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# A Potential Role for the Analytical Ultracentrifuge in the Experimental Measurement of Protein Valence

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**A method is described whereby sedimentation velocity is combined with equilibrium dialysis to determine the net charge (valence) of a protein by using chromate as an indicator ion for assessing the extent of the Donnan redistribution of small ions. The procedure has been used in experiments on bovine serum albumin under slightly alkaline conditions (pH 8.0, *I* 0.05) to illustrate its application to a system in which the indicator ion and protein both bear net negative charge and on lysozyme under slightly acidic conditions (pH 5.0, *I* 0.10) to illustrate the situation where chromate is a counterion.** 

Despite being a fundamental parameter of which we would like to know the magnitude, the valence (or net charge) of a protein has rarely been measured because of the difficulty of making such determinations. Interest in this problem, which surfaced sporadically at various stages of the 20th century, has been rekindled by the requirement of a magnitude for the net charge of a protein in the assessment of effects of thermodynamic nonideality (1–5)—a phenomenon that assumes importance in concentrated solutions.

Introduction of the moving boundary electrophoresis apparatus (6) greatly facilitated the measurement of electrophoretic mobility, from which the net charge could be determined by resorting to a microscopic model of electric migration (7, 8). Clearly, the same basic principles apply to the interpretation of mobilities obtained by gel electrophoresis (9) and capillary electrophoresis (10). A problem with that approach stems from the dependence of electrophoretic mobility upon frictional coefficient as well as net charge, whereupon a magnitude for the latter may only be inferred by assigning a size and shape to the macroion. A similar difficulty is encountered in the determination of net charge by equilibrium electrophoresis (11, 12).

Model dependence of the estimated net charge of a protein by moving boundary electrophoresis can be eliminated by its evaluation either from the size of the stationary boundary (13) or from the supernatant buffer concentration required for its elimination (14–16). However, the demise of moving boundary electrophoresis has effectively eliminated these methods as viable options.

A simple approach to unequivocal determination of the net charge of a protein is to monitor the extent of the Donnan redistribution of ions in equilibrium dialysis of a protein solution—either by measuring the Donnan potential (17) or by measuring the difference between the concentrations of small ions in the protein solution and the diffusate (18, 19). However, measurement of the Donnan potential is only an option at low ionic strengths (17). An adaptation of the alternative approach (18, 19) is now described in which sedimentation velocity replaces ultrafiltration for assessing the Donnan redistribution of ions—a modification which renders the method far more attractive by decreasing substantially the amount of protein required for net charge determination.

## MATERIALS AND METHODS

Solutions (0.5 ml, 12 mg/ml) of salt-free bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, MO) were prepared by dissolving the protein directly in Tris–chloride buffer, pH 8.0, *I* 0.05 (0.02 M Trizma base–0.01 M HCl–0.04 M NaCl), supplemented with 0.2 mM $K_2CrO_4$ to provide an indicator ion. After dialysis at 20°C against the same buffer (3 ×

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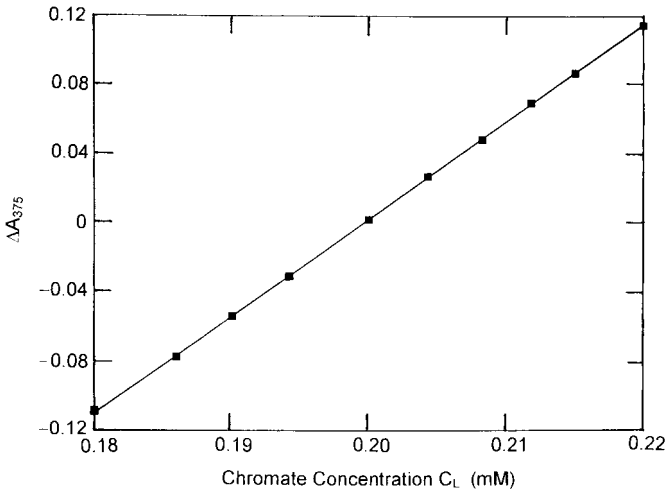


