector D2). Variation in the absorptivities of different proteins (in detectors D1 and D2) may also be a factor.

#### Design of the assay system

Initial attempts to develop an FIA system utilizing piston (LC), peristaltic or syringe pumps to pump the carrier and reagent solutions were unsuccessful because of pulsation. Even the syringe piston advance is not smooth, as was first assumed. It was therefore decided to develop a pulse-free prototype system utilizing gas-pressure pumping, with the expectation that the gas-pressure drive would later be replaced with adequately pulse-damped piston, peristaltic or syringe pumps. However, the gas-driven system worked so well that replacement was not considered desirable.

For design and development purposes, the flow of liquid in the general layout in fig. 1(a) was treated as strictly analogous to the flow of current in an electrical circuit [Fig. 5(a)]. (It is recognized that this approach is a reversal of the historical comparison of the flow of electricity in a wire to the flow of liquid in a pipe.) Thus, in analogy with Ohm's law, the rate of liquid flow  $Q$  ( $cm^3\ s^{-1}$ ) in any section of the system under consideration across which the pressure drop is  $P$  ( $dyn\ cm^{-2}$ ) can be considered as given by

$$Q = P/R \quad (1)$$

where  $R$  is the resistance to flow of the liquid. However, by Poiseuille's law [17], for a tube of narrow bore

$$Q = \pi Pr^4/8\eta l \quad (2)$$

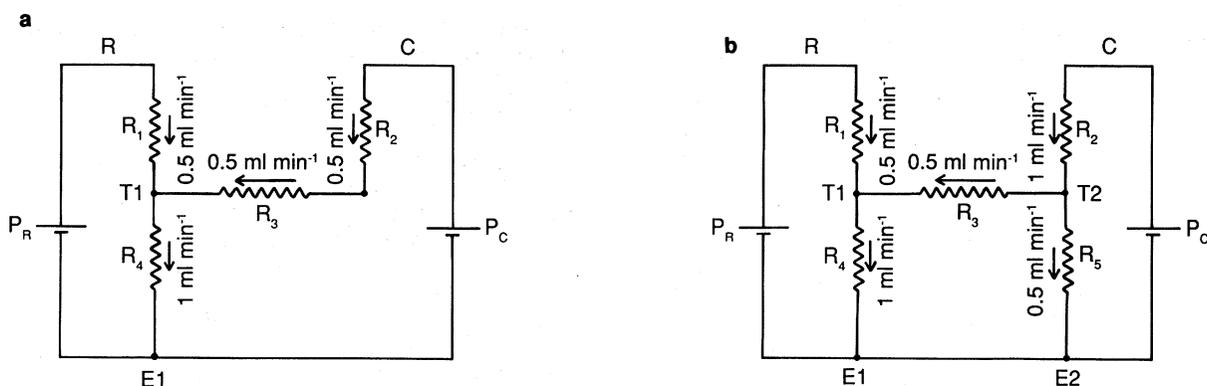


Fig. 5. (a) Circuit analogue of the off-line FIA system in Fig. 1(a). (b) Circuit analogue of the on-line system in Fig. 1(b).  $P_R$  and  $P_C$  are the pressures applied to drive the reagent and carrier solutions; each is a combination of gas pressure and hydrostatic pressure.  $R_i$  is resistance to flow. Other symbols as in Fig. 1.

where  $r$  (cm) is the radius of the tube,  $l$  (cm) is its length and  $\eta$  (poise =  $\text{g cm}^{-1} \text{s}^{-1}$ ) is the viscosity of the liquid flowing through it. Taken together, Eqns. 1 and 2 define the resistance:

$$R = 8\eta l / \pi r^4 \quad (3)$$

the units being  $\text{dyn s cm}^{-5} = \text{g s}^{-1} \text{cm}^{-4}$ .

