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Sedimentation equilibrium and the foundations of protein chemistry

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1. Introduction

David Yphantis is best known for his truly outstanding contributions to the development and application of the sedimentation equilibrium method. In his hands, this method has become one of the most sophisticated and versatile techniques available for the study of macromolecules and their interactions. It seems fitting, therefore, to begin this volume, dedicated to David, with a brief retrospective look at the very beginnings of the sedimentation equilibrium technique, over three-quarters of a century ago. It is also appropriate, I think, to remind ourselves of how important a role this method played in the formative years of protein chemistry.

2. The background

Svedberg published the first edition of his monograph 'Colloid Chemistry' in 1924 [1]. In it, he paid scant attention to the proteins, including only a brief description of their coagulation properties in the chapter titled 'The Colloid Particle as a Micelle'. In this regard, Svedberg was following the view held by most chemists of his time, few of whom considered it possible that proteins were molecules; most thought them to be heterogeneous colloidal aggregates of small peptides or cyclic peptide-like substances. Although the few osmotic pressure studies that had been carried out at that time, such as Sørenson's studies on ovalbumin [2] indicated high molecular weights, these were widely disregarded. Indeed, Svedberg himself [1] described Sørenson's osmometer, but then stated that 'determination of size of particles with this method have not given any appreciable results'.

It is ironic that at the moment of publication of 'Colloid Chemistry', Svedberg himself was perfecting the instrument that was to completely revolutionize science's view of proteins. Together with his collaborator, H. Rinde, Svedberg had for several years been analyzing the size distribution in colloids by following their sedimentation under gravity. They had developed an optical system to record such sedimentation, and mathematical procedures for its analysis [3]. However, the very fine 'amicroscopic' colloids could not be studied in this way. Not only was their gravitational sedimentation exceedingly slow, but problems of thermal convection become serious in the weak gravitational field. Accordingly, Svedberg and Rinde suggested in 1923 the use of a centrifugal field [3]. Later that year, during a sabbatical visit to the University of Wisconsin, Svedberg and Nichols constructed a primitive version of the ultracentrifuge [4]. The first measurements made were essen-

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tially sedimentation velocity studies (although the term was not yet used or even defined) on gold sols. A much improved instrument was constructed when Svedberg returned to Sweden in the next year, and is described as 'The Ultracentrifuge, A New Instrument for the Determination of Size and Distribution of Size of Particle in Amicrosopic Colloids' [5]. Note that even in 1924, Svedberg's orientation is still that of a colloid chemist. Again, first tests of the instrument were made using gold sols. However, within a year, Svedberg's interests were to undergo a radical change.

3. The first sedimentation equilibrium experiment

It is remarkable that the first major application of the new ultracentrifuge—the study of hemoglobin by Svedberg and Fåhreus [6]—should produce one of the most important advances in all of protein chemistry. Not only did it represent the first time that the technique of sedimentation equilibrium had been applied to any macromolecule, but it also provided the unexpected demonstration that proteins were very large molecules of homogeneous molecular size. Thus, this paper more than any other, led to the demise of 'colloid' theories of protein structure, and opened the way to visualizing proteins as giant molecules of defined structure.

The experiment examined the sedimentation equilibrium of horse hemoglobin (actually CO–Hb and met–Hb) under a variety of solution conditions. It is presented as a demonstration of a new method for the 'determination of the molecular weight of the proteins'; the very wording of this title suggests a remarkable shift in Svedberg's attitude toward proteins in a very few years. The use of the term 'molecular weight' is in itself noteworthy. Svedberg and Fåhreus explain the choice of hemoglobin on the basis of its physiological importance [7], but it seems likely that its special optical absorbance properties, and earlier indications of relatively high molecular weight were equally important. At this point, they were relying on absorption optics in the visible region of the spectrum, and the top rotational speed of the available ultracentrifuge was approximately 10 000 rev./min. This latter fact dictated the use of sedimentation equilibrium rather than sedimentation velocity experiments, although the theory for both methods was already well advanced (see [8]).

It is a beautiful paper. Svedberg and Fåhreus mention a few preliminary trials with oxyhemoglobin, but its instability led them to do the final work with CO-hemoglobin and met-hemoglobin. The studies were done with remarkable care. The partial specific volume was determined pycnometrically, and parameters of the sedimentation equilibrium experiments such as solute concentration, column height, and rotor speed were varied, without significant effect on the results. The same equilibrium distribution was found when approached by acceleration from rest or deceleration from a higher speed, an important test at this point in the development of the method.

The data were analyzed using the conventional sedimentation equilibrium equation in a form in which integration is carried out between pairs of points x_1 and x_2 in the cell:

$$M = \frac{2RT\ \ln\left(C_1/C_2\right)}{\omega^2(1-\bar{v}\rho)(x_1-x_2)(x_1+x_2)} \qquad (1)$$

This is not the way we would do it today and in fact, is a poor way to get an average value over the cell, for averaging such values almost eliminates the effect of all but the end points. However, as we shall see, it proved to be a very useful way to represent the data at the time. It is notable that Svedberg and Fåhreus show all of their data, a model that more contemporary researchers could follow! One representative set of the data are shown in Table 1. There are two important conclusions from this, and the other data in the paper.