**FIG. 1.** Calibration plot for the conversion to chromate concentration, $C_L$, of measured absorbance differences at 375 nm between the sample and reference sectors of a 12-mm double-sector ultracentrifuge cell: the reference sector contained 0.200 mM sodium chromate and the sample sector the indicated chromate concentration in the range 0.18–0.22 mM. Ordinate values reflect measurements of $\Delta A_{375}$ corrected by subtracting the corresponding $\Delta A$ at 500 nm, a wavelength at which chromate does not absorb.

250 ml) the protein solutions were spun at 60,000 rpm and 20°C in a Beckman XL-I analytical ultracentrifuge. The progress of these sedimentation velocity runs was monitored spectrophotometrically at 375 nm to determine the magnitude of the difference in chromate concentration between the reference diffusate and the solvent plateau generated by protein migration. In that regard the standard setting of 5 for data averaging applied to the acquisition of each absorbance scan. Location of the baseline ($\Delta A_{375} = 0$) in these experiments was based on scans at 500 nm, a wavelength at which neither the protein nor the chromate ion exhibit absorbance. To calibrate the difference in absorbance in terms of chromate concentration the solution side of the double sector cell was filled with chromate solutions prepared either by dilution of the reference diffusate with unsupplemented Tris–chloride buffer, pH 8.0, $I$ 0.05, or by its supplementation with a more concentrated stock solution of potassium chromate in buffer (Fig. 1). These chromate solutions were prepared by weight rather than volume to specify their concentrations more accurately.

Results of the sedimentation velocity experiments were analyzed in terms of the expression (20)

$$(C_L^\alpha/C_L^\beta)^{1/Z_L} = 1 - Z_P C_P^\alpha/(2I), \qquad [1]$$

where $C_L^\beta$ is the concentration of the indicator chromate anion (the noncounterion) in the reference sector and $C_L^\alpha$ is the corresponding concentration in the protein-depleted region of the sample sector: $Z_L$ and $Z_P$ are the respective valences of chromate (−2) and protein. Because the Donnan redistribution of chromate ion refers to the protein solution in dialysis equilibrium with buffer (ionic strength $I$), allowance for the effect of radial dilution was made in the calculation of the initial protein concentration, $C_P^\alpha$, from the Rayleigh interference record of the sedimentation velocity pattern. Specifically, the fringe count, $J_r$, for the sedimenting boundary at radial distance r was corrected to its value prior to sedimentation, $J_{r_a}$, by means of the relationship

$$J_{r_a} = J_r(r/r_a)^2, \qquad [2]$$

where $r_a$ denotes the radial position of the air–liquid meniscus (21). $J_{r_a}$ was then converted to the corresponding molar concentration, $C_P^\alpha$, on the basis that

$$C_P^\alpha = J_{r_a}/(M_P\, dJ/dc). \qquad [3]$$

The fringe conversion factor, $dJ/dc$, has been taken as 3.33 fringes for a 1 mg/ml solution (22); and $M_P$, the molecular mass of bovine serum albumin, as 66 kDa (23). Subject to the availability of sufficient dialyzed protein solution, this allowance for radial dilution can be avoided by interferometric measurement of the dialyzed protein solution directly in a separate synthetic boundary experiment.

An analogous experimental protocol was followed in experiments with lysozyme (Sigma) dissolved in acetate–chloride buffer, pH 5.0, $I$ 0.10 (0.05 M potassium acetate–0.05 M KCl, pH adjusted with acetic acid), supplemented with 0.2 mM $K_2CrO_4$. However, allowance needs to be made for the fact that the cationic nature of the protein at pH 5 renders chromate a counterion. Consequently, the above definition of $C_L^\alpha$ refers to the potassium ion concentration required for neutralization of the indicator ion in the protein-depleted plateau region of the sedimentation velocity pattern at 60,000 rpm. $C_L^\alpha$ has thus been taken as twice the concentration of chromate ion in this region. The only other departure from the above procedure has been the value of $M_P$, taken as 14.4 kDa for lysozyme (24).

