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Note

A method for estimating deuterium oxide density gradients from shifts in the glass-electrode potential

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In a previous paper from this laboratory¹, the utility of deuterium oxide (D_2O) as a density-gradient solute in small isoelectrofocusing columns was demonstrated. A D_2O density gradient created directly in a 1.5-ml column by free interdiffusion of three D_2O solutions for 3 min (ref. 2) was sufficiently strong and sufficiently stable with time to stabilize protein zones of the usual concentrations against convection during isoelectric focusing.

Density gradients made from D_2O have also proved useful for the separation of biological species by centrifugation. According to a recent paper by Trinick and Rowe³, zonal-velocity sedimentation in such gradients seems to be the only known successful way of fractionating thick filaments from vertebrate skeletal muscle.

In work with H_2O – D_2O gradients, it is generally of interest to know the initial density course in the gradient and its stability with time. Previously¹, I obtained this information by *in situ* measurement of the refractive-index gradient as described by Rilbe and Pettersson², whereas Trinick and Rowe³ evaluated the density gradient by pycnometry of the fractions. It is a unique feature of the former technique that the H_2O – D_2O gradient may be repeatedly recorded at desired intervals of time. On the other hand, it is a serious drawback that apparatus for direct photography of refractive-index gradients is nowadays available only to a limited number of biochemical laboratories.

The pycnometer method has proved useful also for very small samples⁴. It is self-evident, however, that accurate evaluation of the density of a H_2O – D_2O fraction containing only 0.06 ml (as in ref. 1) is a difficult task, requiring not only a sensitive balance but also manual skill and patience. Overflow will be an additional complication if the filling of the micro-pycnometer has to be made at a temperature lower than room temperature (*e.g.* at 5° as in the cited works).

The present paper suggests an alternative method for evaluating D_2O density gradients; this method is based on the shift in the asymmetry potential of a glass electrode in D_2O as compared with H_2O .

PRINCIPLE OF THE METHOD

Several workers have reported that, when a glass electrode is used to measure the acidity of a D_2O solution at 25°, the observed pH-meter reading is about 0.4 pH

unit lower than for a H_2O solution of equal acidity⁵. For mixtures of H_2O and D_2O , the deviation in pH-meter reading, $\Delta\text{p}(\text{DH})^*$, is almost proportional to the atom fraction, n , of deuterium in the mixture. This is evident from pH-meter readings made by Glasoe and Long⁶, and by Salomaa *et al.*⁷, on solutions containing the same amount of strong acid (*e.g.*, 0.01 M hydrochloric acid) but different amounts of D_2O . In principle, therefore, it should be possible to estimate the value of n (and hence the density) of an unknown H_2O - D_2O mixture by making the mixture (or a sample of it) 0.01 M in hydrochloric acid and reading its "pH". A comparison with the pH-meter reading of D_2O -free 0.01 M hydrochloric acid and a calibration curve of $\Delta\text{p}(\text{DH})$ vs. n would then give the value of n .

EXPERIMENTAL

A series of D_2O density gradients was prepared in the 1.5-ml column described elsewhere^{1,8,9} by free interdiffusion of three aqueous solutions that contained 0.0, 50.0 and 97.5% of D_2O by volume as previously¹, but, in addition, were each 0.01 M in hydrochloric acid. The solutions were prepared by adding, from a constriction pipette, 0.200 ml of 0.5 M hydrochloric acid in H_2O to 9.8 ml of either H_2O or H_2O mixed with D_2O in the appropriate volume ratio. The resulting repeatability in acidity as obtained from pH-meter readings on three preparations of the D_2O -free 0.01 M hydrochloric acid was ± 0.004 pH unit.

The prepared density gradient was left in the column (kept in upright position) for 2 h, then the column contents were fractionated into twenty-five 0.06-ml fractions¹⁰, which were subjected to pH analysis.

The pH measurements were made with a micro electrode unit (type E 5021; Radiometer, Copenhagen, Denmark) coupled to a precision pH meter with a built-in 10-fold scale expander (Radiometer, type PHM26). The glass electrode (G297/G2) in the micro electrode unit was of the capillary type and required about 0.02 ml of sample to give a pH reading. The temperature of the water-jacketed electrodes was kept constant at 25.0°. The pH meter was standardized with conventional buffer solutions of pH 2 and pH 4.