1. The molecular weight of hemoglobin is approximately 68 000, or four times the molecular weight associated with a single mole of iron. This is an important conclusion, but in fact had already been reported from the osmotic studies of Adair [9] a few months earlier. Svedberg and Fåhreus do not mention the Adair paper. Given the slowness of communication in 1925, it seems unlikely they would have seen it in time

Table 1
Sedimentation of CO-Hemoglobin (Data of Svedberg and Fåhraeus, 1926 [6])

x_1 (cm)	x_2	C_1 (g/100 ml)	C_2	$M \times 10^{-3}$	M/16 700
4.61	4.56	1.220	1.061	71.30	4.27
4.56	4.51	1.061	0.930	67.67	4.05
4.51	4.46	0.930	0.832	58.33	3.49
4.46	4.41	0.832	0.732	67.22	4.02
4.41	4.36	0.732	0.639	72.95	4.37
4.36	4.31	0.639	0.564	60.99	3.65
4.31	4.26	0.564	0.496	75.57	4.59
4.26	4.21	0.496	0.437	69.42	4.16
4.21	4.16	0.437	0.388	66.40	3.98
			Ave	67.87	4.06

Conditions: Initial conc 1.0 g/100 cc, $T = 293.3°$, column length = 0.6 cm, 8708 rev./min, time of expt = 39 h.

to even add a 'Note in Proof', because the Adair paper appeared October 1, 1925, whereas Svedberg and Fåhreus' paper was submitted August 4, 1925 and published February 5, 1926. Clearly, we should give them the benefit of the doubt. Both results are reasonably close to the exact value of 64 715 that we now calculate from the amino acid sequence of horse hemoglobin (plus hemes). The agreement by two quite different methods, osmotic pressure and sedimentation equilibrium, must have been impressive to scientists of the time. However, the sedimentation equilibrium experiment had much more to tell.

2. The more important observation was the *homogeneity* of the hemoglobin molecules with respect to molecular weight. The rather awkward way in which the data are presented has the great advantage that this is immediately clear to every reader. At each interval across the solution column, roughly the same value of M is obtained. This same result was found in every one of the four experiments reported. If the authors had done as we are likely to do today, and graphed ln C vs. x^2, they would have indeed obtained a nice straight line (I have checked this and it gives a very slightly better result), but this does not make nearly so clear a demonstration of homogeneity to the uninitiated.

The homogeneity of hemoglobin was an observation of the very greatest importance. It seems to have been surprising to Svedberg, for the last paragraph of the paper makes the point almost with reluctance, and this most vital conclusion is not included in the summary! However, it is just this finding—that proteins are large molecules of precisely defined molecular weight—that eventually shifted the whole paradigm of protein chemistry away from the 'colloid' perspective that had so long encumbered it. Colloidal aggregates are rarely if ever truly homogeneous. The idea of *molecular* homogeneity could now make connection with the polypeptide hypothesis of Hofmeister [10] and Fischer [11] and the high polymer concept being developed by Staudinger [12]. Protein chemistry was finally on a firm foundation.

4. Further studies

Of course, one paper could not convince the scientific world of the molecular nature of proteins. However, over the few years following the 1926 paper, Svedberg and his colleagues presented an overwhelming mass of evidence, all to the same point—the majority of proteins were high molecular weight, homogeneous substances. The Svedberg laboratory developed this evidence along two tracks. The first depended upon Svedberg's rapid development of high-speed ultracentrifuges, which allowed sedimentation velocity studies of even small proteins. An early landmark result was the 1927 [13] demonstration of the velocity sedimentation of hemoglobin with results strongly supporting the 1926 sedimentation equilibrium paper.

The second line of research involved further use of the sedimentation equilibrium method, aided by UV absorption optics or refractometric optical systems permitting the study of substances colorless in the visible spectral region. By 1930, sedimentation equilibrium results for ovalbumin [14–16] serum albumin and serum globulin [17] and a Bence–Jones protein [18] had been reported. Where we can make comparisons, the results are mostly in good agreement with sequence data, and demonstrate homogeneity. The puzzling exception is ovalbumin. The early osmotic pressure studies of Sørenson [2] reported a value of approximately 34 000 Da, which agreed with a 'minimal' molecular weight calculated from amino acid composi-

tion by Cohn et al. [19]. The initial studies of electrolyte-free ovalbumin in Svedberg's laboratory [14] gave indications of heterogenteity, with a major component of 35 000 Da and a small amount of a much larger component that could be removed by electrodialysis. Repetition of the study, using purified material in the presence of electrolytes [15,16] gave results in the same range.

The problem is that we now know, from sequence analysis, that the correct value for the molecular weight of ovalbumin is nearly 43 000 Da. A sedimentation equilibrium result close to this was later found by Pedersen, and reported as ''unpublished work'' in '*The Ultracentrifuge*' [20]. The explanation proposed therein for the early erroneous values was that sufficient time for equilibrium to be attained had not been allowed.

It is remarkable that so many early studies of ovalbumin, using a variety of techniques, faithfully reproduced the same erroneous value. Strangely, it seems probable that this coincidence of errors actually increased confidence in the new methods and new ideas, whereas contradiction would have sowed doubt. Science proceeds in mysterious ways.

Acknowledgments

It is with pleasure I dedicate this note to my old friend and colleague, David Yphantis, who taught us all much about doing science properly.

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