## RESULTS AND DISCUSSION

In the envisaged determination of protein charge from the extent of the Donnan redistribution of ions the analytical ultracentrifuge is essentially being used as a double-beam spectrophotometer. In that regard a standard spectrophotometer could certainly be used to quantify the difference in indicator ion between the dialyzed protein solution and the equilibrium diffusate. However, the consequent calculation of $C_L^\alpha$ from

the chromate concentration in the dialyzed protein solution involves the inherent assumption that chromate does not bind to the protein. That incorporation of the sedimentation velocity experiment into the analysis removes the necessity for that assumption/approximation is first illustrated by determining the net charge borne by bovine serum albumin at pH 8.

*Determination of the Net Charge on Bovine Serum Albumin (pH 8.0, I 0.05)*

Figure 2a presents scans at 375 nm (●) and 500 nm (○) taken after attainment of a rotor speed of 3000 rpm in an experiment with 12.6 mg/ml bovine serum albumin (pH 8.0, $I$ 0.05). Although there is certainly a pronounced difference between the absorbances of chromate in the sample and reference sectors (the trace at 500 nm is regarded as the baseline), the chromate concentration in the dialyzed albumin solution is greater than that in the reference diffusate, despite the fact that serum albumin is also anionic at alkaline pH (25). Such an observation signifies that chromate must be binding to the protein. The advantage of using the ultracentrifuge as the spectrophotometer is that in sedimentation velocity any bound chromate should migrate with the protein, leaving behind a protein-depleted solvent plateau with a lower concentration of indicator ion. That chromate does, indeed, bind to bovine serum albumin is confirmed by increasing the centrifugal speed to 60,000 rpm, which gives rise to a boundary in chromate constituent (Fig. 2b). The corresponding Rayleigh pattern for the assessment of $C_P^{\alpha}$ is shown in Fig. 3.

Of importance from the viewpoint of charge determination is the finding that the absorbance due to chromate in the protein-depleted zone is less than that of the reference diffusate, as predicted by Eq. [1] for a system in which the indicator ion and protein both bear net negative charge. Identification of this chromate concentration as $C_L^{\alpha}$ entails the reasonable approximation that albumin and the albumin–chromate complex should comigrate because of their similar size (26, 27). A disconcerting feature of the absorbance scans is the amount of scatter evident in Figs. 2a and 2b. For the region $6.1 < r < 6.2$, shown in further detail in Fig. 4, the mean values of $\Delta A_{375}$ (corrected for baseline) are +0.034 (±0.010) and −0.099 (±0.008) in the distributions at 3000 and 60,000 rpm, respectively. Because this level of uncertainty is unacceptably large for the evaluation of $Z_P$, three repetitive scans were recorded to ascertain the reproducibility of the mean plateau absorbances. From those values, recorded in column 2 of Table 1, it is evident that the mean absorbances are being defined with greater precision than that associated with the mean value for a given scan. Also summarized in Table 1 are the magnitudes of other parameters required for the evaluation of $Z_P$ via Eq. [1], as are the results (column 3) from a second experiment with bovine serum albumin under the same conditions (pH 8.0, $I$ 0.05). In each experiment the penultimate line gives the magnitude of the binding function $\nu$ (28) obtained by assuming identical absorption coefficients for bound and free chromate. The final line of Table 1 presents the values of the net protein charge after correction for chromate binding. There is good agreement between the two estimates of the net charge on