The pH measurements were started by filling the glass capillary several times with the D_2O -free initial solution. (Henceforth, the constant value thus obtained will be denoted by pH_0 .) Then the capillary was filled twice with each of the 25 fractions in order of increasing D_2O content, and the pH-meter reading was taken 1 min after the filling. Finally, additional readings were made on the D_2O -free hydrochloric acid. In conformity with earlier observations¹, the first of the additional readings was about 0.02 pH unit higher than pH_0 , whereas subsequent ones slowly decreased to pH_0 . Since the observed positive shift (δ) in pH-meter reading is probably due to a successive penetration of D_2O into the glass membrane, and since the fractions were measured in the order of increasing D_2O concentration with no rinse in between, a negative correction equal to 0.04δ multiplied by the fraction number was applied on the second pH-meter reading for each fraction. The corrected reading is denoted by pH_f (f for fraction).

* The value of $\Delta\text{p}(\text{DH})$ is defined as the meter reading in H_2O minus the reading in the mixed solvent.

The volume fraction, x_V , of D_2O in the solvent of each fraction was calculated from the difference $pH_0 - pH_r = \Delta p(DH)$ by means of the empirical equation:

$$\Delta p(DH) = 0.325 x_V + 0.060 x_V^2 \quad (1)$$

This equation was derived by the method of least squares from pH-meter readings made earlier¹ on ten solutions of hydrochloric acid prepared as described above and containing 0–90% of D_2O .

RESULTS AND DISCUSSION

The volume percentages of D_2O ($100x_V$) resulting from two identical density-gradient preparations are plotted in Fig. 1 vs. fraction number and vs. column level. For comparison, Fig. 1 also contains the corresponding concentration course as obtained by measurement of the refractive-index gradient (*i.e.*, the curve marked ● —● in Fig. 2 of ref. 1).

The density scale of Fig. 1 was calculated from the D_2O -concentration scale as follows. Since mixtures of H_2O and D_2O are ideal, their densities (ρ) vary linearly with x_V . At 5°, the densities of pure H_2O and pure D_2O are 1.000 and 1.106 g/ml, respectively¹¹; this gives the relation ρ (g/ml) = 1.000 + 0.106 x_V .

It can be seen from Fig. 1 that the concentration courses estimated by the

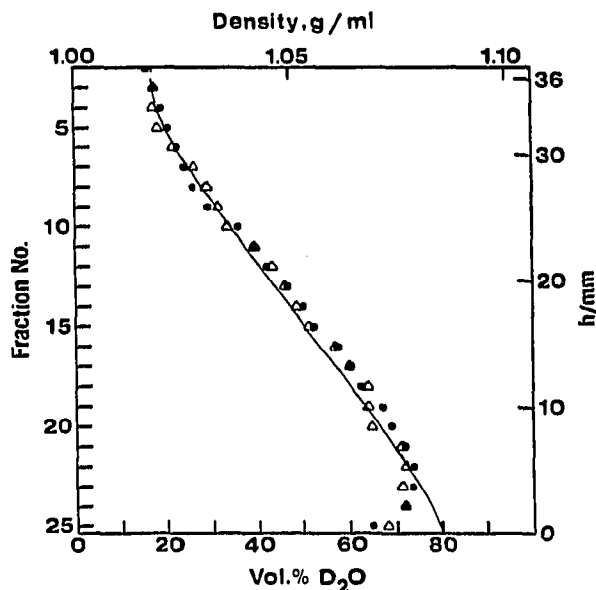


Fig. 1. Analysis of D_2O density gradients prepared at 5° in a 1.5-ml column by free interdiffusion² of three initial solutions containing 0.0, 50.0 and 97.5% of D_2O by volume, respectively. The solid curve was obtained by measurement of the refractive-index gradient². The filled circles and the triangles refer to the D_2O concentrations of 0.06-ml fractions from separate experiments and are derived by comparing pH-meter readings with a calibration curve (for details see text). The prepared density gradients had been left in the column (kept in the vertical position) for 2 h before the analysis. The density scale was calculated from the D_2O -concentration scale by means of the relation ρ (g/ml) = 1.000 + 0.106 x_V (see text). The vertical distance from the bottom of column is indicated by h .

method described agree well with the one evaluated by refractometry. The deviation is less than ± 4 vol. % of D_2O except for fractions No. 24 and 25. The concentration values obtained for these fractions are not relevant, however, as the fractions were contaminated by the D_2O - and hydrochloric acid-free sucrose solution¹⁰ used for displacement of the column contents at the fractionation.