Estimates of suitable values for the resistances [Fig. 5(a)] were made as follows:  $P_R$  was taken (initially) to be ca.  $1.5 \times 10^5 \text{ dyn cm}^{-2}$ . A convenient value of  $R_1/R_4$  was estimated by consideration of physical requirements and the presumed requirements of the reaction coils. From Eqn. 1,  $P_1 = Q_1 R_1$  and  $P_4 = Q_4 R_4$ , where  $P_i$  and  $Q_i$  are the pressure drop across and the flow-rate through resistor  $R_i$ .  $Q_1 = 8.333 \times 10^{-3} \text{ cm}^3 \text{ s}^{-1}$  (0.5 ml  $\text{min}^{-1}$ ) and  $Q_4 = 1.667 \times 10^{-2} \text{ cm}^3 \text{ s}^{-1}$  (1 ml  $\text{min}^{-1}$ ). However,  $P_R = P_1 + P_4$ ; hence  $P_R = Q_1 R_1 + Q_4 R_4$ . Having chosen  $R_1/R_4$ ,  $R_1$  and  $R_4$  can be calculated. Choosing  $P_C = P_R$ , and noting that  $Q_2 = Q_3 = Q_1$ , it follows that  $R_2 + R_3 = R_1$ .

Each of the three resistance values  $R_1$ ,  $R_2 + R_3$  and  $R_4$  was divided into two portions, one to be provided by 20 and the other by 24-gauge tubing. Thus, for example, for  $R_4$  [T1-E1 in Fig. 1(a)], 24-gauge tubing ( $r = 0.025 \text{ cm}$ ) was used for the segment which includes the reaction coils; the remainder of  $R_4$  was provided by 20-gauge ( $r = 0.040 \text{ cm}$ ) tubing. Knowing  $r$  for each portion of resistance, the length of tubing for that portion was calculated from Eqn. 3 (Table 1). For these calculations the viscosities of the reagent ( $\eta_R$ ) and

carrier ( $\eta_C$ ) were taken to be  $0.013 P$ ; the viscosities actually differed slightly,  $\eta_R$  being  $0.0129$  and  $\eta_C$   $0.0135 P$ .

The actual values of each of the three resistances were determined by measuring the rate of flow, at a set hydrostatic pressure, through the resistor when detached from the remainder of the system. The resistance values were about 25% higher than expected from the calculations. This can be attributed to the tubing diameter being slightly smaller than the nominal value. The presence of components, such as V and D1 [Fig. 1(a)], whose resistance was neglected, may also have contributed. In any case, knowing the measured values of the resistances,  $P_R$  and  $P_C$  were recalculated:  $P_R = Q_1 R_1 + Q_4 R_4$  and  $P_C = Q_2 (R_2 + R_3) + Q_4 R_4$ .

The transformation of the off-line system in Fig. 1(a) into the on-line system in Fig. 1(b) involves addition of the fractionator F, the stream splitter T2, the detector D2 and the tubing between T2 and E2. The tubing between C and T1 in Fig. 1(a) was also redistributed [Fig. 1(b)]. The electrical analogue of Fig. 1(b) is given in Fig. 5(b).  $R_1$ – $R_4$  have the same values in Fig. 5(a) and (b);  $P_R$  is also unchanged. The field flow fractionator (F) was assumed to have negligible resistance (this assumption could not be made for other fractionators, such as a chromatographic column). Outlets E1 and E2 were set at the same vertical level, and are therefore shown as con-

nected in the circuit diagram [Fig. 5(b)]. As  $P_5 = P_3 + P_4$ ,  $Q_5 R_5 = Q_3 R_3 + Q_4 R_4$ . Now,  $Q_5 = Q_3 = 1/2 Q_4$ , and  $R_3$  and  $R_4$  are known.  $R_5$  can therefore be calculated. Being large,  $R_5$  was obtained by using a combination of 24- ( $r = 0.025$  cm) and 30-gauge ( $r = 0.013$  cm) tubing (Table 2, T2-D2 and D2-E2).

$P'_c$  can now be calculated:  $P'_c = Q_5 R_5 + Q'_2 R_2$  [the primes refer to values for the on-line system in Fig. 5(b), which are different from the corresponding values for the off-line system in Fig. 5(a)]. The increase in pressure,  $P'_c - P_c$ , was provided by raising vessel C 16 cm.

### Conclusion

Procedures have been developed for the off-line and on-line assay of the protein + peptide content of subcellular particle (membrane) preparations, which scatter light. The procedures utilize the principles of FIA to yield a measurement of the concentration of the unknown in less than 1 min after addition of the reagent (OPA + ME). All pumping is done by gas pressure. Design principles and details of the assay systems are provided.

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