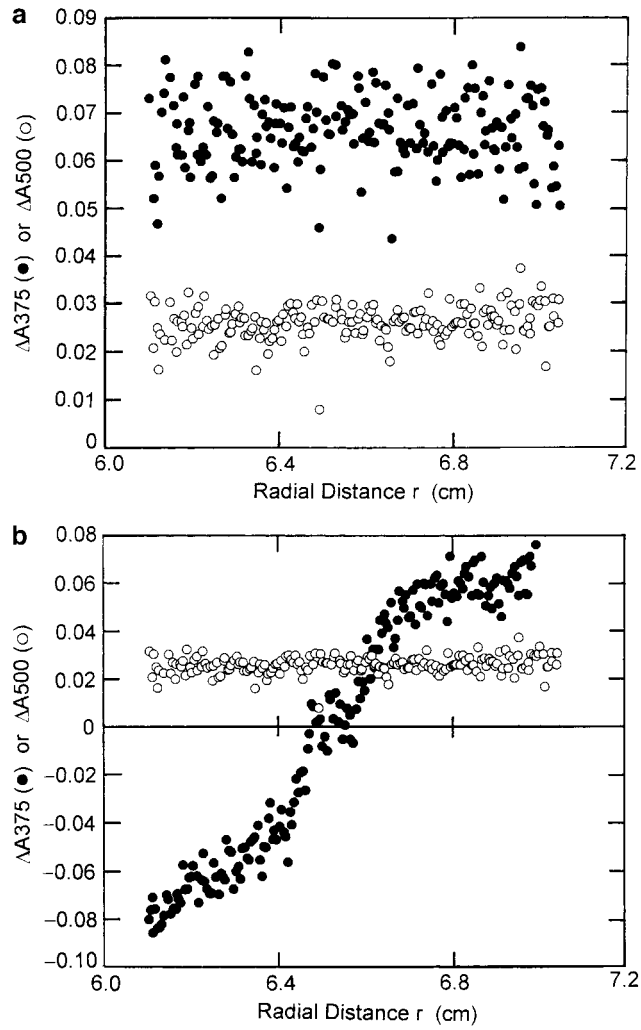


**FIG. 2.** Illustrative ultracentrifuge scans used for determining the net charge on bovine serum albumin in Tris–chloride buffer, pH 8.0, $I$ 0.05. (a) Scan of the $\Delta A_{375}$ distribution at 3000 rpm (●) reflecting the difference in chromate concentrations between dialyzed protein solution ($C_P$ = 0.191 mM) and the diffusate with which it was in dialysis equilibrium, the chromate concentration in the diffusate ($C_L^{\beta}$) being 0.200 mM: ○, scan at 500 nm, taken as baseline in the calculation of corrected absorbance differences. (b) Corresponding scan of the distribution at 375 nm taken after centrifugation at 60,000 rpm to generate a protein-free plateau region in the vicinity of the air–liquid meniscus, located at radial distance $r_a$: the baseline estimate from (a) is also included.

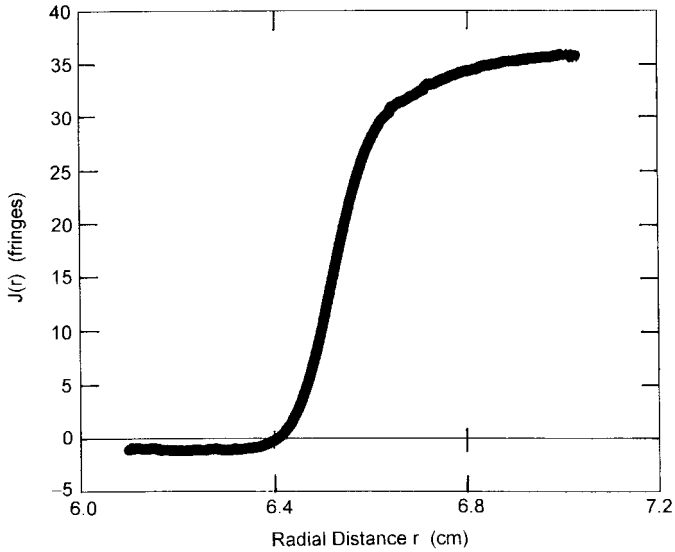


**FIG. 3.** Rayleigh interferometric record of the solute distribution at 60,000 rpm, which is used for the determination of the molar protein concentration, $C_P^\alpha$, via Eqs. [2] and [3].