If the regression straight lines are calculated from the resulting densities of fractions No. 3–22 as given in Fig. 1, density gradients of 0.0218 g/ml·cm (filled circles) and 0.0211 g/ml·cm (triangles) are obtained. The corresponding density gradient pertinent to the solid curve shown in Fig. 1 is 0.0206 g/ml·cm. Thus, both the density-gradient values estimated by "pH" measurements are higher than the value evaluated by refractometry (by 6 and 2.5 %, respectively). This result may be due to an inaccuracy in the solid curve of Fig. 1, but it is also conceivable that eqn. 1 is not pertinent (*cf.* below) or that the corrections applied to the pH-meter readings to give pH_r are not adequate. In any event, the observed uncertainty in the density-gradient strength should normally be insignificant when D_2O density gradients are being used for stabilization of isoelectrofocusing systems or for preparative sedimentation runs.

Likewise, the observed uncertainty in the D_2O concentrations estimated by the present method should normally be unimportant if the goal is to evaluate isoelectric points from runs in D_2O density gradients. The "absolute" pI value that can be calculated¹ for a protein component focused in such a gradient is certainly influenced by the value of x_V assigned to the focusing level, as the former value is obtained by adding the pertinent value of $\Delta p(DH)$ to the apparent pI primarily evaluated from the measured pH course. However, according to eqn. 1, an assumed error of ± 0.04 in x_V would induce an error of only ± 0.013 pH unit in $\Delta p(DH)$.

The potential accuracy of the present method of estimating D_2O concentrations is dependent on the validity of the determined values of $\Delta p(DH)$ and of the equation used for calculating x_V from these values. As was mentioned earlier, the $\Delta p(DH)$ value corresponding to 100 vol. % of D_2O is about 0.4 pH unit, which means that the average increase in $\Delta p(DH)$ is only 0.004 pH unit per 1 vol. % of D_2O . Consequently, a precision pH meter is required to obtain sufficient accuracy in pH_0 and pH_r . However, it is also important to control accurately the temperature of the pH cell by means of a thermostat. Around 25°, the pH-meter reading for 0.01 *M* hydrochloric acid decreased about 0.02 pH unit as the temperature of the cell was raised by 1°.

The equation used here for calculating x_V from measured values of $\Delta p(DH)$ may be compared with the data of Glasoe and Long, and of Salomaa *et al.* If the pH-meter readings for 0.01 *M* hydrochloric acid solutions given by Glasoe and Long in Fig. 1 of their paper⁶ are transformed into an equation by the method of least squares, the following relation is obtained:

$$\Delta p(DH) = 0.319x_V + 0.072x_V^2 \quad (2)$$

In the interval $0 \leq x_V \leq 0.8$, the $\Delta p(DH)$ values obtained from eqns. 1 and 2 for a given value of x_V , agree to within ± 0.002 pH unit.

The equation proposed by Salomaa *et al.*⁷

$$\Delta p(DH) = 0.3317n + 0.0766n^2 \quad (3)$$

gives values of $\Delta p(\text{DH})$ that are always higher than those obtained from eqn. 1, the difference increasing as n increases. It should be noted, however, that, if eqn. 3 is used instead of eqn. 1 to evaluate the percentages (by volume) of D_2O in fractions No. 3–22 in Fig. 1, agreement with the solid curve is considerably better. For example, the arithmetic mean of the deviation from the said curve is -0.4 vol. % if eqn. 3 is used, whereas the corresponding figure for eqn. 1 is $+3.1$ vol. %.

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