bovine serum albumin in 0.05 *I* Tris–chloride buffer, pH 8.0, and also between the present valence of −24 and that (−23 to −25) inferred from the pH titration curve for bovine serum albumin (29) and the likely isoelectric point of pH 4.7–4.9 (25).

### *Determination of the Net Charge on Lysozyme (pH 5.0, I 0.10)*

The corresponding study of lysozyme in acetate–chloride buffer (pH 5.0, *I* 0.10) has been undertaken to illustrate features of the approach with a cationic protein, whereupon the chromate indicator ion distributes as a counterion during the equilibrium dialysis step. Consequently, the positive value of $A_{375}$ observed for this system in the initial distribution recorded at 3000 rpm (line 2 of Table 2) cannot be taken as indicating the existence of chromate binding by the enzyme. We therefore determine the net charge by taking $C_L^\alpha$ as the concentration of noncounterion ($K^+$) associated with chromate in the protein-depleted region of the sedimentation velocity pattern recorded at 60,000 rpm. Interpretation of the negative value of $\Delta A_{375}$ in this region (line 3 of Table 2) in terms of Fig. 1 yields the corresponding concentration of chromate in this region, which is doubled to obtain the concentration of noncounterion associated with the chromate, $C_L^\alpha$ (line 4 of Table 2). The following line summarizes the application of Eq. [1] with $Z_L = +1$ to obtain $Z_P$. As with the experiments on serum albumin, the precision of $\Delta A_{375}$ values from a given scan again left much to be desired, but the mean values from a series of scans of the same distribution exhibited much less scatter. Reasonable agreement is observed between the magnitudes of $Z_P$ determined from the two experiments. Furthermore, the essential absence of chromate binding to lysozyme in the present experiments is established by calculating $Z_P$ from Eq. [1] with $Z_L = -2$ and $C_L^\alpha$ taken as the chromate ion concentration inferred from $\Delta A_{375}$ for the dialyzed protein solution (line 2 of Table 2)—an approach that presumes no anion binding. Clearly, the magnitudes of $Z_P$ so determined (final line of Table 2) are indistiguishable from their counterparts calculated without any such approximation.

The currently determined valence of +7 for lysozyme at pH 5 is smaller than the net charge of +9 that may be inferred from the pH titration data of Tanford and Wagner (30), but such an estimate neglects the conse-

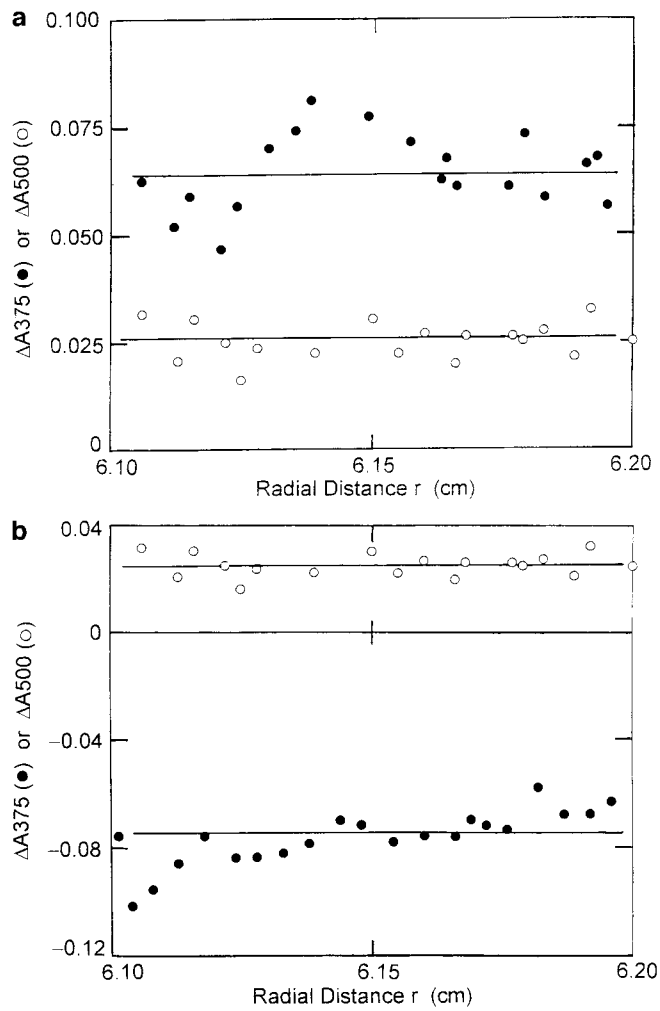


**FIG. 4.** Region of the distributions over which data were averaged to determine $\Delta A$ values (—) for the scans: (a) $\Delta A_{375}$ (●) and $\Delta A_{500}$ (○) measurements in scans of distributions at 3000 rpm; (b) corresponding scan of the $\Delta A_{375}$ distribution at 60,000 rpm (●) together with the baseline estimate from (a). Values that are reported in lines 2 and 3 of Table 1 reflect the mean of average corrected $\Delta A_{375}$ estimates from three scans of the same distribution.

quences of proton release during dissociation of the oligomeric states of lysozyme existing at neutral pH (31, 32). An even higher net charge of +14 has been obtained at pH 4.5 on the basis of the ionic strength dependence of the osmotic second virial coefficient for lysozyme and an assumed lack of chloride binding (3, 4). Inasmuch as two additional groups are protonated in the 0.5 pH increment (30), a net charge of +12 is thereby indicated for lysozyme at pH 5. The discrepancy between that estimate and the present value of $Z_P$ calls into question the validity of the assumed absence of chloride binding that was inherent in the earlier determinations of lysozyme valence from second virial coefficients obtained by sedimentation equilibrium (3, 4).

## CONCLUDING REMARKS

The present investigation has certainly illustrated the feasibility of combining sedimentation velocity with equilibrium dialysis in order to determine the net charge (valence) of a protein (or other macroion). However, a disappointing aspect has been the large uncertainty inherent in the estimate (± SD) of $Z_P$—a reflection of the noise in the absorption scans. The fact that replicate scans and duplicate experiments yield values of the net charge that are in substantial agreement clearly offers encouragement for the pursuit of endeavors to decrease the extent and hence consequence of experimental scatter in Beckman XL-I absorption scans. Increasing the magnitude of $\Delta A_{375}$ by employing a higher protein concentration offers one possible solution to decreasing the consequences of experimental scatter—a possibly obligatory modification inasmuch as a relatively low ionic strength (0.05 $I$ and 0.1 $I$ for albumin and lysozyme studies, respectively) has been used here to enhance the magnitude of $\Delta A_{375}$. Unfortunately, the alternative option of increasing the indicator ion concentration is precluded because the absorbance at 375 nm in the reference sector is already unity for 0.2 mM chromate. Major improvements in precision are thus likely to require modification of the data analysis rather than of the experimental design. In that regard there is always the possibility of introducing further means of data regularization, an approach already incorporated into ultracentrifuge applications (33, 34).

Although this investigation has clearly not achieved its goal of developing a ready means of protein charge determination, the approach seemingly has potential provided that technical aspects of absorbance delineation can be improved in the Beckman XL-I ultracentrifuge. It is hoped that this scientific rationale for protein charge determination may stimulate interest in the attainment of those technological developments and hence render feasible the routine measurement of protein valence.

**TABLE 1**

Calculation of the Net Charge (Valence) of Bovine Serum Albumin (pH 8.0, $I$ 0.05) by Using Sedimentation Velocity to Determine the Donnan Distribution of an Indicator ion (L, Chromate) in Equilibrium Dialysis (Fig. 2)

| Parameter | Expt 1 | Expt 2 |
|---|---|---|
| $C_P^\alpha$ (mM) | 0.191 | 0.181 |
| $\Delta A_{375}$ (3,000 rpm)[a] | +0.0349 (±0.0100)[b] | +0.0287 (±0.0053) |
| $\Delta A_{375}$ (60,000 rpm)[a] | −0.0956 (±0.0039) | −0.0914 (±0.0040) |
| $C_L^\alpha$ (mM)[c] | 0.1829 (±0.0007) | 0.1836 (±0.0007) |
| $(C_L^\alpha/C_L^\beta)^d$ | 0.9145 (±0.0035) | 0.9180 (±0.0035) |
| $Z_P{}^e$ | −23.9 (±1.0) | −24.1 (±1.0) |
| $\nu^f$ | 0.12 (±0.01) | 0.12 (±0.01) |
| $Z_P$ (corrected)[g] | −23.7 | −23.9 |

[a] Means of corrected absorbance differences in the range $6.1 < r < 6.2$ cm from three replicate scans.
[b] Numbers in parentheses denote the uncertainty (±SD) in the estimate.
[c] Obtained from $\Delta A_{375}$ at 60,000 rpm and the relationship $\Delta A_{375} = 1.134 + 5.678\ C_L$ (Fig. 1).
[d] $C_L^\beta = 0.200$ mM (the chromate concentration in the diffusate).
[e] Calculated from Eq. [1] with $Z_L = -2$ for chromate ion.
[f] $\nu = (\bar{C}_L^\alpha - C_L^\alpha)/C_P^\alpha$, where $\bar{C}_L^\alpha$, the total ligand concentration, is obtained from Fig. 1 in the same way as $C_L^\alpha$ but with $\Delta A_{375}$ (3000 rpm) as the absorbance difference.
[g] $Z_P$ (corrected) $= Z_P - Z_L \nu C_P^\alpha$.

**TABLE 2**

Calculation of the Net Charge (Valence) of Lysozyme (pH 5.0, $I$ 0.10) by Using Sedimentation Velocity to Determine the Donnan Distribution of an Indicator Ion (Chromate) in Equilibrium Dialysis

| Parameter | Expt 1 | Expt 2 |
|---|---|---|
| $C_P^\alpha$ (mM) | 0.746 | 0.0.746 |
| $\Delta A_{375}$ (3,000 rpm)[a] | +0.0.0632 (±0.0006)[b] | +0.0635 (±0.0026) |
| $\Delta A_{375}$ (60,000 rpm)[a] | −0.0283 (±0.0023) | −0.0307 (±0.0018) |
| $C_L^\alpha$ (mM)[c] | 0.3894 (±0.0008) | 0.3846 (±0.0006) |
| $Z_P{}^d$ | +7.1 (±0.5) | +7.6 (±0.4) |
| $Z_P{}^e$ | +7.0 (±0.1) | +7.1 (±0.3) |

[a] Means of corrected absorbance differences in the range $6.1 < r < 6.2$ cm from three replicate scans.
[b] Numbers in parentheses denote the uncertainty (±SD) in the estimate.
[c] Obtained from $\Delta A_{375}$ at 60,000 rpm by doubling the chromate concentration inferred from Fig. 1.
[d] Calculated from Eq. [1] with $Z_L = +1$ for potassium ion.
[e] Calculated from Eq. [1] with $Z_L = -2$ and $C_L^\alpha$ taken as the concentration of chromate inferred from the magnitude of $\Delta A_{375}$ reported in line 2.

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